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Effects of Uridine and Thymidine on the Degradation of 5-Fluorouracil, Uracil, and Thymine by Rat Liver Dihydropyrimidine Dehydrogenase[1]

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ABSTRACT

The kinetic properties and control mechanisms of 5-fluorouracil (5-FU), uracil, and thymine degradation by rat liver dihydropyrimidine dehydrogenase were studied *in vitro*. The calculated Michaelis constant (K_m) for 5-FU was 3.49 ± 0.41 (SE) μM, similar to those for uracil (2.26 ± 0.28 μM) and for thymine (2.23 ± 0.34 μM). However, the reduction of 5-FU appears to be most sensitive to the inhibitory effects of increased substrate concentration. The specific activities of dihydropyrimidine dehydrogenase (nmol/min/mg of protein) for 5-FU, uracil, and thymine were 0.82, 0.68, and 0.56, respectively. Uridine was found to be a potent noncompetitive inhibitor of pyrimidine base degradation *in vitro*, displaying an inhibition constant (K_i) for 5-FU of 0.71 μM. Total inhibition of 5-FU degradation occurred at a uridine concentration of 10 μM, whereas thymidine was found to be a much less potent noncompetitive inhibitor of pyrimidine base degradation (K_i 24 μM). This paper provides the first documentation of *in vitro* inhibition of dihydropyrimidine dehydrogenase activity by nucleosides. The concomitant utilization of uridine and 5-FU in clinical situations might prove useful by decreasing 5-FU catabolism to toxic metabolites as well as enhancing 5-FU cytotoxicity.

INTRODUCTION

The pyrimidine bases uracil and thymine are degraded by the liver to CO_2 and to the amino acids β-alanine and β-aminoisobutyric acid (6, 10). The rate-limiting enzyme in this pathway is the NADPH-dependent enzyme DPD[3] (EC 1.3.1.2) which catalyzes the conversion of uracil and thymine to their dihydro derivatives (1, 7).

5-FU, the fluorinated analogue of uracil used in chemotherapy for solid tumors, is believed to be degraded by the same metabolic pathway as uracil and thymine (15). *In vivo* studies have demonstrated that 90% of the parent drug is eliminated by hepatic degradation (3, 20). However, previous investigators have not examined the kinetic characteristics of 5-FU degradation *in vitro* by DPD. Furthermore, little information is available concerning the effects of endogenous regulators upon pyrimidine base or pyrimidine base analogue catabolism. It is known, however, that the pyrimidine degradation pathway is diminished in growing or malignant cells and that the activity is diminished in livers of newborn animals (5, 18).

We have recently described two siblings from a family who excreted high levels of uracil and thymine in their urine. One sibling developed severe toxicity when treated with 5-FU for breast cancer. We proposed a genetic defect in DPD as the etiology of the clinical and biochemical findings (17). The investigation of this family prompted further studies of 5-FU degradation *in vitro* and an examination of possible mechanisms which could modulate the enzymatic reduction of 5-FU by DPD.

MATERIALS AND METHODS

Materials. Uracil, thymine, 5-FU, NADPH, uridine, and thymidine were purchased from Sigma Chemical Company, St. Louis, MO. The other chemicals and reagents were obtained from local commercial sources.

Livers of adult Sprague-Dawley rats, weighing 200 to 300 g, were used in these studies. The animals were killed by cervical dislocation, and their livers were quickly removed, placed in ice-cold 0.25 M sucrose solution (pH 7.30, containing 0.5 mM EDTA), and weighed. All further procedures were performed at 4°C. The livers were minced and homogenized by hand using a glass homogenizer. The homogenized tissue was reconstituted to 1 g (wet weight) per 3.3 ml of solution and was centrifuged at 105,000 × *g* for 60 min. The supernatant was decanted, and the proteins were precipitated by the addition of a 32% volume of saturated ammonium sulfate solution. The precipitate was removed by centrifugation at 1500 × *g* for 20 min. The supernatant was then reprecipitated by the addition of a 15% volume of saturated ammonium sulfate solution. The resulting precipitate was removed by centrifugation and resuspended in ice-cold distilled water to a final volume of 5 ml per 10 g of liver (wet weight). Protein concentration of the precipitate was determined by the method of Lowry *et al.* (13). One g of fresh liver yielded about 5 mg of enzyme extract. DPD activity in the enzyme extract remained virtually unchanged when stored at −20°C. Repeated freezing and thawing resulted in a decrease in enzyme activity. Accordingly, the specific activity of each thawed enzyme sample was determined immediately prior to each individual experiment.

DPD Assay. DPD was assayed spectrophotometrically using a modification of the method of Fritzson (8). The incubation mixture contained 0.5 mg of protein, 50 μM NADPH, 25 mM phosphate buffer, pH 7.35, and various concentrations of substrate and inhibitors in a final incubation volume of 0.5 ml. The blank reaction mixtures contained all reagents including the inhibitors but without the substrate. Net oxidation of NADPH was followed at 340 nm for 10 min in a quartz cuvet with a 1-cm light path, using a Beckman DU-8 recording spectrophotometer equipped with an enzyme kinetics module. One unit of enzyme activity is defined as nmol of NADPH oxidized in 1 min using an extinction coefficient for NADPH of μM = 0.0062. Specific activity of DPD is expressed as units/mg of protein.

Enzyme activity was tested with 5-FU, uracil, or thymine as substrates over concentrations ranging from 0.5 to 100 μM. Results of reaction velocity against substrate concentrations were plotted graphically. In a preliminary series of experiments, 24 different nucleosides and their

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[3] The abbreviations used are: DPD, dihydropyrimidine dehydrogenase; 5-FU, 5-fluorouracil.

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mono, di, and triphosphate derivatives at concentrations of 5 to 10 μM were tested for inhibition of DPD activity at substrate concentrations of 50 μM. Of the compounds tested, only uridine and thymidine showed significant inhibitory activity.

The possibility that uridine could be degraded to uracil by a contaminating nucleoside phosphorylase activity in the DPD preparation was assessed as follows. A reaction mixture containing 50 μM uridine, partially purified DPD (10 mg of protein), 500 μM NADPH, and 50 mM phosphate buffer, pH 7.35, was incubated for 10 min in a total volume of 5 ml. The reaction was terminated by the addition of 100 μl of 10% perchloric acid, and uracil was extracted and quantitated by capillary gas chromatography using the procedure previously described by this laboratory (17). Control reaction mixtures containing combinations of uridine plus NADPH, DPD plus NADPH, and uracil alone were also incubated under identical conditions to those described above. No uracil could be detected after 10 min of incubation in any reaction mixture except in that containing uracil alone. Under these conditions, the extraction efficiency for uracil was consistently 70% or greater. These results clearly indicate that the partially purified preparation of DPD contained no measurable uridine phosphorylase activity.

Data Analysis. Results of inhibition studies were tested for significance using an unpaired Student *t* test, plotted graphically, and expressed mathematically using double-reciprocal plot intercepts as determined by the Wilkinson regression analysis (19). Inhibitory constants (K_i) were calculated using the method of Dixon (4).

RESULTS

The partially purified DPD catalyzed the NADPH-dependent degradation of all three substrates (5-FU, uracil, and thymine). Chart 1 (control plots) shows reaction velocity plotted against substrate concentration for 5-FU, uracil, and thymine. The V_{max} was attained for 5-FU and uracil at concentrations of 10 μM and for thymine at 20 μM. 5-FU showed apparent substrate inhibition at concentrations above 20 μM (data not shown). The substrate inhibition by 5-FU at concentrations of 100 μM was 28 ± 7% (SE, $n = 3$). Table 1 lists the specific activities at apparent V_{max} and the apparent Michaelis constants (K_m) for DPD with 5-FU, uracil, and thymine as substrates. In spite of similar Michaelis constants, DPD showed the highest specific activity when using 5-FU as substrate, while thymine showed the lowest activity. The reaction velocity diminished after 2 min of incubation with all substrates, possibly due to product inhibition (Chart 3).

Uridine was found to be a potent inhibitor of DPD-catalyzed reduction of all three substrates (Chart 1). Thymidine was found to be much less potent as an inhibitor (Chart 2). Inhibition of pyrimidine base reduction by uridine and thymidine appears to occur by a noncompetitive mechanism (Chart 4). Table 2 summarizes the calculated inhibition constants (K_i) for uridine and thymine. Compared to 5-FU and uracil, the reduction of thymine by DPD is the most susceptible to inhibition by uridine and thymidine.

DISCUSSION

In vivo studies using radioactively labeled 5-FU have demonstrated that this compound is eliminated mainly by hepatic metabolism (15). It is believed that 5-FU is degraded in a similar manner as uracil and thymine; *i.e.*, it is initially reduced to 5,6-dihydrofluorouracil by DPD (3, 15). This report documents and characterizes the parameters of 5-FU reduction by DPD as well as those for uracil and thymine.

Although the hepatic DPD preparation used in this study was not pure, the specific activity of this preparation obtained by ammonium sulfate precipitation was 5-fold higher than the activity of liver supernatant (9). A nucleoside phosphorylase contaminant in this enzyme preparation could, if present, generate uracil and thymine from uridine and thymidine, resulting in a decrease in the inhibitor concentration and an increase in the effective

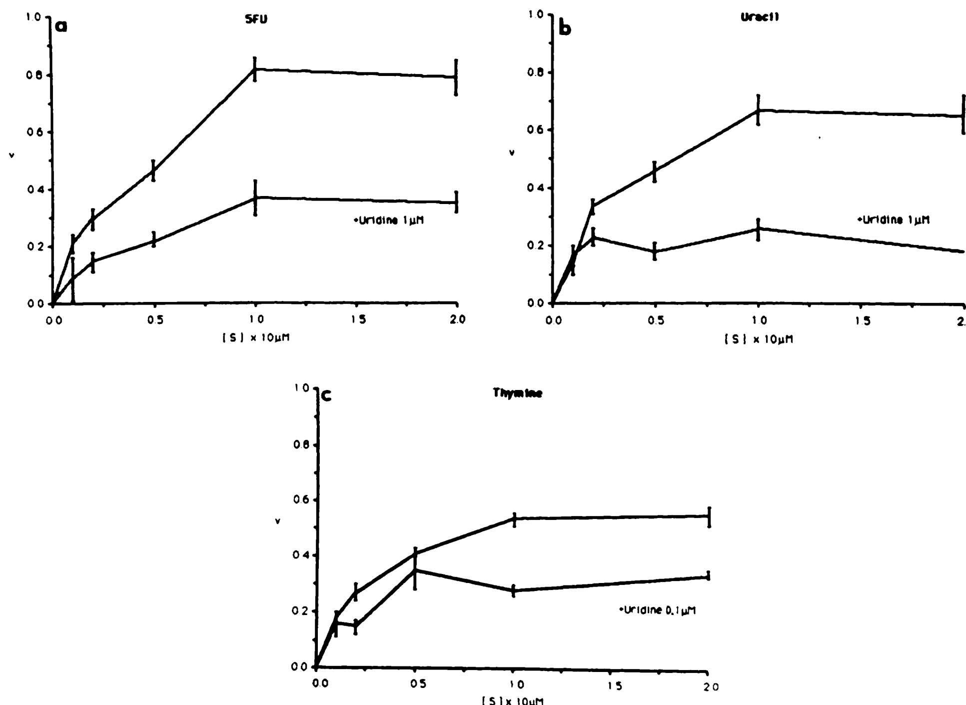

Chart 1. Effect of uridine on the concentration-dependent activity of DPD for 5-FU (*a*), uracil (*b*), and thymine (*c*). Reaction velocity (*v*) is expressed as nmol of NADPH oxidized per mg of protein per min. [*S*], concentration of substrate; *points*, mean number of individual liver preparations assayed; *bars*, SE. Between 15 and 19 (*a*), 15 and 20 (*b*), and 11 and 14 (*c*) values were used for each point of the noninhibited reactions; and 3 and 5 (*a*), 2 and 4 (*b*), and 3 (*c*) values were used for each point of the inhibited reactions.

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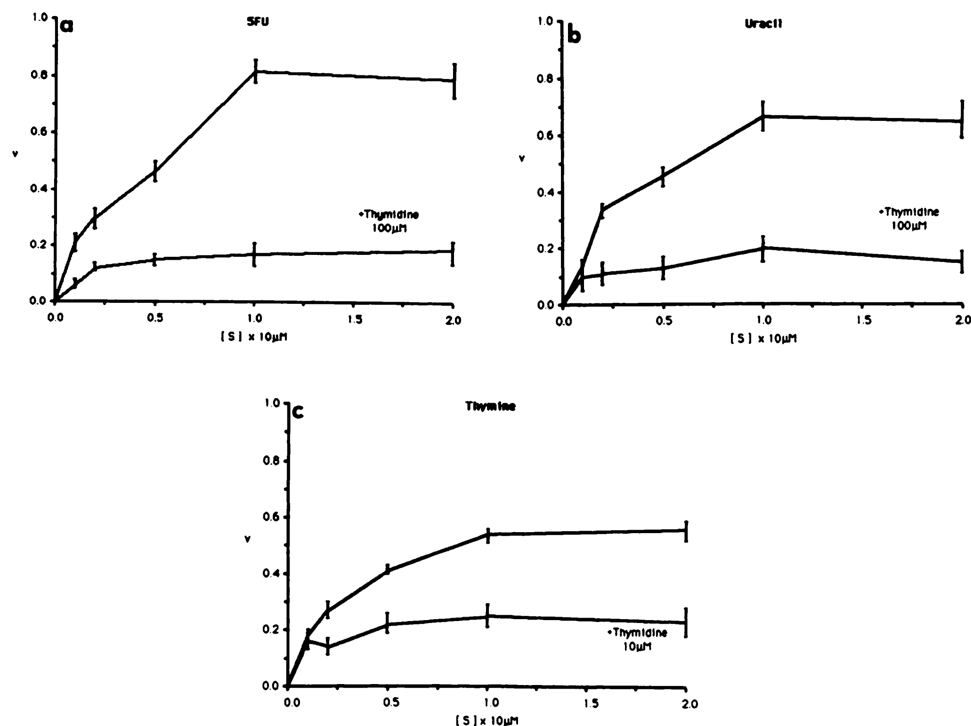

Chart 2. Effect of thymidine on the concentration-dependent activity of DPD for 5-FU (*a*), uracil (*b*), and thymine (*c*). Reaction velocity (*v*) is expressed as nmol of NADPH oxidized per mg of protein per min. *Points*, mean number of individual liver preparations assayed; *bars*, SE. Between 15 and 19 (*a*), 15 and 20 (*b*), and 11 and 14 (*c*) values were used for each point of the noninhibited reactions and 3 and 4 (*a*), 3 and 4 (*b*), and 4 (*c*) values were used for each point of the inhibited reactions.

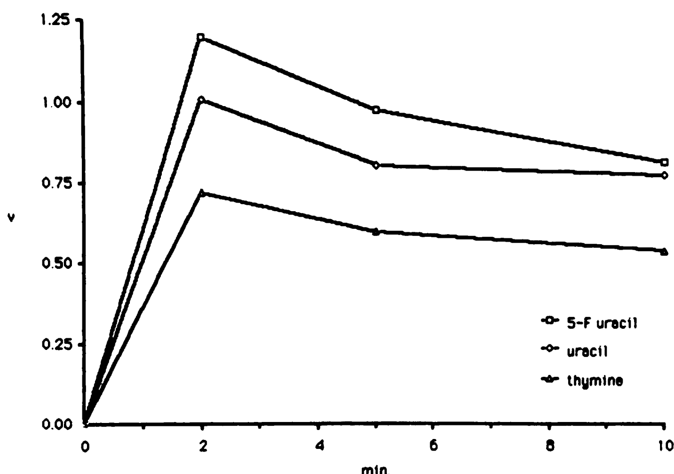

Chart 3. Change in reaction velocity of DPD with time for 5-FU, uracil, and thymine. Velocity (*v*) is expressed as nmol of NADPH oxidized per mg of protein per min at maximal substrate concentrations (10 μM). The *points* on each *graph* represent mean values obtained from 13 to 20 separate rat liver preparations.

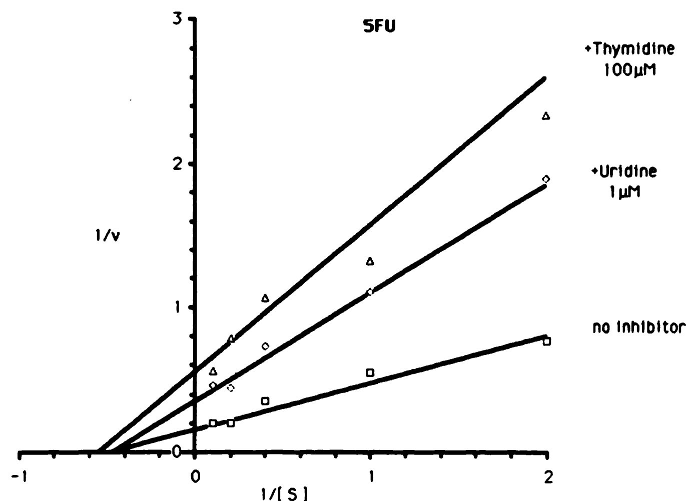

Chart 4. Lineweaver-Burk plot for the inhibition of 5-FU reduction by uridine and thymidine. □, 5-FU with no inhibitor, average of 15 to 19 experiments/*point*; ◇, 5-FU + 1 μM uridine, average of 3 to 4 experiments/*point*; △, 5-FU + 100 μM thymidine, average of 3 to 4 experiments/*point*. The *intercepts* for each line were determined using the Wilkinson regression analysis (19). The calculated kinetic constants for each experimental group are as follows: noninhibited reaction, K_m = 4.2 μM, V_{max} = 1.02 units/mg of protein; uridine inhibition, K_{app} = 4.3 μM, V_{app} = 0.46 units/mg of protein, K_i = 0.84 μM; thymidine inhibition, K_{app} = 3.6 μM, V_{app} = 0.28 units/mg of protein, K_i = 39 μM. The reduction of DPD activity with each inhibitor is statistically significant ($P < 0.01$).

substrate concentration. However, no uridine phosphorylase activity was detected in the partially purified hepatic DPD preparation. Furthermore, the majority of the nucleoside phosphorylase enzymes should precipitate in the 32% ammonium sulfate fraction discarded during the DPD purification (2).

Our results show that the affinity of 5-FU for DPD is similar to those of uracil and thymine (Table 1). However, the specific activity of DPD with 5-FU as a substrate at maximal velocity is higher than that obtained with uracil or thymine. Possibly the fluorine radical favors reduction of the carbons at positions 5 and 6.

Previously, no information was available in regards to the control mechanisms of pyrimidine base degradation *in vitro*. It was previously demonstrated that thymidine administered with 5-FU prolonged the half-life of 5-FU in blood (14), though the amounts of thymidine used in those studies were very large. In contrast, we observed that uridine is a very potent inhibitor of DPD *in vitro*, whereas thymidine is a much less potent inhibitor. Uridine has been shown to potentiate the cytocidal effect of 5-FU in AKR leukemia cells *in vivo* (16).

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Table 1

The specific activity at V_{max} and Michaelis constants (K_m) for 5-FU, uracil, and thymine with rat liver DPD

The K_m was calculated from the formula

$$K_m = [s]\left(\frac{V_{max}}{v} - 1\right)$$

using 2 to 3 substrate concentrations for each determination. Specific activity is expressed per mg of purified protein. One unit of enzyme activity is defined as nmol of NADPH oxidized per min.

Substrate	K_m (μM)	Specific activity (units/mg)
5-FU	3.5 ± 0.4[a] (13)[b]	0.82 ± 0.04 (18)
Uracil	2.3 ± 0.3 (11)	0.68 ± 0.05 (20)
Thymine	2.2 ± 0.3 (10)	0.56 ± 0.03 (14)

[a] Mean ± SE.
[b] Numbers in parentheses, number of individual liver preparations.

Table 2

Inhibition constants (K_i) for uridine and thymidine with rat liver DPD

The K_i values were calculated according to the method of Dixon (4) using the formula for noncompetitive inhibition

$$V_{app} = \frac{V_{max}}{1 + \frac{[i]}{K_i}}$$

The experiments were performed in triplicate and the average values used.

Substrate	Uridine K_i (μM)	Thymidine K_i (μM)
5-FU	0.71	24
Uracil	0.62	41
Thymine	0.16	7.9

It is possible therefore that the administration of 5-FU with uridine may permit the usage of smaller 5-FU doses, thereby avoiding central nervous system toxicity thought to be caused by fluorinated metabolites of 5-FU (11, 12). Additional detailed studies will be needed in order to characterize the theoretical beneficial effects of uridine on 5-FU metabolism and toxicity.

REFERENCES

1. Canellakis, E. S. Pyrimidine metabolism—enzymatic pathways of uracil and thymine degradation. J. Biol. Chem., *221:* 315–322, 1956.
2. Canellakis, E. S. Pyrimidine catabolism. II. Enzymatic pathways of uracil anabolism. J. Biol. Chem., *227:* 329–338, 1957.
3. Chabner, B. A. Pyrimidine antagonists. *In:* Pharmacologic Principles of Cancer Treatment, pp. 183–210. Philadelphia: W. B. Saunders Company, 1982.
4. Dixon, M. The determination of enzyme inhibitor constants. Biochem. J., *55:* 170–171, 1953.
5. Ferdinandus, J. A., Morris, H. P., and Weber, G. Behavior of opposing pathways of thymidine utilization in differentiating, regenerating, and neoplastic liver. Cancer Res., *31:* 550–556, 1971.
6. Fink, R. M., Fink, K., and Henderson, R. B. β-Amino acid formation by tissue slices incubated with pyrimidines. J. Biol. Chem., *201:* 349–355, 1953.
7. Fritzson, P. The catabolism of C^{14}-labeled uracil, dihydrouracil, and β-ureidopropionic acid in rat liver slices. J. Biol. Chem., *226:* 223–238, 1957.
8. Fritzson, P. Properties and assay of dihydrouracil dehydrogenase of rat liver. J. Biol. Chem., *235:* 719–725, 1960.
9. Grisolia, S., and Cardoso, S. S. The purification and properties of hydropyrimidine dehydrogenase. Biochim. Biophys. Acta, *25:* 430–431, 1957.
10. Grisolia, S., and Wallach, D. P. Enzymatic interconversion of hydrouracil and β-ureidopropionic acid. Biochim. Biophys. Acta, *18:* 449, 1955.
11. Koenig, H., and Patel, A. Biochemical basis of the acute cerebellar syndrome in 5-fluorouracil chemotherapy. Trans. Am. Neurol. Asoc., *94:* 290–292, 1969.
12. Koenig, H., and Patel, A. The acute cerebellar syndrome in 5-fluorouracil chemotherapy: a manifestation of fluoroacetate intoxication (abstract). Neurology, *20:* 416, 1970.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265–275, 1951.
14. Martin, D. S., Nayak, R., Sawyer, R. C., Stolfi, R. L., Young, C. W., Woodcock, T., and Spiegelman, S. Enhancement of 5-fluorouracil chemotherapy with emphasis on the use of excess thymidine. Cancer Bull., *30:* 219–224, 1978.
15. Mukherjee, K. L., Boohar, J., Wentland, D., Ansfield, F. J., and Heidelberger, C. Studies on fluorinated pyrimidines. XVI. Metabolism of 5-fluorouracil-2-C^{14} and 5-fluoro-2′-deoxyuridine-2-C^{14} in cancer patients. Cancer Res., *23:* 49–66, 1963.
16. Santelli, J., and Valeriote, F. *In vivo* potentiation of 5-fluorouracil cytotoxicity against AKR leukemia by purines, pyrimidines, and their nucleosides and deoxynucleosides. J. Natl. Cancer Inst., *64:* 69–72, 1980.
17. Tuchman, M., Stoeckeler, J. S., Kiang, D. T., O'Dea, R. F., Ramnaraine, M. L., and Mirkin, B. L. Familial pyrimidinemia and pyrimidinuria associated with severe 5-fluorouracil toxicity. N. Engl. J. Med., *313:* 245–249, 1985.
18. Weber, G., Queener, S. F., and Ferdinandus, J. A. Control of gene expression in carbohydrate, pyrimidine, and DNA metabolism. Adv. Enzyme Regul., *9:* 63–95, 1971.
19. Wilkinson, G. N. Statistical estimations in enzyme kinetics. Biochem. J., *80:* 324–332, 1961.
20. Woodcock, T. M., Martin, D. S., Damin, L. A. M., Kemeny, N. E., and Young, C. W. Combination clinical trials with thymidine and fluorouracil: a Phase I and clinical pharmacologic evaluation. Cancer (Phila.), *45:* 1135–1143, 1980.

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