Irreversible Enzyme Inhibitors. LXXIII.[1,2] Inhibitors of Guanine Deaminase. I. Mode of Binding of Guanine

B. R. BAKER

Department of Chemistry, University of California, Santa Barbara, California 93106, and the Department of Medicinal Chemistry, State University of New York at Buffalo, Buffalo, New York

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Investigation of guanine and 17 related compounds as substrates or inhibitors of guanine deaminase has led to a suggested mode of binding of guanine. It is highly probable that the 1- and 9-hydrogens of guanine are complexed to the enzyme as electron acceptors and the 6-oxo and 7-nitrogen are complexed as electron donors. It appears that the π cloud, as well as the 2-NH_2 group of guanine, does not complex to the enzyme. It is possible that the 3-nitrogen of guanine as an electron donor is complexed to the enzyme.

Guanine deaminase (guanase) is a catabolic enzyme that converts guanine (I) to xanthine (II);[3] the enzyme reaction appears to be irreversible. 8-Azaguanine (III),[3] thioguanine (IV),[4] and 1-methylguanine[5] are known substrates. The most potent known inhibitor is 5-aminoimidazole-4-carboxamide (V).[6] The partially purified enzyme from rabbit liver is commercially available; it is readily assayed by the rate of decrease in optical density at 245 mμ, where guanine has an absorption maximum and xanthine has a minimum.[3] Neither guanosine nor 5′-guanylic acid are substrates.[3]

Some years ago it was proposed[4b,7] that the selective action of thioguanine (IV) on certain tumors could be due to the lack of guanine deaminase in these susceptible cell lines; thus, when thioguanine could not be detoxified to thioxanthine[4] within a cell, cell death resulted. If guanine deaminase could be inhibited selectively in a tumor cell line with minimum blockade of this enzyme in normal tissues, then such an inhibitor could be used in conjunction with thioguanine with cell lines otherwise less affected by thioguanine. Ordinary reversible inhibitors rarely show tissue or species specificity unless some nonfunctional part of the enzyme is employed for binding, such as the hydrophobic region of dihydrofolic reductase.[8] In contrast, active-site-directed irreversible enzyme inhibitors[9,10] can show tissue specificity; for example, little selectivity with reversible inhibitors of lactic dehydrogenase from skeletal muscle or heart could be shown, but the active-site-directed irreversible inhibitors could be constructed that did show selective inactivation between these two isozymes.[11]

An active-site-directed irreversible enzyme inhibitor operates by first forming a reversible complex, then a slower formation of a covalent linkage between the enzyme and the inhibitor in a facile neighboring group reaction which leads to inactivation. In order to design an active-site-directed irreversible inhibitor, a definitive *modus operandi* has been gradually developed[9,10] which leads to a greater probability of success; if a covalent forming group is placed on the potential irreversible inhibitor in any position that will interfere with reversible complex formation, then the facile neighboring group reaction is automatically negated.[9,10] With an uninvestigated enzyme, such as guanine deaminase, the following *modus operandi* should be employed.[10] (a) The groups on the substrate or an inhibitor necessary for binding should be determined; a binding group can be eliminated if the binding by a second group can be increased sufficiently. (b) An area on the inhibitor should be found that is not in contact with the enzyme when the inhibitor forms a complex—so-called bulk tolerance. (c) Groups of varying lengths terminating in a covalent-forming moiety such as bromoacetamido should be placed on the noncontact area of the inhibitor, then investigated for inactivation. A study of the first phase with guanine deaminase is the subject of this paper; in the following paper[12] a study of the second phase is presented.

The inhibitory properties[6] of 5-aminoimidazole-4-carboxamide (V) were checked; V was complexed to the enzyme about half as well as the substrate, guanine (I) (see Tables I and II). This result indicated that the 2-NH_2C moiety was not necessary for binding.

That the 7-nitrogen of guanine was complexed as an electron donor to the enzyme was indicated by the lack of inhibition of 3-aminopyrazole-4-carboxamide (VI);

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(2) For the previous paper of this series see B. R. Baker, T. J. Schwan, and B.-T. Ho, *J. Pharm. Sci.*, in press. The series on Nonclassical Antimetabolites and the series on Analogs of Tetrahydrofolic Acid have been combined into one series since they have a common objective, namely, the design of active-site-directed irreversible inhibitors. A collected list of references will be sent on request.

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(9) B. R. Baker, *ibid.*, **57**, 347 (1964), a review.

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(12) B. R. Baker and D. V. Santi, *J. Med. Chem.*, **10**, 62 (1967); paper LXXIV of this series.

TABLE I

INHIBITION OF GUANINE DEAMINASE BY GUANINE ANALOGS

No.	Structure	Concn, μM	% inhib[a]	$([I]/[S])_{0.5}$[b]	Source[c]
II	O; HN, N; O=, N, N; H, H	500	0	>150[d]	A
V	H_2N–CO, N·HCl; H_2N, N; H	28[e]	50	2.1	B
VI	H_2N–CO, N; NH_2, N, 0.5 H_2SO_4; H	500[e]	0	>150[d]	B
VII	NH_2; N, N; NH_2, N, N; H	300	0[f]	>20[d]	C
VIII	SCH_3; N, N; NH_2, N, N; H	250	0[f]	>75[d]	C
IX	O; CH_3N, N; NH_2, N, N; H	125	Substrate[g]		D
X	O; HN, N; NH_2, N, N; CH_3	275	50[f,h]	21	D
XI	O, CH_3; HN, N; NH_2, N, N	250	0[f,h]	>75	D

[a] Commercial enzyme from rabbit liver was assayed as described in the Experimental Section with 13.3 μM guanine; compounds were dissolved in 1 N KOH unless otherwise indicated. [b] Ratio of concentration of inhibitor to substrate giving 50% inhibition. [c] A, Nutritional Biochemical Corp.; B, Aldrich Chemical Co.; C, Sigma Chemical Corp.; D, Dr. Roland K. Robins. [d] Since 20% inhibition is readily detectable, the concentration for 50% inhibition is at least four times greater than the concentration investigated. [e] Dissolved in 0.05 M Tris buffer, pH 7.4. [f] No substrate properties at this concentration. [g] One-half the rate of guanine at this concentration, but barely perceptible at 25 μM; previously observed to be a substrate by G. H. Hitchings and E. A. Falco, *Proc. Natl. Acad. Sci. U. S.*, **30**, 294 (1944). [h] Dissolved in $(CH_3)_2SO$ and assay run in 10% $(CH_3)_2SO$.

TABLE II

INHIBITION OF GUANINE DEAMINASE BY

R_6; HN, N, R_8; NH_2, N, N; R_9

No.	R_6	R_8	R_9	Concn, μM	% inhib[a]	$([I]/[S])_{0.5}$[b]	Source[c]
I	O	CH	H	13.3	Substrate[d]		A
III	O	N	H	200	Substrate[e]		A
IV	S	CH	H	100	Substrate[f]		B
XII	O	CBr	H	250[g,h]	0	>75	C
XIII	O	COH	H	250[h]	0	>75	A
XIV	O	CH	CH_3	275[h]	50	21	D
XV	O	CH	C_6H_5	9.8[g]	50	0.73	D
XVI	S	CH	C_6H_5	83[g]	50	6.2	D
XVII	O	CH	p-ClC_6H_4	3.8[g]	50	0.29	D
XVIII	O	N	p-ClC_6H_4	18	50	1.4	D

[a] Commercial enzyme from rabbit liver was assayed with 13.3 μM guanine as described in the Experimental Section; the inhibitors were dissolved in 0.01 N KOH unless otherwise indicated. [b] Ratio of concentration of inhibitor to substrate giving 50% inhibition. [c] A, Nutritional Biochemical Corp; B, Sigma Chemical Co.; C, Calbiochem Co.; D, Dr. Roland K. Robins. [d] $K_m = 3.2 \times 10^{-6}\ M$ was obtained by the reciprocal plot method; A. Roush and E. R. Norris have recorded $K_m = 5 \times 10^{-6}\ M$ for the enzyme from rat liver. [e] About one-half the rate observed with 13.3 μM guanine; A. Roush and E. R. Norris have recorded $K_m = 7 \times 10^{-5}\ M$ for the enzyme from rat liver. [f] Measured by the rate of optical density change at 335 mμ. [g] Inhibitor dissolved in $(CH_3)_2SO$, assay run in 10% $(CH_3)_2SO$. [h] No substrate properties at this concentration.

since the pyrazole ring is a weaker base than imidazole, it is possible that this weakening of the base strength could decrease binding if the π cloud of the ring system were a donor. That the latter interpretation is unlikely is indicated by the following.

(a) 7-Methylguanine (XI) is not an inhibitor. If the π cloud of the imidazole moiety were a donor, then XI should bind to the enzyme at least as well as 9-methyl guanine (X).

(b) If the lone-electron pair of the 7-nitrogen of guanine were a donor, then the more basic this nitrogen is, the better the complexing should be and *vice versa*. Note that 8-hydroxyguanine (XIII) actually has an acidic NH group at the 7 position and 8-bromoguanine (XII) has a base-weakened 7-nitrogen; both compounds are poor inhibitors. Although 2,6-diaminopurine (VII) and 2-amino-6-methylthioguanine (VIII) have a 7-nitrogen that is more basic than that of guanine, VII and VIII are not inhibitors; in these two cases the NHC=O grouping at 1,6, which are probable binding points, has been changed, also indicating that the 1,6 grouping contributes more to binding than the 7-nitrogen.

That the NHC=O moiety at 1,6 complexes to the enzyme with the hydrogen as an acceptor and the oxygen as a donor is indicated by the following.

(a) Removal of the NHC=O moiety, as in 2,6-diaminopurine (VII) and 2-amino-6-methylthiopurine (VIII) results in no detectable binding.

(b) Methylation of the 1-nitrogen of guanine gives a compound (IX) that is still a substrate, but requires a much higher concentration than guanine for saturation of the enzyme, thus indicating that IX does not complex as well as guanine due to loss of the 1-hydrogen in IX.

(c) Conversion of the 6-oxo group of guanine (I) to 6-thione (IV) still allows substrate properties (Table II), as previously noted.[4] That such a structural change leads to a nine-fold loss in binding can be seen by comparing the 9-phenyl derivatives, XV and XVI; this result indicates that the 6-oxo group, as a donor, complexes with the enzyme, since the 6-thione is a poorer donor. If only the 6-oxo group of the NHC=O moiety were complexed, then an even bigger difference in binding might have been anticipated; however, since the 1-hydrogen is apparently binding as an acceptor, it would be more acidic and a better acceptor with a 6-thione group, thus partially compensating for the decrease in binding of the 6-thione group.

That the 3-nitrogen of guanine (I) may complex as a donor to the enzyme is indicated by the lack of binding by xanthine (II) which has an acidic 3-NH group; this interpretation is highly equivocal since the decrease in basicity of the 7-nitrogen of xanthine (II) compared to guanine (I) could also account for this result. If the 5-amino group of the imidazole-4-carboxamide (V) were removed and tested as an inhibitor, lesser binding could also be due to a decreased basicity of the nitrogen corresponding to the 7-nitrogen of guanine; thus, it might be difficult to determine with any greater degree of certainty that the 3-nitrogen of guanine is complexed to the enzyme.

That the 9-hydrogen of guanine is complexed as an acceptor to enzyme is indicated by the 21-fold loss in binding when the 9-hydrogen is replaced by methyl, as in 9-methylguanine (X); removal of 7-methylation, as in XI, gives an even greater loss in binding. These results might also be interpreted as due to a lack of bulk tolerance for either group within the enzyme-inhibitor complex, but such an interpretation is less likely. Replacement of the 9-hydrogen by phenyl (XV) gives an excellent inhibitor; that this result is most probably due to a hydrophobic interaction with the enzyme, combined with some additional forces, is discussed in the accompanying paper.[12]

Replacement of the 8-CH of guanine by nitrogen, as in 8-azaguanine (III) (Table II), gives a sevenfold loss in binding as noted by their relative K_m values. This result is substantiated by comparison of their 9-(*p*-chlorophenyl) derivatives, the guanine derivative (XVII) being a fivefold better inhibitor than the corresponding 8-azaguanine derivative (XVIII). The poorer binding by the 8-azaguanines is more apt to be due to the weaker basicity of the 7-nitrogen, since *v*-triazole is a weaker base than imidazole, than due to binding by the 8-CH group.

The contribution to binding to guanine deaminase by groups on guanine (I) can be summarized in several classes: (a) those groups that most probably complex to the enzyme include the 1- and 9-hydrogens, the 6-oxo, and the 7-nitrogen—the first two as electron acceptors and the last two as donors; (b) those groups that most probably do not complex to the enzyme include the π-cloud system, the 2-NH_2C moiety and the 8-CH group; (c) those groups that might be complexed to the enzyme such as the 3-nitrogen as a donor where insufficient evidence is available to make the probability higher.

Experimental Section

Guanine deaminase (guanase) was a rabbit liver preparation purchased from Sigma Chemical Co. as a 1-mg/ml suspension; at this concentration it was reputed to deaminate 0.1 μmole of guanine/min. The suspension was stable over 4 months at 2–5°; for assay, 50 μl of bulk enzyme was diluted with 1.95 ml of 0.05 *M* Tris buffer (pH 7.4), which could be kept at room temperature for a day's run.

Guanine (15 mg) was dissolved in 1.00 ml of 1 *N* KOH, then diluted to 100 ml with water. For assay, 1 ml was diluted with 14 ml of water to give a 66.7 μM solution. The assay was performed as follows. In a 1-ml cuvette was placed 0.70 ml of 0.05 *M* Tris buffer (pH 7.4) and 200 μl of 66.7 μM guanine. The reaction was started by addition of 100 μl of diluted enzyme solution; the decrease in optical density at 245 mμ was recorded continuously with a Gilford 2000 spectrophotometer, being about 0.005 optical density unit/min. The final concentration of guanine was 13.3 μM.

Inhibitors were dissolved in (a) 0.05 *M* Tris buffer (pH 7.4) if soluble, (b) at 100 m*M* in 1 *N* KOH, then 100-fold dilution with Tris buffer, or (c) if base insoluble, in DMSO. In the latter case the assay was run in 10% DMSO the rate of reaction being identical with or without the presence of DMSO. The concentration for 50% inhibition was determined by testing a series of inhibitor concentrations giving 30–70% inhibition; when V_0/V_I was plotted against the inhibitor concentration, [I], where V_0 = velocity without inhibitor and V_I = velocity with inhibitor, the 50% inhibition concentration was obtained where $V_0/V_I = 2$.[13]

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