value[1] of $1{\cdot}3 \times 10^{-2}$ M. The value for V_{max} was 11·1. K_3, the dissociation constant of the enzyme–inhibitor complex was $1{\cdot}33 \times 10^{-4}$ M. K_4, the dissociation constant of the enzyme–substrate–inhibitor complex was $2{\cdot}54 \times 10^{-5}$ M.

From the data presented, it is concluded that BCME is an inhibitor, albeit an incomplete one, for Tween hydrolase in rat adipose tissue. It appears that the enzyme is inhibited *in vivo*, that a dose–response relationship exists, and that the concentration of inhibitor required to inhibit the enzyme *in vitro* is comparable to that required to induce comparable degrees of inhibition *in vivo*.

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Biochemical Pharmacology, 1966, Vol. 15, pp. 1621–1623. Pergamon Press Ltd., Printed in Great Britain.

Comparative growth-inhibitory activivity of homofolic acid against cell lines sensitive and resistant to amethopterin

(*Received* 15 *March* 1966; *accepted* 13 *June* 1966)

CERTAIN sublines of neoplastic cells selected for resistance to amethopterin *in vitro*[1, 2] or *in vivo*[3] contain increased amounts of folate reductase (dihydrofolate reductase, tetrahydrofolate dehydrogenase). On the basis of this evidence, attention has been directed to the possible exploitation of this metabolic difference for the selective eradication of such amethopterin-resistant cells.[3] Antimetabolites which are substrates of folate reductase, such as N^{10}-methylfolic acid[4] and homofolic acid,[5] are converted to the corresponding analogs of tetrahydrofolate. Thymidylate synthetase is inhibited by tetrahydrohomofolate[5] and by N^{10}-methyltetrahydrofolate.[6] The increase in folate reductase content of amethopterin-resistant sublines of sarcoma-180 cells *in vitro*[1] or in murine leukemia L-1210 cells *in vivo*[7] was not accompanied by any significant change in the kinetic characteristics of this enzyme. Thus, more rapid formation of the tetrahydrofolate analogs would be expected to occur in cells containing larger amounts of folate reductase. The purpose of this study was to compare the growth-inhibitory activity of homofolic acid in cultures of S-180 cells which differed by 200-fold in their content of folate reductase.

EXPERIMENTAL

The development and characteristics of the sublines of sarcoma-180 cells sensitive (AH/S) and resistant to amethopterin (AT/174 and AT/3000) have been described.[1, 8] Flasks (T-15) were inoculated with 200,000 cells in 2 ml of the corresponding maintenance medium.[8] After incubation overnight at 36°, the medium in each flask was replaced by the appropriate experimental medium in which the growth of the cells depends upon folic acid (no thymidine or purines supplied), and which was supplemented with varied concentrations of homofolate. The media were changed thereafter three times so that the total period of growth in the presence of the analog was 7 days. The total protein content was determined according to the method described by Oyama and Eagle.[9] The control cultures grew 12- to 15-fold in 7 days as measured by increase in protein content over the inoculum.

RESULTS AND DISCUSSION

Homofolate[10] differs from folic acid by the presence of a methylene group between C^9 and N^{10} of pteroylglutamic acid. This structural modification does not prevent the enzymatic reduction of the pteridine ring, but does increase the distance between the pteridine ring and the nitrogen atom

corresponding to the N^{10}-position of tetrahydrofolate which is involved in the carbon-transfer functions of the different cofactors. Thus, homofolate is reduced by folate reductase of *Escherichia coli*, and the tetrahydro product of this reaction was found to be an effective inhibitor of thymidylate synthetase.[5] Homofolate is also reduced by the folate reductase of S-180 cells, although at a rate about one fifth of that of folate at pH 7·0; this difference, however, is small in relation to the relative amounts of this enzyme in the cell lines compared in this study. The N^{10}-position of some other folate analogs is blocked by replacement of the hydrogen atom by a methyl group, as in N^{10}-methylfolate and C^9-N^{10}-dimethylfolate. These compounds are also substrates for folate reductase,[4] and their tetrahydro analogs are inhibitors of thymidylate synthetase.[6]

Although N^{10}-methylfolate inhibits the growth of certain bacteria,[11] it was not an effective inhibitor of either the sensitive or resistant S-180 cell lines *in vitro* at concentrations up to 1×10^{-4} M. Homofolate also inhibits the growth of certain bacteria but is much less potent than tetrahydrohomofolate.[5] It is not known whether the growth-inhibitory effect of these antimetabolites is related to competition with folate for cellular uptake or to inhibition of thymidylate synthetase.

One subline of S-180 cells highly resistant to amethopterin (AT/3000) and one subline with an intermediate level of resistance (AT/174) were compared with sensitive cells (AH/S). The amounts of amethopterin in the medium which inhibited growth by 50 per cent and the amounts of folate reductase in the cells are shown in Table 1. The folate reductase content in the extracts of these cells had been determined by titration with amethopterin, as described in other studies.[1, 8] The presence of 40- or 200-fold more folate reductase in the two resistant cell lines was not associated with any marked increase in their sensitivity to homofolic acid (Table 1).

TABLE 1. GROWTH-INHIBITORY ACTIVITY OF HOMOFOLATE AGAINST AMETHOPTERIN-SENSITIVE AND -RESISTANT CULTURES OF SARCOMA 180

Sarcoma 180 cell line	50% Inhibition by amethopterin (μM)	Folate reductase content (μmoles/kg cells)	Homofolate causing 50% inhibition (μM)	Homofolate causing 75% inhibition (μM)
Sensitive, AH/S	0·076 ± 0·032	0·6	7·0	15·0
Resistant, AT/174	9·4 ± 1·1	25·8 ± 0·9	6·6	9·4
Resistant, AT/3000	160	121 ± 11·5	5·1	7·3

In general, cultures of cells can be exposed to relatively constant concentrations of analogs for prolonged periods. In contrast, cells *in vivo* are exposed to blood and tissue concentrations of the antimetabolite that can vary over a wide range during the growth cycle. Since the Michaelis constant and turnover number of folate reductase were similar in the sensitive and resistant S-180 cells,[1] the lack of any direct relationship between the folate reductase content and the potency of homofolate as an inhibitor of these cell lines indicate that the rate of reduction of homofolate is not the factor that limits the inhibitory potency of this compound. The limiting factor might be the rate of cellular permeability. It is known that amethopterin enters cultured S-180 cells very slowly and that the rate of permeability and the requirement for folate are not altered in the cell lines with high levels of folate reductase.[1, 12] If the rate of entry of homofolate is also restricted, then the amount of folate reductase even in the sensitive cell line may be sufficient for the reduction of all intracellular homofolate to tetrahydrohomofolate.

The amount of homofolate available was insufficient for more detailed studies. The evidence presented in this preliminary study demonstrates that the growth-inhibitory activity of homofolic acid was approximately the same in cultures of sarcoma 180 cells which differed greatly (200-fold) in their content of folate reductase and their degree of resistance to amethopterin.

Acknowledgement—We wish to express our thanks to Dr. Roy Kisliuk and Dr. Morris Friedkin for the sample of homofolate used in this study. This work was supported in part by a research grant (CA-04175) from the U. S. Public Health Service.

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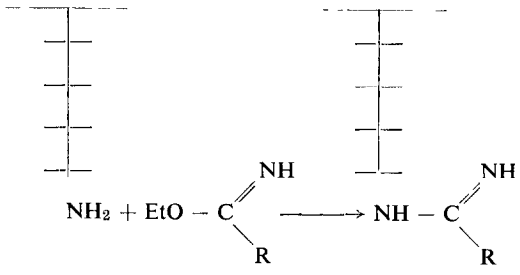
Biochemical Pharmacology, 1966, Vol. 15, pp. 1623–1625. Pergamon Press Ltd., Printed in Great Britain.

Retention of enzymatic activity by egg white lysozyme bearing alkylamidino substituents

(*Received* 8 *June* 1966; *accepted* 14 *June* 1966)

FAVOURABLE modification of biological activity sometimes follows the incorporation of alkyl chains into drug molecules. The principle has been applied to lysozyme (E.C. 3.2.1. 17; N-acetylmuramide glycanohydrolase) while seeking either to modify its limited antibacterial spectrum by imparting a degree of lipid affinity, or conferring a degree of resistance to peptic hydrolysis by steric hindrance. Most chemical reagents attack the free amino groups and cause inactivation, probably by reducing the basic character of the enzyme.[1] Converting the lysine residues to arginine residues, however, leaves activity unimpaired.[2] By reaction with imino esters, the epsilon amino groups of lysine residues in peptides may be converted to alkylamidino groups[3] permitting retention of basic character while introducing alkyl chains (I; R = alkyl).



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EXPERIMENTAL

Amidination of lysozyme

The appropriate nitriles were converted to imino ester hydrochlorides, by the Pinner method and reacted with commercial crystallized egg white lysozyme as described by Wofsy and Singer,[4] using their conditions for both partial and exhaustive amidination. The reaction mixtures were dialysed against water, aliphatic esters removed by filtration and the product freeze-dried. The extent of amidination was determined by condensation of residual free amino groups with fluoro-dinitrobenzene, hydrolysis, and estimation of dinitro-phenyl-lysines as described by Levy.[5] The N-terminal