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INACTIVATION OF THYMIDYLATE SYNTHETASE BY A NOVEL MECHANISM-BASED ENZYME INHIBITOR: 1-(β-D-2'-DEOXYRIBOFURANOSYL)8-AZAPURIN-2-ONE 5'-MONOPHOSPHATE

Thomas I. Kalman and David Goldman

Departments of Medicinal Chemistry and Biochemical Pharmacology,
State University of New York at Buffalo, Amherst, NY 14260

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SUMMARY: Incubation of thymidylate synthetase from *Lactobacillus casei* with the novel substrate analog 1-(2'-deoxyribosyl)8-azapurin-2-one 5'-monophosphate (I) resulted in a time dependent, irreversible, loss of enzyme activity (k_i=1.4 min^{-1}; K_i=1.6x10^{-5} *M*). The presence or absence of the cofactor 5,10-CH_2-tetrahydrofolate did not influence the inhibition, whereas the substrate deoxyuridylate afforded protection against inactivation of the enzyme by I. The destruction of the electrophilic center at position 6 by reduction to the dihydro derivative transformed I into a reversible competitive inhibitor (K_i=1.2x10^{-4} *M*). Mechanistic considerations suggest that I acts as an enzyme generated, covalently bound, transition state analog.

Thymidylate synthetase (EC 2.1.1.45), a key enzyme in the pathway of *de novo* biosynthesis of DNA-thymine, catalyses the formation of thymidylate *via* reductive methylation of deoxyuridylate (dUMP) utilizing 5,10-CH_2-tetrahydrofolate as one-carbon donor and reducing agent (1-3). The currently accepted catalytic mechanism of C-C bond formation (3-6) involves activation of dUMP *via* the nucleophilic attack at position 6 of the pyrimidine ring by an ionized cysteinyl SH-group of the enzyme forming a transient, covalently bound, carbanionic intermediate, which subsequently becomes alkylated at the C-5 position.

As an approach toward the development of new mechanism-based inhibitors of thymidylate synthetase, structural analogs of the substrate were designed incorporating the following desirable chemical properties: 1. strong electrophilic character of the position corresponding to C-6 of

Abbreviations: dAPuMP, 1-(β-D-2'-deoxyribofuranosyl)8-azapurin-2-one 5'-monophosphate; dUMP, 2'-deoxyuridine 5'-monophosphate.

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the substrate dUMP, in order to facilitate covalent bond formation with the functional nucleophilic cysteine residue of the enzyme; 2. resonance stabilization of the anionic Michael adduct formed between the inhibitor and the ionized sulfhydryl group of the enzyme.

In this communication we describe the thymidylate synthetase inhibitory properties of a novel substrate analog (1-(β-D-2'-deoxyribofuranosyl)8-azapurin-2-one 5'-monophosphate (dAPuMP), designed as a mechanism-based inhibitor of this enzyme, using the rational outlined above. Model studies (7) to be published elsewhere, demonstrated the favorable chemical characteristics of dAPuMP required for potent enzyme inhibitory activity.

MATERIALS AND METHODS

Crude thymidylate synthetase of methotrexate resistant *L. casei* was obtained from New England Enzyme Center (Boston, MA), purified essentially as described by Leary and Kisliuk (8) and assayed at 30° by the spectrophotometric method of Wahba and Friedkin (9). The assay mixture contained the following: Tris-acetate, 70 m*M*, pH 7.4; 2-mercaptoethanol, 100 m*M*; $MgCl_2$, 20 m*M*; *d,l*-L-tetrahydrofolate (Sigma), 0.3 m*M*; formaldehyde, 9 m*M*; EDTA, 0.3 m*M*; dUMP, 1.5 m*M* and sufficient enzyme to produce an absorbance change of 0.01-0.02 unit/min at 340 nm.

For inactivation studies the enzyme was preincubated in 70 m*M* Tris-acetate buffer (pH 7.4) containing 1 m*M* dithiothreitol (Sigma) and inhibitor at varying concentrations. At various time intervals aliquots were diluted 10-fold into the regular assay mixture and the remaining enzyme activity was determined as described above.

The synthesis of 1-(β-D-2'-deoxyribofuranosyl)8-azapurin-2-one 5'-monophosphate and 1-(β-D-2'-deoxyribofuranosyl)1,6-dihydro-8-azapurin-2(3H,6H)-one 5'-monophosphate was previously described (7) and will be published elsewhere.

RESULTS AND DISCUSSION

Incubation of *L. casei* thymidylate synthetase with dAPuMP (≥0.1 μ*M*) caused a time-dependent loss of enzyme activity that followed pseudo first order kinetics. The results of a typical experiment are shown in Figure 1. Progressive increase of dAPuMP concentration produced a characteristic rate "saturation" effect, indicating the formation of an enzyme-inhibitor complex prior to inactivation. A double reciprocal plot of the observed

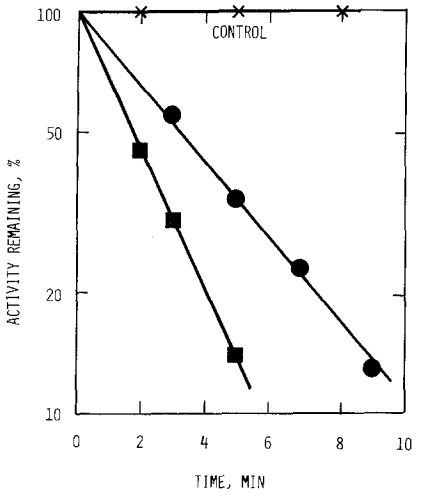

Figure 1. Time course of inactivation of thymidylate synthetase by dAPuMP. The enzyme was preincubated for the indicated times with the following concentrations of dAPuMP: (×), none; (●), 6.7 μ*M*; (■) 27 μ*M*; and the remaining activity was determined as described in materials and methods.

inactivation rate constants *vs.* inhibitor concentrations ($1/k_{obsd}$) *vs.* 1/[dAPuMP]) yielded an inactivation rate constant, k_i = 1.4 min^{-1} and an apparent dissociation constant of the reversible enzyme-inhibitor complex, $K_i = 1.6 \times 10^{-5}$ *M*. The corresponding nucleoside analog of dAPuMP, lacking the 5'-phosphate, was without any inhibitory activity.

The cofactor 5,10-CH_2-tetrahydrofolate was not required for inactivation to occur, and its presence did not affect the observed inactivation rate appreciably. In contrast, the presence of the substrate dUMP afforded protection against inactivation of the enzyme by dAPuMP. The inactivation of thymidylate synthetase by 1.2 μ*M* dAPuMP could be stopped instantaneously by the addition of 2.9 m*M* dUMP. If the substrate was added at a time when 60% of the enzyme activity was already lost, the remaining 40% activity could be maintained for at least an additional 100 min (Figure 2). In the absence of dUMP the catalytic activity of the enzyme was completely lost by that time.

These findings provide evidence for the reversible formation of an enzyme-inhibitor complex *via* specific binding of the inhibitor to the

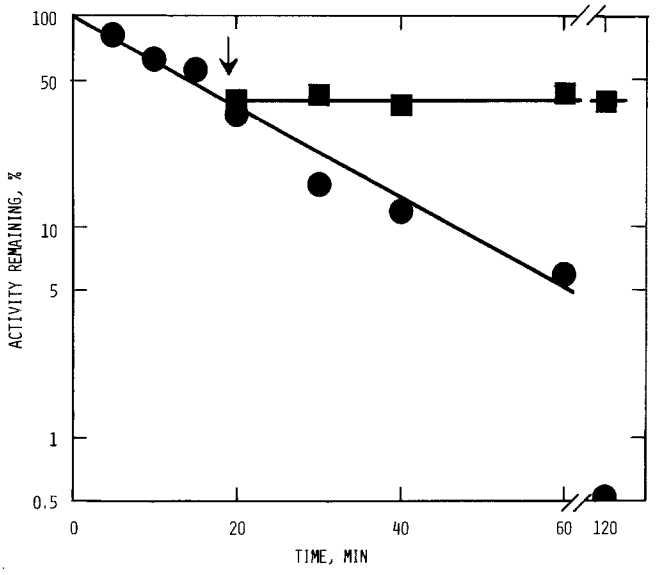

Figure 2. Substrate protection of thymidylate synthetase against inactivation by dAPuMP. The enzyme was preincubated with 1.2 μ*M* dAPuMP, as described in the legend of Figure 1. At ca. 19 min, indicated by an arrow, dUMP (2.9 m*M*) was added in a parallel set of tubes and the remaining activity was determined as described above; (●), no dUMP; (■), 2.9 m*M* dUMP.

active site of the enzyme preceding the inactivation site. Furthermore, the first order rate constant of inactivation (k_i) represents the rate constant of the conversion of the inactive *reversible* enzyme-inhibitor complex to the inactive *irreversible* complex. Formally, the kinetic mechanism of the inactivation of thymidylate synthetase by dAPuMP is indistinguishable from that of an active site directed irreversible inhibition or affinity labelling (10-13):

$$[E + I] \underset{}{\overset{K_i}{\rightleftharpoons}} [E \cdot I] \xrightarrow{k_i} [E\text{-}I']$$

However, the molecular mechanism of the inactivation step must be fundamentally different from that involved in the action of a typical affinity labelling reagent.

In the rationale for the design of dAPuMP the assumption was made that the covalent interaction of the inhibitor with the active site cysteinyl SH-group of the enzyme is dependent on the favorable electrophilicity of the C-6 position of the analog. Thus, it was of interest

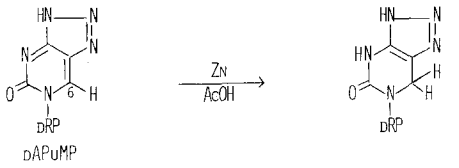

Figure 3. Reduction of dAPuMP to its dihydro derivative.

to examine what effect the removal of the reactive electrophilic center at position 6 of dAPuMP would have on the enzyme inhibitory activity of the analog. A similar approach was used in the study of the inhibition of thymidylate synthetase by showdomycin-5'-phosphate (14). By chemical reduction, dAPuMP was converted to its dihydro derivative 1-(β-D-2'-deoxy-ribofuranosyl)1,6-dihydro-8-azapurin-2(3H,6H)-one 5'-monophosphate, as outlined in Figure 3. The reduced analog was unable to inactivate thymidylate synthetase and behaved as a reversible inhibitor competitive with dUMP. Its K_i-value (1.2×10^{-4} *M*) was an order of magnitude higher than that of dAPuMP. The results demonstrate that the *unsaturated* C-atom at position 6 of dAPuMP is essential for the ability of the analog to inactivate thymidylate synthetase. This is consistent with the mechanistic postulate leading to the development of dAPuMP as a mechanism based enzyme inhibitor.

It is of particular interest that the inactivation of thymidylate synthetase by dAPuMP could not be reversed under conditions which readily regenerate the catalytic activity of the enzyme inactivated by 5-fluoro-dUMP or 5-nitro-dUMP (15). All attempts to reactivate the inactivated enzyme failed. This suggests that dAPuMP is bound extremely tightly to thymidylate synthetase in the inactivated complex and may not be dissociated without the denaturation of the enzyme.

A plausible mechanism for the inactivation of thymidylate synthetase by dAPuMP consistent with the results obtained in this study is outlined in Figure 4. The nucleophilic attack at position 6 of dAPuMP by the functional SH-group of cysteine-198 (16) of the enzyme generates a negative

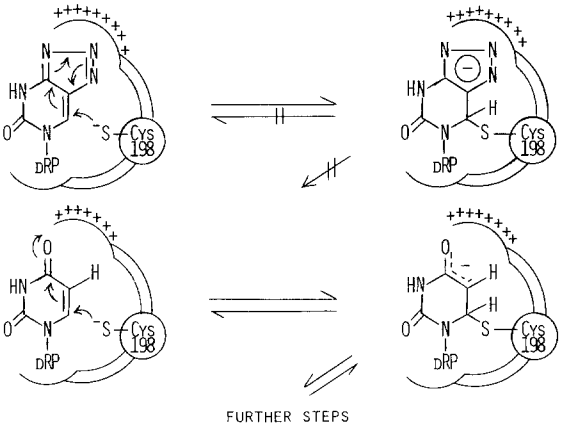

Figure 4. Analogy between the formation of the inactive enzyme-dAPuMP complex and that of the carbanionic intermediate in the normal thymidylate synthetase catalyzed reaction.

charge in the triazole ring, which is very strongly resonance stabilized. The resulting structure closely resembles the postulated transient carbanion intermediate (5) involved in the enzyme catalyzed reaction (Figure 4). The strong interaction of the negative charge of the enzyme-bound dAPuMP with the active site functional group(s) involved in stabilizing the carbanionic intermediate is a very likely explanation for the unique stability of the covalent enzyme-inhibitor complex. The proposed molecular mechanism of the inhibitory action of 5-nitro-dUMP (15, 17-19) is very similar to that of dAPuMP discussed above, but the reversibility of the inactivation (15, 18) indicates a relatively weaker stabilization of the negative charge developed at the 4- and 5-positions of the 5-nitropyrimidine ring in the corresponding enzyme-inhibitor complex.

The requirement for activation of dAPuMP by enzymatic catalysis leading to the loss of enzyme activity is one of the principal characteristics of mechanism-based enzyme inhibitors acting as suicide substrates (20-25). However, no latent, enzyme activated, chemically reactive functional group characteristic also of suicide inactivators (20,22,25) is involved in the action of dAPuMP. Instead, the tight binding of dAPuMP to thymidylate synthetase appears to be the consequence of a

transition state analog (26-30) like activity due to the resemblance of the enzyme-bound inhibitor to the carbanionic intermediate. Thus, dAPuMP shares some of the characteristics of both suicide inactivators and transition state analogs and may be considered as an enzyme generated, covalently bound, transition state analog.

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