Irreversible Enzyme Inhibitors LXVII

2-Amino-6-(*p*-bromoacetamidophenylbutyl)-5-phenylbutyl-4-pyrimidinol, an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase

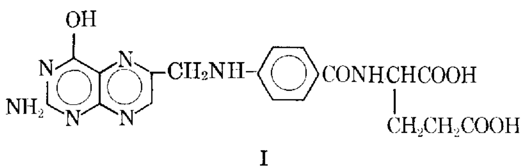
By B. R. BAKER and JOHANNES H. JORDAAN

2-Amino-5-phenylbutyl-4-pyrimidinols substituted by a *p*-bromoacetamidophenylbutyl group (XIII) or a *p*-bromoacetamidophenethyl group (XIV) on the 6-position can inactivate dihydrofolic reductase with a half-life of 18 and 12 min. at 37°, respectively. These inactivations have the kinetic parameters expected for enzyme inactivation by the active-site-directed mechanism, that is, an inactivation proceeding by a neighboring group reaction within the reversible complex formed between dihydrofolic reductase and XIII or XIV. Neither iodoacetamide nor *p*-bromoacetamidophenylbutyric acid showed any inactivation of dihydrofolic reductase at a concentration equal to XIII under conditions where XIII gave 65–70 per cent inactivation. The rate of inactivation of dihydrofolic reductase by XIII and XIV was slowed by the reversible inhibitors, folic acid (I) and 2,4-diamino-5-(3,4-dichlorophenyl)-6-ethylpyrimidine (XV). The inactivation was also slowed by TPNH, but not by DPNH or adenosine diphosphate.

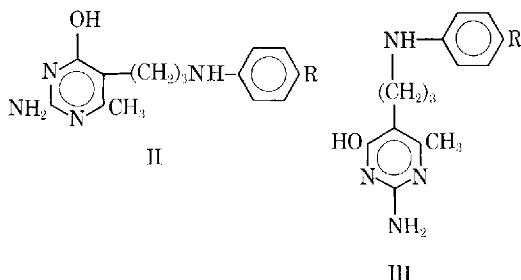
BEFORE THE strong hydrophobic bonding to dihydrofolic reductase was discovered (1)—with its conformational implications (2–8)—more than 30 potential active-site-directed irreversible inhibitors for this enzyme had been synthesized and evaluated on the premises discussed below.

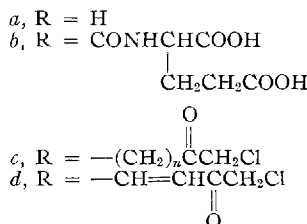
THEORETICAL

It should theoretically be possible to modify folic acid (I) with a chloromethyl ketone group in place of one of the carboxyls in order to obtain an active-



site-directed irreversible inhibitor that would operate by the endo-mechanism (9) by alkylation of the enzymic binding point for one of the carboxyl groups. The synthesis of such a pteridine is fraught with monumental incompatibilities of functional groups. Since the anilino propylpyrimidine (II*a*) (10) without a carboxy-L-glutamate moiety was still





about one-eighth as good an inhibitor as II*b* (10, 11), compounds II*c* (n = 0,2,4,6) and II*d* were synthesized as potential active-site-directed irreversible inhibitors of dihydrofolic reductase (11). Although these five compounds were good reversible inhibitors, they failed to show irreversible inhibition. In retrospect, these failures were most probably due to the possibility that the anilinopropyl group of II*a*, *c*, and *d* was not complexed to the enzyme at the *p*-aminobenzoyl locus for folic acid, but was complexed in the hydrophobic region in a conformation such as III where the pyrimidine moiety has been twisted 60° with respect to the "normal" complexing of the pteridine of folic acid in conformation I. A conformation such as II would destroy the juxtaposition of the nucleophilic groups on the enzyme—which complex the glutamate carboxyls—with the alkylating functions of III*c* and *d;* that is, if the anilinopropyl side chains of III*c* and *d* are complexed in a hydrophobic region, then by definition there are not apt to be hydro-

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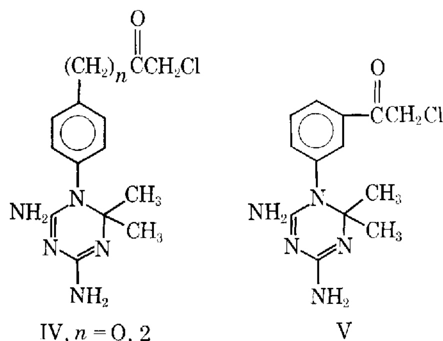
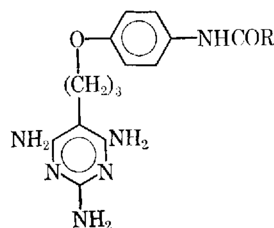
The previously published papers on "Nonclassical Antimetabolites" and on "Analogs of Tetrahydrofolic Acid" have been combined into one series, since they have the common objective of the design of active-site-directed irreversible enzyme inhibitors. A collected list of references on "Irreversible Enzyme Inhibitors" will be sent on request.

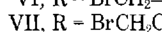
philic nucleophilic groups on the enzyme in this hydrophobic area needed for covalent bond formation in a neighboring group reaction within the enzyme–inhibitor complex.

Some related types of potential active-site-directed irreversible inhibitors were synthesized and evaluated—namely, the 1-phenyl dihydro-*s*-triazine derivatives, IV and V (12). These compounds were excellent reversible inhibitors but failed to show irreversible inhibition since the phenyl moiety is complexed in a hydrophobic region (3). Similarly, compounds V–VIII were good reversible inhibitors, but failed to show irreversible inhibition (13).



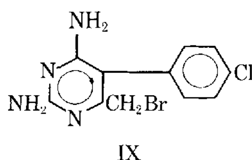
IV, $n = 0, 2$ V



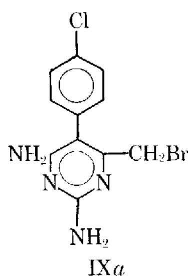


VI, R = $BrCH_2$–
VII, R = $BrCH_2CONH(CH_2)_2$–
VIII, R = $BrCH_2CONH$–C_6H_4–$(CH_2)_3$–

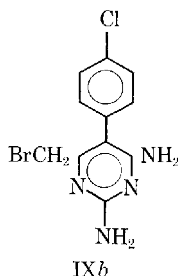
A different type of potential active-site-directed irreversible inhibitor was constructed on the proposition that there might well be an enzymic proton donor, such as imidazole, near the 8-position of folic acid when it is complexed to the enzyme; such a



IX



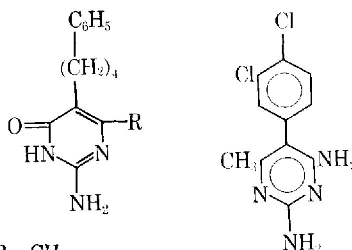
IX*a*



IX*b*

proton donor could aid in the TPNH reduction of folic acid. The pyrimethamine-type analog (IX) was synthesized and found to be an excellent reversible inhibitor, but not an irreversible inhibitor of dihydrofolic reductase (14). Again, in retrospect, it is probable that the 5-phenylpyrimidine (IX) binds its phenyl to the hydrophobic region in a "twist" conformation as depicted in IX*a*; if such were the case, then IX*a* would not have its bromomethyl in close enough proximity to the supposed enzymic proton donor. A similar conclusion could be drawn if IX complexed to dihydrofolic reductase in a "twist-flip" conformation (IX*b*).

As a result of a study on possible pyrimidine conformations when these inhibitors are complexed to



X, R = CH_3
XI, R = C_3H_7-*n*
XII, R = $-(CH_2)_4C_6H_5$
XIII, R = $-(CH_2)_4C_6H_4(NHCOCH_2Br)$-*p*
XIV, R = $-(CH_2)_2C_6H_4(NHCOCH_2Br)$-*p*

XV

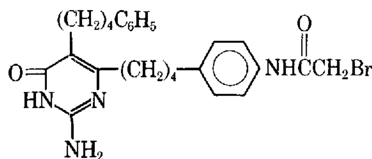
dihydrofolic reductase, it was suggested that a pyrimidine such as X would have the conformation shown (with respect to conformation I for folic acid), since the strong hydrophobic bonding could be determinant for the binding conformation (7). Such a conformation for X suggests that chain lengthening of the R group should project it into a hydrophilic region. That this might be the case was supported by the observation that the *n*-propyl group caused XI to be a thirtyfold less effective inhibitor than the 6-methylpyrimidine (X) (7); this result could be due to the repulsion of a hydrophobic group from a hydrophilic area on the enzyme or to a steric interaction unfavorable to complex formation between the enzyme and the inhibitor. That steric interaction was less likely was indicated by the increase in the size of the R group to phenylbutyl (XII) (15); XII was a fivefold better inhibitor of dihydrofolic reductase than XI, also indicating that the phenyl group on the 6-side chain was complexed to the enzyme. Therefore, the 6-bromoacetamidophenyl butyl pyrimidine (XIII) was selected as a likely candidate for an active-site-directed irreversible inhibitor of dihydrofolic reductase with the premise that the 5-phenylbutyl group would complex in the hydrophobic area, and the 6-phenylbutyl group could project into a hydrophilic area on the enzyme; the corresponding 6-phenethyl derivative (XIV) was also investigated.

RESULTS

The bromoacetamido pyrimidine (XIII) was about equal to XII when assayed (16) as a reversible inhibitor of dihydrofolic reductase; that is, XIII showed 35% inhibition at a concentration of 100 μM—the maximum solubility in 10% *N,N*-dimethylformamide—in the presence of 6 μM di-

hydrofolate. It was estimated (1) that the K_i was about 4×10^{-5} M. When XIII was incubated with dihydrofolic reductase at 37° in the absence of TPNH, inactivation occurred with a half-life of about 18 min. When XIII, iodoacetamide, and *p*-bromoacetamidophenylbutyric acid (17), all at 40 μM, were simultaneously incubated with the enzyme at 37° for 25 min., XIII gave 65% inactivation, but the other two compounds gave only 2–3% inactivation (Table I, *A*); thus, the random bimolecular inactivation of dihydrofolic reductase was ruled out (21).

TABLE I.—INACTIVATION OF DIHYDROFOLIC REDUCTASE[a] BY



XIII

Expt.	Incubation Mixture	Inactivation, %
A	40 μM XIII	65
	40 μM ICH_2CONH_2	3
	40 μM *p*-$BrCH_2CONHC_6H_4$-$(CH_2)_3COOH$	2
B	40 μM XIII	73
	40 μM XIII + 15 μM Folic acid	50
C	40 μM XIII	68
	40 μM XIII + 0.15 μM XV	32
D[b]	40 μM XIII	62
	40 μM XIII + 12 μM TPNH	23
	40 μM XIII + 60 μM TPNH	0
F	40 μM XIII	76
	10 μM XIII	43

[a] Dihydrofolic reductase from pigeon liver in 0.05 M Tris buffer (pH 7.4) plus 10% *N,N*-dimethylformamide was incubated in the absence of TPNH at 37° for 25 min. with the additions indicated; the amount of remaining enzyme was then assayed (14). In each experiment an enzyme control was run simultaneously with the two or three other solutions; only 0–4% thermal inactivation of the enzyme occurred. [b] In similar simultaneous experiments, adenosine diphosphate or DPNH at 36 μM showed no protection, whereas 36 μM TPN protected about half as well as 36 μM TPNH.

Table I summarizes experiments on the protection against this inactivation. The reversible inhibitors, folic acid (18) and 2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine (XV) (19), slowed the rate of inactivation, evidence that the active site was involved in the inactivation (20, 21). Surprisingly, TPNH also protected against inactivation; since the reversible inhibition by XIII was found to be independent of TPNH concentration, the protective effect of TPNH was not due to the complexing of the bromoacetamidophenyl moiety of XIII to the enzymic region normally complexing TPNH. Two other possible explanations are that (*a*) the complex between XIII and dihydrofolic reductase juxtaposes the bromoacetyl group to a TPNH binding point or (*b*) TPNH causes a conformational change in the enzyme which moves the enzymic nucleophilic group from juxtaposition with the bromoacetyl group in the XIII–enzyme complex. These two possibilities cannot as yet be distinguished.

It was noted that 36 μM adenosine diphosphate or DPNH showed no protection. TPN at 36 μM showed half the protection given by 36 μM TPNH at this pH of 7.4. These results exclude the destruction of the inhibitor by the phosphate mono ester group. DPNH binds poorly to dihydrofolic reductase at pH 7.4 (22); the binding of TPN is quite good at pH 5.5 (22), but its binding at pH 7.4 has not been measured. Thus, these protection experiments are best explained on the relative binding of the various phosphates to dihydrofolic reductase.

Increasing incubation time did not lead to total inactivation, but inactivation leveled off at 80–90%. This result is similar to that observed (23) with the action of *p*-nitrophenyl α-bromoacetamidoisobutyrate on chymotrypsin where 80–90% inactivation occurs; the remaining activity was shown to be due to a modified enzyme where K_m was increased, but $V_{max.}$ remained nearly the same (23, 24). It will be of interest in the future to determine if $V_{max.}$ or K_m has changed for dihydrofolate or TPNH or both after dihydrofolic reductase has been inactivated with XIII.

If the inactivation of dihydrofolic reductase proceeds through an obligatory complex between the enzyme and the inhibitor (XIII), then a "rate-saturation" effect should be observed, that is, the rate of inactivation is dependent upon the concentration of enzyme–inhibitor complex, EI (21), as shown in Eq. 1.

$$[EI] = \frac{[E_t]}{\frac{K_i}{[I]} + 1} \qquad \text{(Eq. 1)}$$

The amount of reversible complex can be calculated from Eq. 1, previously derived (21), where [EI] = concentration of complex, $[E_t]$ = total active enzyme, and [I] = inhibitor concentration. Since XIII has $K_i = 4 \times 10^{-5}$ M and the incubation is performed with 4×10^{-5} M of XIII, then $[EI] = 0.5\ [E_t]$. If $[I] = 1 \times 10^{-5}$ M, as in experiment *F* (Table I), then $[EI] = 0.2\ [E_t]$. Note that XIII at 4×10^{-5} M gave 69% inactivation in 25 min., but that XIII at 1×10^{-5} M gave 43% inactivation. It can be calculated that 1×10^{-5} M of XIII would have given 23% inactivation in 25 min. if the reaction were proceeding by a bimolecular reaction, but 40% if inactivation occurred through the EI complex as in Eq. 1. Thus, the observed 43% at 25 min. with 1×10^{-5} M represents a rate-saturation effect as a first approximation.

The 5-phenylbutyl-4-pyrimidinol (XIV) with the shorter phenethyl side chain containing a *p*-bromoacetamido group was also found to be an active-site-directed irreversible inhibitor of dihydrofolic reductase. XIV was a reversible inhibitor with $K_i = 3 \times 10^{-5}$ M; when incubated with dihydrofolic reductase at 37°, inactivation occurred with a half-life of about 12 min. With extended time the enzyme became 80–90% inactivated, but did not inactivate completely, as also noted with XIII. The phenethyl pyrimidine (XIV) showed the same protection pattern as observed with XIII in Table I.

Whether XIII and XIV attack the same amino acid on dihydrofolic reductase must await inactivation experiments with the pure enzyme; that the same amino acid would be attacked is unlikely, due

to the conformational changes that either XIII or the enzyme would have to undergo. Such conformational changes on XIV would most likely be energetically unfavorable, but the energetics of such a conformational change in the enzyme are unknown.

DISCUSSION

An active-site-directed irreversible enzyme inhibitor theoretically operates by first forming a complex with the enzyme, then the complex undergoes a facile neighboring group reaction between an enzymic nucleophilic group and a leaving group on the inhibitor (9, 25, 26). There are two types of these irreversible inhibitors: (*a*) those that operated by covalent bond formation inside the active-site—the so-called endo-mechanism—and (*b*) those that operate by covalent bond formation outside of the active-site—the so-called exo-mechanism. Examples of both types are known (9, 25, 26).

The endo-type of irreversible inhibitor is preferred by protein structure chemists since they wish to "label" amino acids in the active-site by attachment of a covalently linked moiety that can be identified; the specific "labeled" amino acid is determined by total hydrolysis of the protein and its position in the amino acid sequence by partial hydrolysis (26). It has been anticipated (26) that the endo-type would be much less effective for chemotherapy than the exo-type since the active site of an enzyme can vary only little from species to species and still be operational. In contrast, the exo-type could be expected to be far more species and tissue specific since covalent bond formation is taking place outside the active site where numerous changes can be made without disrupting the protein's function (26, 27).

At the time our program on active-site-directed irreversible inhibitors for dihydrofolic reductase was initiated in 1961, the factors in the design of irreversible inhibitors for enzymes was just beginning to emerge (21, 25, 28); since then the field has developed considerably in both concept and practice (9, 26). Therefore, at the start we set goals toward both the endo-type and exo-type of irreversible inhibitors (25) for dihydrofolic reductase. It soon became apparent that the active-transport system for cell wall penetration by folic acid and its derivatives (10, 29) was sensitive to the wrong positioning of bulky groups. It was noted that the folic acid analog (XVIII) (30), was cytoxic to S-180 cells in culture, but the 6-phenylpyrimidine analog of folic acid (XVI) (31) was inert; however, when the carboxy-L-glutamate moiety of XVI was not present, as in XVII (31), XVII was cytoxic.[1]

OH
NH_2 N N $(CH_2)_3NH$ —R

XVI, R = $-CONHCH(CH_2CH_2COOH)COOH$

XVII, R = H

SH
NH_2 N N CH_3 $(CH_2)_3NH$ $CONHCH(CH_2)_2COOH$ COOH

XVIII

The carboxy-L-glutamate moiety has been implicated as being essential for active transport of folic acid and its close analogs (10), but less related dihydrofolic reductase inhibitors such as pyrimethamine (XIX) entered cells by passive diffusion (10, 29). It then follows that perhaps XVI was inert because the 6-phenyl group was too bulky to be

NH_2
NH_2 N N C_2H_5 Cl

XIX

accommodated by the active transport system for folic acid, but, in addition, the ionized carboxy-L-glutamate aborted any possible passive diffusion. For this reason all of our subsequent work avoided the presence of the carboxy-L-glutamate moiety so that the inhibitors would be able to penetrate the cell membrane by passive diffusion. Furthermore, certain 2,4-diamino heterocycles without the carboxy-L-glutamate moiety can complex to dihydrofolic reductase even better than folic acid (I) (16, 19, 26, 29), and such molecules without carboxy-L-glutamate moiety are considerably easier to synthesize and purify.

The removal of the carboxy-L-glutamate moiety led to the discovery of the useful hydrophobic bonding area on dihydrofolic reductase (1) with its species differences in conformational tolerances (4, 8, 29). Although the presence of the hydrophobic region on dihydrofolic reductase aborted the success of more than 30 candidate active-site-directed irreversible inhibitors, knowledge now developed on the hydrophobic region (1–8) should be usable for the design of species- or tissue-specific irreversible inhibitors of this enzyme.

Note that the irreversible inhibition of dihydrofolic reductase by XIII (Table I) is slowed by the presence of TPNH. If XIII is alkylating a point on the enzyme that normally complexes TPNH, then by definition XIII is operating by the endo-mechanism; if XIII is alkylating some other point not essential for enzyme activity, but TPNH causes a conformational change in the enzyme which removes this attackable function on the enzyme from juxtaposition with the alkylating function of XIII, then the exo-mechanism is operating. In either case the hydrophobic region can be used to introduce a new parameter for tissue- or species-specificity.

The maximum specificity should be observed if an alkylating function on the inhibitor can be bridged back from the hydrophobic region on the enzyme to the more polar nucleophilic region. In this way the ability of an irreversible inhibitor to bridge between the pyrimidine locus in the active-site and the enzymic nucleophilic site can be controlled by the nature of the hydrophobic site, which is outside the active site and where the greatest

[1] The authors thank Dr. M. Hakala for these assays.

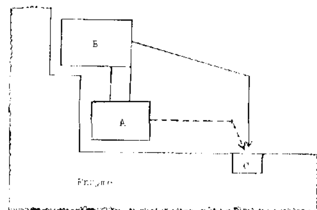


Fig. 1.—Schematic representation of an active-site-directed irreversible inhibitor that utilizes hydrophobic bonding for specificity. Key: A, the pyrimidine of an inhibitor; B, the hydrophobic group of an inhibitor; C, an enzymic nucleophilic group. The solid arrow represents a covalent-forming group on one inhibitor that can bridge properly from the hydrophobic bonding region to a nucleophilic site on the enzyme; the dotted arrow represents a covalent-forming group on a second inhibitor that can bridge from the pyrimidine region to a nucleophilic site on the enzyme (see *Discussion*).

evolutionary differences can occur (26, 27) (Fig. 1). It should also be possible to obtain specificity by varying the group that complexes in the hydrophobic region; this could alter the position of the alkylating function (dotted arrow, Fig. 1) by positioning the pyrimidine slightly differently; this can be likened to a fulcrum where a slight shift on the hydrophobic side will shift the alkylating side in the opposite direction where a part of the pyrimidine is the axis of the fulcrum.

Although studies of this type for tissue specificity could be pursued with the 2-amino-4-pyrimidinol type of inhibitors such as XIII, an additional important problem should be solved before such studies are initiated. Since XIII and XIV have K_i's near 3×10^{-5} M, it is unlikely that these inhibitors would be effective below 3×10^{-6} M since the amount of intracellular enzyme in the enzyme-inhibitor complex would only be about 10% of the total enzyme. There is a definite practical limitation on the intracellular concentration of inhibitor that can be obtained in a whole animal system, which is in turn partially dependent upon dosage. A reasonable maximum limit for intracellular inhibitor concentration is 10^{-6} M; the more dilute the inhibitor can be and still form 50% reversible complex with the available enzyme, the more potent the inhibitor will be irreversibly (32) and the less dosage will have to be used. 2,4-Diaminopyrimidines are 300–1000-fold more potent reversible inhibitors than the corresponding 2-amino-4-pyrimidinols (1, 16). For example, pyrimethamine (XIX) has $K_i = 7 \times 10^{-9}$ M with the folic reductase from rat liver (10); therefore, an active-site-directed irreversible inhibitor of dihydrofolic reductase that has a K_i approaching the 10^{-8} M of pyrimethamine (XIX) would at 10^{-8} M complex half the enzyme. Since the inactivation rate is dependent upon the concentration of reversible enzyme-inhibitor complex, such a hypothetical irreversible inhibitor should be effective at 10^{-8} M or less; such a concentration is readily achieved within a cell by passive diffusion.

The solution to such a problem may not be simply the conversion of XIII or XIV[2] to the corresponding 2,4-diamino pyrimidines since there seems to be some conformational differences in the manner in which 2,4-diaminopyrimidines and 2-amino-4-pyrimidinols complex to dihydrofolic reductase (7). The vigorous pursuit of the 2,4-diaminopyrimidine and 4,6-diamino-1,2-dihydro-*s*-triazine types of active-site-directed irreversible inhibitors for dihydrofolic reductase is continuing in this laboratory.

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[2] The synthesis of XIII and XIV have been described previously (15, 17, 33).