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5-NITRO-2'-DEOXYURIDINE 5'-MONOPHOSPHATE IS A POTENT IRREVERSIBLE INHIBITOR OF LACTOBACILLUS CAESI THYMIDYLATE SYNTHETASE

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SUMMARY

5-Nitro-2'-deoxyuridine 5'-monophosphate was found to be an active site-directed irreversible inhibitor of thymidylate synthetase from Lactobacillus caesi. It's K_I was determined as 2.9 x 10^{-8} M from a double-reciprocal plot of velocity vs substrate concentration.

INTRODUCTION

5-Nitro-2'-deoxyuridine ($NO_2{}^5dU$), first prepared by an enzymatic synthesis (1) and more recently by a chemical synthesis (2), shows potent in vitro antiviral activity against vaccinia virus and herpes simplex virus (1,3). Toward uninfected replicating primary rabbit kidney cells, $NO_2{}^5dU$ exhibits toxicity comparable to 5-iodo-2'-deoxyuridine (4), but $NO_2{}^5dU$ is significantly less toxic than 5-fluoro-2'-deoxyuridine (4). The nitro nucleoside, $NO_2{}^5dU$, retains its potent anti-herpes virus activity against only those viral strains that induce thymidine kinase in the host cell (3). In this respect, it behaves similarly to a number of other analogs of thymidine (5). The incorporation of [^{14}C]-deoxyuridine into the DNA of uninfected primary rabbit kidney cells is blocked by $NO_2{}^5dU$ at a much lower concentration than is the incorporation of [^{3}H]-thymidine (3); furthermore, the anti-vaccinia activity of $NO_2{}^5dU$ is reversed by extremely low concentrations of thymidine (3). These latter three observations suggested (3) that $NO_2{}^5dU$ may owe its antiviral activity

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to a blockade of thymidylate synthetase by the 5'-monophosphate of $NO_2{}^5dU$. In this study, we have explored the ability of the 5'-monophosphate of $NO_2{}^5dU$ ($NO_2{}^5dUMP$) to function as an inhibitor of the thymidylate synthetase of Lactobacillus casei.

MATERIALS AND METHODS

5'-Nitro-2'-deoxyuridine 5'-monophosphate ($NO_2{}^5dUMP$) was prepared as previously described (2).

The purified enzyme, thymidylate synthetase of Lactobacillus casei, was purchased from the New England Enzyme Center, Tufts University, Boston, Mass. The enzyme was diluted 1:100 with 0.05 M tris-acetate buffer (pH 6.8). The diluted enzyme was activated prior to use by dialysis at 5° for 4 days against 0.1 M potassium phosphate (pH 6.8) containing 50 mM 2-mercaptoethanol. The substrate, [5-^{3}H]-2'-deoxyuridine 5'-monophosphate was purchased from Moravek Biochemicals, Industry, California, and the cofactor, dl-L-tetrahydrofolic acid was the product of Sigma Chemical Co. (St. Louis, MO).

Enzyme Assay

The spectrophotometric assay for thymidylate synthetase activity was used to determine the K_m and K_I values for substrate and inhibitor. The assay conditions were those of Daron and Aull (6) with the exception that (0.05M) tris-acetate buffer at pH 6.8 was used.

For the spectrophotometric assay, 1 ml of solution contained 25 mM mercaptoethanol, 0.222 mM dl-tetrahydrofolic acid, 6.75 mM formaldehyde, 5.0 mM sodium bicarbonate, 0.1 M Tris-acetate buffer pH 6.8, 25 µl of the diluted enzyme solution and substrate and inhibitor at the stated concentrations when necessary. Control reactions lacked the substrate 2'-deoxyuridine 5'-phosphate. The velocity of the reaction at 30° was measured by the change in absorbance at 340 mm. At higher substrate concentrations, the change in absorbance with time was linear for several minutes. At low substrate concentrations the initial velocity was linear for less than a minute.

The second assay method used for preincubation studies was a modification of the radioisotope released from tritiated substrate as reported by Roberts (7). The assay concentrations are the same as those used in the spectrophotometric assay for mercaptoethanol, dl-tetrahydrofolic acid, sodium bicarbonate, Tris-acetate buffer pH 6.8 and formaldehyde. Assays were run in a total volume of 0.1 ml containing 5 µl of diluted enzyme and the stated amounts of [5-^{3}H]-dUMP (500 µCi/µmole) and inhibitor when required; control reactions lacked the cofactor.

Assays were run at 30° for 7 min. The reaction was stopped by the addition of 50 µl of 20% trichloroacetic acid and the unreacted substrate was removed by adding 0.25 ml of activated charcoal in a 20% aqueous suspension. After standing 15 min, the mixture was filtered through a cotton plugged Pasteur pipet and 0.1 ml of the filtrate was counted in a scintillation fluid containing 0.5% 2,5-diphenyloxazole and 10% Beckman BBS-3 solubilizer in toluene.

Enzyme Preincubation Studies

Preincubation of 5 µl of the enzyme with the inhibitor was done for periods up to 9 minutes at 30° in a solution containing the varying amounts of $NO_2{}^5dUMP$,

6 mM $MgCl_2$, 0.25 mM EDTA, and 12 mM tris-acetate buffer (pH 6.8) in a total of 50 µl. Substrate protection was evaluated by the addition of varying amounts of [5-^{3}H]-2'-deoxyuridine 5'-monophosphate. After preincubation, the solution was assayed for remaining enzyme activity by the addition of 10 µl of the cofactor solution described above, substrate to give 5 x 10^{-6} M [5-^{3}H]-dUMP (500 µCi/ mole), and sufficient buffer to maintain the concentration at 6 mM $MgCl_2$, 0.25 mM EDTA and 12 mM tris-acetate buffer (pH 6.8). Control reactions lacked the cofactor. The assay was conducted as described above. Under these conditions, the enzyme was found to be stable during the preincubation period.

Results

The inhibition of L. casei thymidylate synthetase by $NO_2{}^5$dUMP was monitored by measuring dihydrofolic acid formation. The results were analyzed by the double-reciprocal plot of velocity vs substrate concentration (Figure 1). The calculated K_m for substrate is 1.5 x 10^{-6}M. Dunlap et al., (8), Crusberg et al., (9) and Daron and Aull (6), using the same assay procedure, reported K_m's ranging from 5.2 to 0.7 µM. In Figure 1 the results show competitive inhibition and the K_I calculates to a value of 2.9 x 10^{-8}M for inhibition by $NO_2{}^5$dUMP.

The data of Table 1 on the results of preincubation of the enzyme and inhibitor show that $NO_2{}^5$dUMP inactivates L. casei thymidylate synthetase and that the inactivation is dependent on the concentration of $NO_2{}^5$dUMP. In addition, these data suggest that $NO_2{}^5$dUMP may inactivate the enzyme through reaction at the active site since the natural substrate, dUMP, can partially prevent the inactivation.

Discussion

The results suggest that $NO_2{}^5$dUMP is a potent active site directed irreversible inhibitor of thymidylate synthetase.

An estimate of the rate of inactivation was determined by preincubation of the enzyme with the inhibitor for nine minutes. As shown in Table 1, at a concentration of 1.4 x 10^{-7}M $NO_2{}^5$dUMP 17% of the original activity remained; at this concentration of inhibitor the half life is 3.4 minutes. Comparable results are observed for the lower concentrations of inhibitor. If this irreversible inhibition is active site directed and is dependent on formation of the reversible E·I complex, the presence of substrate should provide protection against inactivation and this protection should be concentration dependent. This was con-

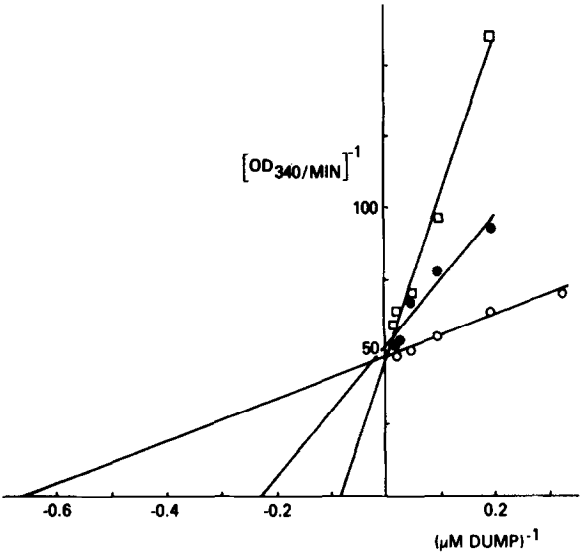

Figure 1. Double reciprocal plot of velocity in change in absorbance per minute vs substrate concentration with varying amounts of the inhibitor, 5-nitro-2'-deoxyuridine 5'-phosphate (0 = no inhibitor; ● = 0.07 μM; ■ = 0.14 μM).

Table 1

Thymidylate Synthetase Inactivation by 5-Nitro-2'-deoxyuridine 5'-phosphate and Substrate Protection[a]

5-Nitro-DUMP (M)	DUMP (M)	% Remaining Activity
1.4×10^{-7}	——	17
1.4×10^{-7}	6×10^{-7}	28
1.4×10^{-7}	10×10^{-7}	33
0.84×10^{-7}	——	43
0.84×10^{-7}	6×10^{-7}	73
0.84×10^{-7}	10×10^{-7}	79
0.28×10^{-7}	——	83
0.28×10^{-7}	6×10^{-7}	95
0.28×10^{-7}	10×10^{-7}	96

[a]The enzyme was preincubated for nine min at 30° and subsequently assayed for remaining activity as described in the text.

firmed by the slower rate of inactivation in the presence of substrate. At the higher inhibitor concentration using substrate levels 4 and 7 times that of inhibitor, the half life for inactivation was 4.9 min and 5.7 min.

When the K_I values calculated for $NO_2{}^5$dUMP, 5-fluoro- and 5-trifluoromethyl-2'-deoxyuridylates are compared, $NO_2{}^5$dUMP emerges as an equally powerful

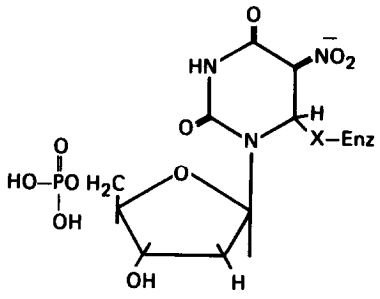

Figure 2. A hypothetical mode of inactivation of thymidylate synthetase by $NO_2{}^5$dUMP.

inhibitor of thymidylate synthetase intermediate between the fluoro- or trifluoromethyl analogs. From this study, the K_I for $NO_2{}^5$dUMP is 2.9×10^{-8}M. From the data of Wataya et al., (11) the K_I for 5-fluoro- and 5-trifluoromethyl-2'-deoxyuridylates are: 1.4×10^{-8} and 3.9×10^{-8}M, respectively.

From studies of quantitative structure-activity relationships, Wataya et al. (11) concluded that electron withdrawal from the uracil heterocycle increased the affinity of 5-substituted-2'-deoxyuridylates for the L. casei thymidylate synthetase. The nitro group is a more powerful electron-withdrawing substituent than is either the fluorine substituent or the trifluoromethyl group; for instance, the Hammet σ_m values, a measure of field effects, for the nitro, fluoro and trifluoromethyl substituents are 0.71, 0.34 and 0.43, respectively (12).

5-Fluoro-2'-deoxyuridine 5'-monophosphate inhibits thymidylate synthetase by binding to a region believed to be the active site (13,14). Various studies (15) have suggested that one of the enzymes' cysteine residues may react at the C6 position of the uracil ring to give a covalent adduct. It is well established that the potent electron-withdrawing properties of the nitro group allows facile addition of a variety of nucleophiles to α-nitro olefins (16) as is embodied in the 5-nitrouracil structure. The addition of azide (17) and alkoxide (18) at C6 of 5-nitrouracil derivatives has been documented previously. It is, therefore, quite possible that the irreversible inhibition of thymidylate synthetase by

$NO_2{}^5$dUMP may proceed in an analogous fashion through an intermediate such as that in Figure 2.

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