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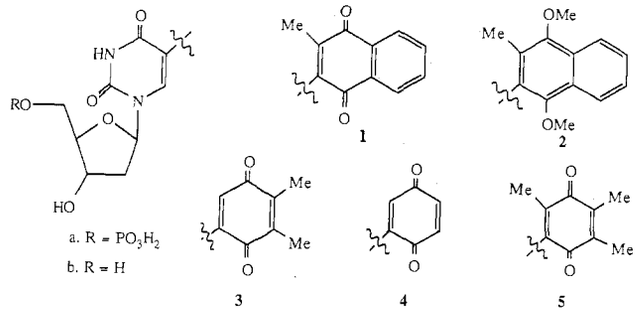
Communications to the Editor

Inactivation of Thymidylate Synthase at an Alternate High-Affinity Binding Site

Sir:

Studies in these laboratories have been directed to the development of substrate analogues for the title enzyme that have high affinity and employ the catalytic features of the enzyme in the conversion of the analogue to a chemically reactive intermediate. Such reagents, termed mechanism-based inhibitors,[1] should prove useful in the study of the catalytic features of the enzyme.

One approach for the development of mechanism-based inhibitors of thymidylate synthase is to enhance the addition of the active-site thiol nucleophile[2] to carbon 6 of the pyrimidine ring by incorporation at carbon-5 of groups that can delocalize the developing charge in the initial catalytic reaction.[3] For this reason 5-(3-methyl-1,4-naphthoquinon-2-yl)-2′-deoxyuridine 5′-phosphate (**1a**) was synthesized.[4] This compound was found to be a potent substrate-competitive inhibitor of thymidylate synthase (K_i = 0.61 μM, substrate K_m = 6.4 μM).



Examination of the naphthoquinone nucleotide **1a** for inactivation of thymidylate synthase showed much the same pattern observed for other affinity labels.[3-5] With use of five concentrations of **1a**, a plot of k_{obsd} vs. [**1a**] showed a negative deviation (Table I). From the double-reciprocal plot a k_2 of 0.025 s^{-1} was calculated from the intercept (eq 1). The K_a calculated from the data and eq

$$1/k_{obsd} = \left[\frac{1}{K_a k_2}\right]\frac{1}{[\mathbf{1a}]} + \frac{1}{k_2} \qquad (1)$$

1 was 1.1 × 10^5 M^{-1} (K_d = 9.1 μM). The fact that this value was significantly different from the inhibitory constant for active-site binding[4] (K_i = 0.61 μM) was the first indication that inactivation at an alternate binding site of the enzyme was occurring. A minimal mechanism that satisfies the initial results is shown in Scheme I.

Scheme I

$$\mathrm{E} + \mathbf{1a} \overset{K_a}{\rightleftharpoons} [\mathrm{E}\cdot\mathbf{1a}] \xrightarrow{k_2} \mathrm{E}\text{-}\mathbf{1a}$$

To test this hypothesis various concentrations of compound **1a** were examined for time-dependent loss of en-

Table I. Kinetic Constants for the Inactivation of *L. casei* Thymidylate Synthase by 5-(3-Methyl-1,4-naphthoquinon-2-yl)-2′-deoxyuridine Nucleotide **1a** and Nucleoside **1b**[a]

no.	concn, μM	substrate concn,[b] μM	inhibitor concn,[c] μM	k_{obsd} s^{-1}	k_2,[d] s^{-1}
1a[e]	2.0	0		0.0051	
	3.0	0		0.0066	
	6.0	0		0.0093	
	10	0		0.013	
	30	0		0.016	0.025[f]
	6.0	15		0.010	
	10	15		0.014	
	30	15		0.017	
	3.0	30		0.0074	
	6.0	30		0.010	
	10	30		0.016	
	6.0	100		0.0097	0.026[g]
	3.0	0	10	0.0067	
	3.0	0	30	0.0071	
	6.0	0	30	0.010	0.026[h]
1b	60	0		5×10^{-5}	

[a] The compounds were incubated with enzyme (sp act. 3.2 μM mg^{-1} min^{-1}) in 0.1 M phosphate buffer at pH 6.8 at 30 °C. Aliquots were removed at various times and assayed for remaining enzyme activity by using 50 μM substrate in a 60-s assay (ref 5). [b] K_m = 6.4 μM. [c] The inhibitor used was 5-(1,4-dimethoxy-3-methyl-2-naphthyl)-2′-deoxyuridine 5′-phosphate (**2a**), which is a (substrate) competitive inhibitor with a K_i of 2.4 μM (ref 4). [d] Calculated from the double-reciprocal plots of k_{obsd} vs. inhibitor concentration according to eq 1. [e] The concentration of enzyme was 0.15 μM. [f] The k_2 was calculated from duplicate data points obtained from incubation in the absence of substrate (n = 10, r = 0.97). [g] The k_2 was calculated from all the data points (n = 17, r = 0.97). [h] The k_2 was calculated from all the data points (n = 20, r = 0.96).

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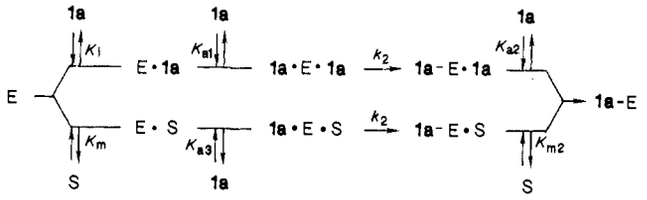
0022-2623/87/1830-1705$01.50/0

Scheme II



zyme activity in the presence of 15, 30, and 100 μM of substrate. The observed rates of enzyme inactivation and the calculated k_2 were unaffected by the presence of substrate (Table I). Given the affinity of the substrate for the active site (K_m = 6.4 μM),[4] a significant decrease in rate[6] would have been observed if either **1a** caused inactivation at the active site or the substrate also had affinity at the site of inactivation.

Given the unique structural specificity for binding and reaction at the site of inactivation, the chemical precursor to compound **1a**, 5-(1,4-dimethoxy-3-methyl-2-naphthyl)-2′-deoxyuridine 5′-phosphate (**2a**), was examined in this assay. Compound **2a** has reasonably high affinity for the active site (K_i = 2.4 μM) and does not inactivate thymidylate synthase.[4] The structural similarity to **1a** suggests that compound **2a** also could have affinity at the site of inactivation. However, the presence of 6 and 30 μM of compound **2a** in the assay for enzyme inactivation by **1a** failed to decrease the rate of inactivation (Table I).

It is recognized that active-site binding of substrate or substrate analogues requires the presence of the 5-phosphate group. Examination of the corresponding nucleoside **1b** in the same assay at a higher concentration (50 μM) showed the rate of enzyme loss was the same as the control (k_{obsd} = 5 × 10^{-5} s^{-1}). The chemical reactivity of the nucleoside **1b** is the same as the nucleotide **1a**, and since the former is inactive, a nonspecific bimolecular mechanism for the enzyme inactivation afforded by **1a** is unlikely. Furthermore, binding to the site of inactivation by **1a** also requires the 5′-phosphate group.

In summary, the kinetic results satisfy the proposal that (a) the rate of inactivation is dependent on the concentration of the binary noncovalent complex formed from E and **1a**, (b) the rate-determining step is k_2, and (c) there is no detectable substrate affinity at the alternate site. Both **1a** and substrate have high affinity for the active site. At the concentrations of **1a** and substrate used, an initial complex of E·**1a** (K_i) or, in the presence of substrate, E·S (K_m) are formed by association at the active site as shown in the kinetic model in Scheme II. Furthermore, since the inactivation appears to take place at an alternate site, the inactivation is dependent on the concentration of the ternary complex of **1a**·E·**1a** or, in the presence of substrate, **1a**·E·S, according to Scheme II. The determined K_a could be a composite of K_{a1} and K_{a2}. However, k_2 is identical with or without substrate. The association values of the inactivated enzyme with either **1a** or substrate cannot be determined with the current data.

Dialysis studies with quinone nucleotides that inactivate thymidylate synthase have shown that active-site inactivation by the dimethylbenzoquinone[4] **3a** was reversible and by the benzoquinone **4a** was irreversible.[5] Dialysis studies using enzyme inactivated with 30 μM of **1a** to give 18% of the original activity showed that a maximum of 55% of the control activity could be recovered by dialysis in 50 mM 2-mercaptoethanol at 2 °C for 16 h.

One potential chemical mechanism for inactivation of the enzyme by **1a** is addition of a nucleophile to carbon 6 of the pyrimidine ring to form a covalent complex. The lack of reaction at this site in chemical model studies[4] and the absence of inactivation by the nucleoside **1b** argue against this as the mechanism. Model studies[4] did show that the naphthoquinone **1b** was an effective oxidizing agent for thiols, and a similar oxidation of the enzyme could lead to inactivation. If this were the case, the trimethylquinone **5a** would have inactivated the enzyme at a faster rate since both nucleotides have high affinity and the rate of oxidation of a model thiol by the trimethylquinone **5b** was over 10 times faster than oxidation by the naphthoquinone **1b**.

Given the above results, it appears that inactivation of the enzyme requires the formation of a complex. One possible explanation that remains to be verified is that **1a** binds and reacts at the cofactor binding site. If this were the case, **1a** would have shown noncompetitive inhibition in the initial studies with the substrate. An unprecedented proposal is that inactivation results from reaction of **1a** with the enzyme at an alternate binding site that apparently has affinity for the substrate analogue **1a**, but not for substrate. The affinity of **1a** for this alternate site is considerably less than that of **1a** for the active site. However, formation of the complex at the alternate site leads to inactivation that is unaffected by the presence of substrate. Such an alternate affinity site that does not appear to bind substrate has not been recognized for thymidylate synthase. Further studies could uncover regulatory or control roles for this region of the enzyme.[7]

Note Added in Proof: Two additional inactivation experiments were completed with the conditions reported in Table I that offer further support for the proposed mechanism. After subtraction of the appropriate control rates of inactivation, incubation of the enzyme with 6 μM of compound **1a** afforded the same rate of inactivation of thymidylate synthase in the absence and presence of 220 μM *dl*-H_4folate. In the second experiment incubation of the enzyme with 23 μM of the nucleoside, compound **1b**, in the absence or presence of 30 μM of substrate did not cause enzyme inactivation.

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Diketopiperazines as a New Class of Platelet-Activating Factor Inhibitors

Sir:

Recent hypothesis of the involvement of platelet-activating factor (PAF) in allergic, inflammatory, and other disease states has generated considerable impact in

