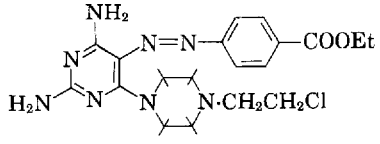
2,4-Diamino-6-substituted-5-(4-carbethoxyphenylazo)pyrimidines as Irreversible Inhibitors of Folic Acid Reductase

Sir:

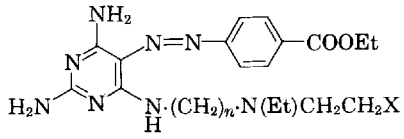
The theory of competitive, or active-site-directed, irreversible inhibition of enzymes has been discussed by Baker (1) and others (2–5). To achieve this type of inhibition, the inhibitor must form an initially reversible enzyme–inhibitor complex and, while in this form, be able to form a covalent bond between reactive groups located on the enzyme and on the inhibitor to yield a covalently bound enzyme–inhibitor complex.

Previous studies (6–9) have shown that 5-arylazopyrimidines possess antifolic properties. Evidence has been presented which suggests that a series of 2,4-diamino-6-substituted-5-arylazopyrimidines are competitive antagonists of rat liver folic acid reductase (10). Thus, it seemed possible that irreversible antagonists might be designed by the incorporation of appropriately located alkylating groups into the 5-arylazopyrimidine molecule.

Some irreversible inhibition was noted (10) with *N*-2-chloroethyl-*N'*-[2,4-diamino-5(4-carbethoxyphenylazo)-6-pyrimidyl]piperazine (I), but this was not particularly marked since lengthy preincubation (8 hr.) of the inhibitor and enzyme was necessary to produce a significant level of irreversible inhibition (10). It seemed probable that the ineffectiveness of I was due to the conformational rigidity of the alkylating side chain which would preclude, save in the most favorable circumstances, effective interaction between the alkylating group and a corresponding nucleophilic site on the enzyme. Accordingly, we have prepared (Table I) a series of 2,4-diamino-5-(4-carbethoxyphenylazo)pyrimidines (II) bearing alkylating 6-substituents of varying chain length and with increased flexibility relative to I. (The synthesis of these compounds and a more detailed analysis of their behavior will be published together in a later paper.)



I



II

The alkylating inhibitors (VIII–XII, Table I) produced irreversible inactivation at rates which were dependent on the length of the 6-alkyl chain despite the similar chemical reactivities of the alkylating groups (11). In order to exclude the possibility that these compounds inactivate folic reductase by random alkylation processes, we have studied the activity of *N*-(2-chloroethyl)-*N*-ethyl-*n*-butylamine (XIII), a compound that represents the alkylating moiety of X. When incubated with folic reductase at the same concentration as X, XIII required 90 min. to produce 50% inactivation and is clearly much less efficient than X which required only 6 min. to produce the same degree of inactivation. Furthermore, incubation of the enzyme with XIII and 2,4,6-triamino-5-phenylazopyrimidine [a competitive reversible antagonist, $K_I = 1.4 \times 10^{-8} M$, (10)] did not alter the time for 50% inactivation, suggesting (*a*) that any conformational change induced by the enzyme–inhibitor interaction does not expose a nucleophilic group that can be alkylated by XIII and (*b*) that XIII produces enzyme inactivation by a nonspecific process.

The data in Table I show that the alkylating

TABLE I.—INHIBITION OF FOLIC ACID REDUCTASE[a] BY 2,4-DIAMINO-6-SUBSTITUTED-5-(4-CARBETHOXYPHENYLAZO)PYRIMIDINES (II)

Compd.	*n*	X	$[I/S]_{50}$[b]	Time for 50% Inactivation, min.[c]
III	2	OH	2.9	>200
IV	3	OH	5.0	>200
V	4	OH	5.6	>200
VI	5	OH	4.8	>200
VII	6	OH	3.6	>200
VIII	2	Cl	2.0	50
IX	3	Cl	3.0	12
X	4	Cl	2.0	6
XI	5	Cl	2.75	10.5
XII	6	Cl	1.9	10
XIII	$CH_3(CH_2)_3N(Et)CH_2CH_2Cl$			90

[a] Folic acid reductase activity was determined at 37° in the presence of 20 μM NADPH, 10 μM sodium citrate, 10 μM $MgCl_2$, 100 μM dimethyl glutarate buffer (pH 6.1), 80 μM folic acid, rat liver homogenate supernatant and inhibitor in water to a final volume of 0.5 ml. (12). [b] $[I/S]_{50}$ is the ratio of the concentrations of inhibitor and substrate required to produce 50% inhibition of enzyme activity. Since $K_I/K_M \approx [I/S]_{50}$ these figures provide an approximate guide to the relative affinities of substrate and inhibitor. Because of the possibility of irreversible inactivation by VIII–XII during the determination of enzyme activity, the $[I/S]_{50}$ values for these compounds are only approximate. [c] Time for 50% inactivation of the enzyme in the absence of inhibitor was 240 min. Inactivation studies were done with an inhibitor concentration of $2 \times 10^{-4} M$.

and nonalkylating inhibitors bind less efficiently to the enzyme than folic acid ($K_M = 6.2 \times 10^{-6}$ M). Variation of the chain length of the 6-substituent has a relatively small effect on the $[I/S]_{50}$ ratios for both the alcohols (III–VII, $[I/S]_{50}$ range, 2.9–5.6) and the chloro compounds (VIII–XII, $[I/S]_{50}$ range, 1.9–3.0). However, incubation of the enzyme with the inhibitor before addition of the substrate resulted in varying degrees of irreversible inactivation of the enzyme. Enzyme inactivation in the presence of the reversible inhibitors (III–VII) was identical to that occurring in the absence of these inhibitors.

The data presented in this communication seem to establish the existence of a nucleophilic site on the enzyme folic acid reductase that can be specifically alkylated by appropriately designed antagonists. Further work is in progress, using analogs of these antagonists, that may further establish the position of this nucleophilic site relative to other binding sites of folic acid reductase.

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Books

REVIEWS

Accepted Dental Remedies 1966. Council on Dental Therapeutics. American Dental Association, 222 E. Superior St., Chicago, Ill. 60611, 1965. xvi + 275 pp. 15 × 22.5 cm. Price $3.00. Paperbound.

The 1966 edition is the thirty-first edition of this well-known handbook of dental therapeutics. Revision once again has resulted in changes in the organization of the material and division into five sections; the previous edition had three. Of the five sections, one represents new material—Therapeutic Guides. The indexes have been incorporated into the fifth section, and the first three—General Principles of Medication, Therapeutic Agents, and Therapeutic Aids—remain the same.

Therapeutic Guides includes a chapter in which conditions such as bleeding, hairy tongue, moniliasis, leukoplakia, etc., are discussed and suggestions for treatment given. Another new chapter is Current Therapeutic Trends in which brief monographs on recently marketed drugs are presented. These drugs are widely advertised, and although some may eventually be accepted by the Council, others are included especially to emphasize the hazards associated with their use.

The discussion on reporting drug reactions has been expanded. The Council on Therapeutics is now cooperating with the FDA in its program on adverse reactions and to further aid the practitioner, brief adverse reaction report forms have been included inside the back cover of the book.

Basic Biochemistry. By M. E. Rafelson, Jr., and S. B. Binkley. The Macmillan Co., 60 Fifth Ave., New York 11, N. Y., 1965. xi + 350 pp. 15.5 × 24 cm. Price $8.50 hardbound; $6.50 paperbound.

This brief textbook provides a basic outline of the principles of biochemistry. The material is presented in a clear and readable style. Discussion is limited but this fact is acknowledged by the authors who suggest more complete reference texts which can be consulted for supplementation of this material. The first few chapters are devoted to descriptive chemistry of acids, bases, and buffers; carbohydrates; lipids; proteins; nucleic acid and nucleoproteins; enzymes; and high energy compounds and oxidative phosphorylation. The remaining chapters are devoted to the metabolic pathways which utilize these compounds. The text