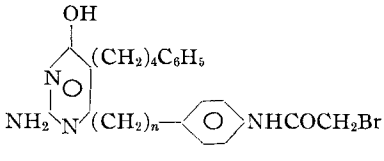
Irreversible Enzyme Inhibitors LXXXVIII. Differential Irreversible Inhibition of Dihydrofolic Reductase from Different Species

Sir:

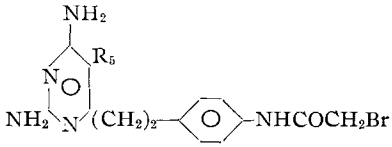
A variety of 2,4-diaminoheterocycles, known to be reversible inhibitors of dihydrofolic reductase, are sufficiently tissue- or species-specific to be useful chemotherapeutic agents (1, 2). Examples are amethopterin for leukemia (3), 5-(*p*-chlorophenyl)-2,4-diamino-6-ethylpyrimidine (4), and 1-(*p*-chlorophenyl)-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine (5) as antimalarials; 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine as an antibacterial (6); and 1-(*p*-chlorophenyl)-4,6-diamino-1,2-dihydro-2,2-(2′-methyltetramethylene)-*s*-triazine as an anthelmintic (7, 8). The selectivity of amethopterin on certain tissues can be attributed to a difference in the efficiency of the folic acid active-transport system (9). The selectivity of the remaining compounds is most probably due to differences in binding by the aryl groups to the hydrophobic bonding region of dihydrofolic reductase (2, 10–12); these compounds appear to enter cells by passive diffusion (13, 14).

Although numerous 2,4-diaminoheterocycles of the type that enter cells by passive diffusion have been screened in a variety of tumors by the CCNSC, none have shown sufficient selectivity to warrant medical use; thus, to date, differences in the hydrophobic bonding region of dihydrofolic reductases from normal and tumor tissue have not been sufficiently great to be exploitable. Since with active-site-directed irreversible inhibitors an extra dimension of specificity can be obtained which is not present with reversible inhibitors (15–17), such irreversible inhibitors might exhibit more selectivity than reversible inhibitors. That selective irreversible inhibition can be obtained with inhibitors of the dihydrofolic reductases from different sources is the subject of this paper.

Compounds I–IV were evaluated as reversible and irreversible inhibitors of the dihydrofolic reductases from pigeon liver and *E. coli* B; the results are presented in Table I. The 2-amino-4-pyrimidinols (I and II) inactivated both enzymes at rates not differing more than a factor of 2 when compared at equal concentrations of reversible enzyme-inhibitor complex—the rate-determining species (16, 18). In contrast, the 2,4-diaminopyrimidine (III) analog of the 2-amino-4-pyrimidinol (I) showed no inactivation of either enzyme.



I, $n = 2$
II, $n = 4$



III, R = $-(CH_2)_4C_6H_5$
IV, R = $-C_6H_4Cl$-*p*

Although the 2,4-diamino-5-*p*-chlorophenylpyrimidine derivative (IV) was equally effective as a reversible inhibitor of both enzymes, irreversible inhibition showed dramatic selectivity; only the *E. coli* B enzyme was inactivated.

Also noteworthy is the comparative irreversible inhibition of the *E. coli* B dihydrofolic reductase with III and IV; the structural difference resides only in the hydrophobic bonding group at the 5-position, yet IV inactivated the enzyme, but III did not. Apparently the nature of binding of the hydrophobic bonding group (R_5) to the enzyme can change the position of the terminal leaving group, thus effecting the juxtaposition of the leaving group to the enzymic nucleophilic group—as previously predicted (12, 19).

The search for specificity with irreversible inhibitors between dihydrofolic reductases from selected animal tumors and a normal tissue, such as liver, from the same animal is currently being pursued.

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TABLE I—INHIBITION OF DIHYDROFOLIC REDUCTASES FROM PIGEON LIVER AND *E. coli* B

No.	Reversible Inhibition, K_i (M)[a] Pigeon Liver	Reversible Inhibition, K_i (M)[a] *E. coli* B	Irreversible Inhibition[b] Pigeon Liver μM Concn. Inhib.	Pigeon Liver μM Concn. TPNH	Pigeon Liver % $E\cdots I$	Pigeon Liver Time, min.	Pigeon Liver % Inactivation	*E. coli* B μM Concn. Inhib.	*E. coli* B μM Concn. TPNH	*E. coli* B % $E\cdots I$	*E. coli* B Time, min.	*E. coli* B % Inactivation
I[c]	3×10^{-5}[d]	1×10^{-3}	40	0	57	12	50[d]	100	0	10	120	59
II[c]	4×10^{-5}[d]	5×10^{-4}	40	0	50	12	50[d]	50	0	10	120	22
III	7×10^{-9}[e]	1×10^{-7}	0.01	12[f]	59	60	0[e]	0.10	30[f]	50	120	0
IV	1×10^{-7}[e]	1×10^{-7}	0.25	12[f]	72	60	0[e]	0.10	30[f]	50	120	33

The technical assistance of Barbara Baine with these assays is acknowledged. [a] K_i was estimated (18) from the concentration of inhibitor necessary to give 50% inhibition in the presence of 6 μM dihydrofolate as previously described (10); K_m = 2.5×10^{-5} M was used for *E. coli* B (20) and 1×10^{-6} M for pigeon liver (18). [b] Inactivation at 37° and pH 7.4 was measured as previously described (21); for the assay of remaining *E. coli* enzyme, 60 μM each of dihydrofolate and TPNH were used to make the rate of reaction dependent on enzyme concentration. [c] All assays contained 10% *N,N*-dimethylformamide to aid solubility. [d] Data from *Reference 19*. [e] Data from *Reference 22*. [f] TPNH is required for optimum reversible complexing of 2,4-diaminoheterocycles, but not for 2-amino-4-hydroxy heterocycles; see Perkins, J. P., and Bertino, J. R., *Biochemistry*, **5**, 1005(1966).

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Revised Structure of Columbianin

Sir:

Columbianin is a naturally-occurring glycosidic coumarin isolated from *Lomatium columbianum* Math. and Const. (1) and from *L. dissectum* var. *multifidum* (Nutt.) Math. and Const. (2). Willette and Soine (1) suggested structure I for columbianin on the basis that, on acid hydrolysis, it yielded D-glucose and a tertiary alcohol (*i.e.*, columbianetin). The structure of the aglycone (II) was firmly established by spectral studies as well as by chemical degradative evidence and has since then been confirmed (3) and extended (4) by Nielsen and Lemmich to show that the absolute configuration is 8(*S*). Since no quantitative estimation of the D-glucose content was made in the earlier study and because the analytical data (*i.e.*, assuming water of crystallization) corresponded within limits for I and its tetraacetate, the structure assignment was considered adequate.

Recently, in connection with another study, we had occasion to examine the nuclear magnetic resonance (NMR) spectrum of acetylated columbianin and noted that the *O*-acetyl methyls were to be observed as four peaks at τ 7.92, 8.00, 8.04, and 8.07; whereas the *gem*-dimethyls of columbianetin were found as a singlet at τ 8.69. To our surprise, the peak ratio between the *O*-acetyl methyls and the *gem*-dimethyls was found to be 3.52:1 instead of the expected 2:1. This led us to re-examine the structural assignment for columbianin by means of additional analytical data as well as by further degradative studies.

An ebullioscopic determination on the acetate suggested the molecular weight to be about 800 and an acetyl analysis indicated 34.92%, both values being incompatible with the tetraacetate formulation. On the other hand, the results pointed strongly toward a disaccharide moiety in columbianin and a heptaacetate as the acetylated form. Furthermore, additional elemental analyses on the acetate derivative gave much closer correspondence with the values expected for $C_{40}H_{48}O_{21}$ than for the previously suggested $C_{28}H_{32}O_{13} \cdot 2H_2O$. The question of the presence of sugars other than D-glucose had been ruled out in the earlier work (1).

Partial hydrolysis of the glycoside was carried out using an ion exchange resin[1] in the acid cycle (5). Examination of the hydrolysate by paper chromatography was carried out using an ethyl acetate–pyridine–water mobile phase and developing the chromatogram by using the silver nitrate technique (6) for visualization of the

[1] Marketed as Amberlite IR-120 by Rohm and Haas Co., Philadelphia, Pa.