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Cytotoxicity of 5-Fluoro-5′-*O*-nitro-2′-deoxyuridine, a New Fluorinated Pyrimidine Derivative, in L1210 Cultures

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SUMMARY

5-Fluoro-5′-*O*-nitro-2′-deoxyuridine (FdUMN), a neutral isostere of 5-fluoro-2′-deoxyuridine 5′-monophosphate, inhibited the growth of L1210 cultures. The inhibition of L1210 cultures by FdUMN was prevented by thymidine, but not by 2′-deoxyuridine. Like 5-fluoro-2′-deoxyuridine (FdUrd), FdUMN inhibited the incorporation of 2′-deoxyuridine into DNA, but the onset of this inhibition was not immediate, as was seen with FdUrd. FdUMN did not inhibit the activity of purified thymidylate synthetase from *Lactobacillus casei* and was a poor inhibitor of thymidylate synthetase activity in homogenates of L1210 ascites cells. However, after incubation with homogenates of these cells and subsequent addition of ATP, FdUMN inhibited this enzyme effectively. These results indicate that intracellular activation of FdUMN is required for its inhibition of thymidylate synthetase.

INTRODUCTION

FUra[1] is used for the treatment of transplantable animal tumors and human neoplasms of the breast or colon (1–3). The primary, although not exclusive, antineoplastic effect of FUra involves the inhibition of thymidylate synthetase. Inhibition of this enzyme results from the metabolic conversion of this drug to FdUMP (1, 4), a nucleotide that forms a ternary complex with the enzyme in the presence of 5,10-methylene tetrahydrofolate (1, 5, 6). This complex prevents the synthesis of thymidine 5′-monophosphate and leads to "thymine-less" death of cells (4). Several enzymes are required to activate FUra (1), and the development of resistance in murine (7–15) as well as human neoplasms (16) frequently results from either a deletion or mutation of these anabolic enzymes. Drug resistance cannot be overcome by the administration of preformed FdUMP because, at physiological pH, cellular membranes are essentially impermeable to this negatively charged nucleotide; the formation of FdUMP must occur intracellularly (17).

We reasoned that, if an analogue of FdUMP lacked these negative charges, it might readily enter neoplastic cells and might not require metabolic activation. Accordingly, a neutral isostere of FdUMP (Fig. 1) was synthesized by adding a nitrate group at position 5′ of FdUrd. The product, FdUMN, should be more lipid-soluble than either FdUrd or FdUMP (18), and therefore might cross the blood-brain barrier more readily (19). Indeed, FdUMN not only inhibits the proliferation of tumor cells in culture, but also produces substantial increases in lifespans for mice with L1210 leukemia (20). The intent of this study was to investigate the biochemical basis for cytotoxicity of FdUMN in cultured L1210 cells.

MATERIALS AND METHODS

Chemicals. The preparation of FdUMN from FdUrd has been reported elsewhere (21). The radioactive [6-^{3}H] dUrd (18 Ci/mmole), [5-^{3}H]dUrd (12 Ci/mmole), and [5-*methyl*-^{3}H]dThd (6 Ci/mmole) and [5-^{3}H]dUMP (13 Ci/mmole) were purchased from Schwarz/Mann (Orangeburg, N. Y.). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid was obtained from Calbiochem-Behring Corporation (La Jolla, Calif.). *dl*-L-Tetrahydrofolic acid was a product of Sigma Chemical Company (St. Louis, Mo.).

The FdUMN used with cell cultures and homogenates was purified twice by isocratic high-performance liquid chromatography with a 5-μm ultrasphere-ODS reverse-phase column [25 cm × 10 mm inner diameter, Altex, Inc. (Berkeley, Calif.)] and with 2.5% methanol in water for elution. The compound was purified further by crystallization twice from ethyl acetate and benzene. No contaminating FdUrd, the starting material, was detected in the final product. Commercial FdUrd, from Hoffmann-La Roche, Inc. (Nutley, N. J.), was crystallized twice from absolute ethanol: m.p. 148° [m.p. 149–150° (22)].

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[1] The abbreviations used are: FUra, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMN, 5-fluoro-5′-*O*-nitro-2′-deoxyuridine.

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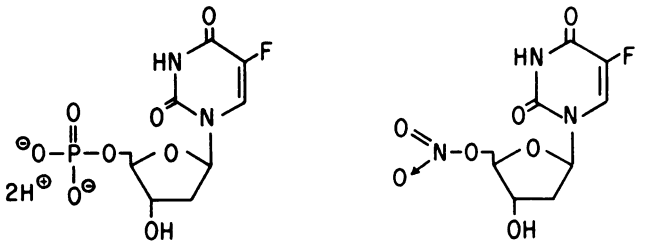

FIG. 1. *Structural formulae of FdUMP and FdUMN*

Culture conditions. Stationary suspension cultures of L1210 cells were grown in Eagle's minimal essential medium containing modified Earle's salt solution and supplemented with both L-glutamine and 10% horse serum. The cultures were grown in plastic flasks at 37° and in an atmosphere of 5% CO_2. Cytotoxicity was determined at 24 hr of cell growth and recorded as the concentration of drug required to inhibit growth by 50% (IC_{50}). The cell line was tested periodically to assure absence of mycoplasma.

Incorporation of deoxyribonucleosides into DNA. Ten-milliliter cultures of L1210 cells in the logarithmic phase of growth were supplemented with 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), 1 μCi of either [5-*methyl*-^{3}H]dThd or [6-^{3}H]dUrd, and either 10 μM FdUMN or 0.1 μM FdUrd. After incubation for various intervals of time, cells from 1-ml aliquots of the cultures were collected on 2.4-cm diameter Whatman GF/A filters, which were washed once with 0.9% NaCl solution and twice with 5% trichloroacetic acid before being dried and placed into ACS aqueous counting scintillant (Amersham Corporation, Arlington Heights, Ill.).

Enzyme assays. The purified enzyme, thymidylate synthetase, of *Lactobacillus casei* was purchased from the New England Enzyme Center, Tufts University Medical School (Boston, Mass.). The spectrophotometric method of Myers *et al.* (23) was used for the assay of this bacterial enzyme.

Tritium-release assays of the activity of thymidylate synthetase in L1210 cells were essentially those reported previously (24). Two hundred microliters of L1210 cultures (100,000 cells) were incubated with 11 μl of [5-^{3}H] dUrd (0.5 μCi), with or without FdUrd or FdUMN, for 30 min in a shaking incubator at 37°. The reaction was terminated by the addition of 11 μl of 50% trichloroacetic acid, and the residual substrate was absorbed by 1 ml of a charcoal suspension (100 mg/ml). After centrifugation, a portion of the supernatant fluid was placed into 10 ml of ACS aqueous counting scintillant, and the tritiated water formed from the release of tritium at position 5 of dUrd was measured.

Thymidylate synthetase in homogenates of L1210 ascites cells was assayed by the tritium-release method as described to monitor the possible conversion of FdUMN to an inhibitor of the enzyme.

RESULTS

Growth inhibition of L1210 cultures. FdUMN inhibited the proliferation of L1210 cells, although less strongly than FdUrd. The IC_{50} value for FdUMN was 550 ± 89 nM (mean ± standard error) for 10 determinations; the value for FdUrd was 2.0 ± 0.2 nM for 13 determinations.

Effect of FdUMN on incorporation of deoxyribonucleosides into DNA. Neither 10 μM FdUMN nor 0.1 μM FdUrd inhibited the incorporation of [5-*methyl*-^{3}H]dThd into DNA when the drug and thymidine were added simultaneously to cultures (data not shown). On the contrary, both agents slightly stimulated the incorporation of dThd into DNA.

As expected, FdUrd was a potent inhibitor of the incorporation of [6-^{3}H]dUrd into DNA (Fig. 2). By contrast, FdUMN, at concentrations toxic to L1210 cells, inhibited incorporation only slightly when added simultaneously with dUrd. After 4 hr of incubation, however, FdUMN inhibited the incorporation of [6-^{3}H]dUrd into DNA (Fig. 3).

Effects on thymidylate synthetase. In the presence of the cofactor, *dl*-L-tetrahydrofolic acid, FdUMN, at 10 mM, did not inhibit purified thymidylate synthetase from *L. casei* after incubation at 37° for 45 min.

The time-dependent effect of FdUMN on thymidylate synthetase activity of L1210 cells was studied by measuring the release of tritium from [5-^{3}H]dUrd, which resulted from the methylation of dUMP (Fig. 4). The inhibition of enzyme activity by FdUrd was almost instantaneous, as indicated by the 0-hr time point. Incubation of cells for 1 hr with 1 nM FdUrd inhibited about 80% of the thymidylate synthetase activity, whereas under similar conditions only about 10% of the enzyme activity was inhibited by 200 nM FdUMN. However, after cells were incubated for 3 hr with FdUMN (200 nM), the enzyme activity was inhibited by about 70%, and remained near this level for the next 2 hr.

Enzymatic assay for the possible contamination of FdUrd in FdUMN. To test for the possibility that FdUMN delayed the onset of enzyme inhibition because of traces of contaminating starting material (FdUrd), both agents, either singly or together, were added simultaneously with [5-^{3}H]dUrd to L1210 cells (Table 1). The addition of 5 nM FdUrd inhibited the activity of thymidylate synthetase by about 65%, whereas 1 μM FdUMN did not inhibit the enzyme. When both agents (with the molar concentration of FdUMN being 200-fold in excess over that of FdUrd) were added to suspensions of L1210 cells, the inhibition of thymidylate synthetase was similar to that observed after the addition of FdUrd alone. Therefore, the delay in onset of the inhibition of thymidylate synthetase in intact L1210 cells did not seem to result from a block of the uptake of possible contaminating FdUrd in FdUMN. Moreover, excess FdUMN did not appear to interfere with either the phosphorylation of FdUrd to FdUMP or the inhibition of thymidylate synthetase by FdUMP.

FdUMN was further examined for possible contamination by the starting material, FdUrd, by assaying the effect of FdUMN on the activity of thymidylate synthe-

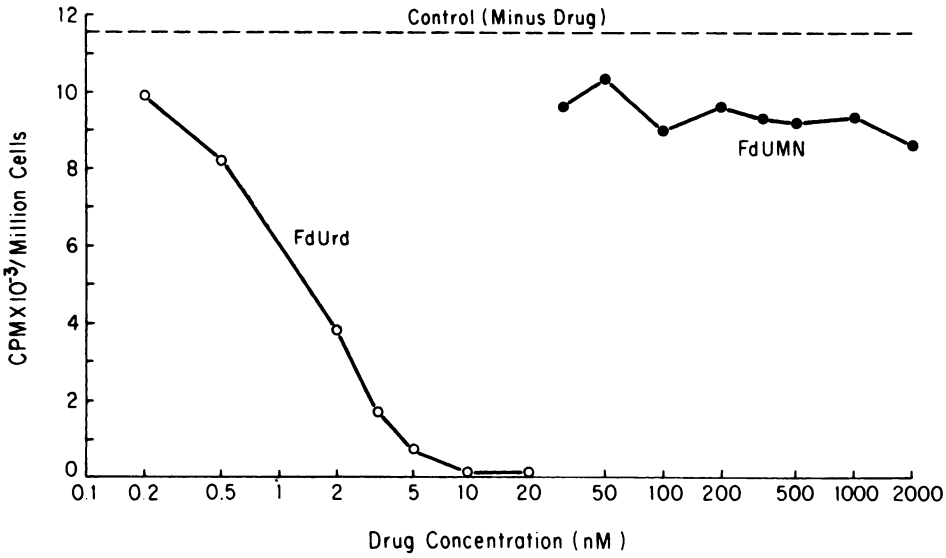

FIG. 2. *Incorporation of [6-^{3}H]dUrd into DNA of L1210 cells*
Cultures of L1210 cells were incubated for 30 min with [6-^{3}H]dUrd in the presence of various concentrations of either FdUrd or FdUMN before assaying the incorporation of radioactivity into DNA, expressed as counts per minute × 10^{-3}/million cells.

tase in a homogenate of L1210 ascites cells (Table 2). The addition of 75 μM FdUMN with 0.68 mM ATP caused a 47% inhibition of the activity of thymidylate synthetase. This inhibition was similar to that produced by 7.5 nM FdUrd plus ATP. These observations indicate that, if the FdUMN was contaminated with FdUrd, the contamination amounted to 0.01% or less.

Reversal of growth inhibition. Cultures of L1210 cells were incubated for 24 hr with various concentrations of FdUMN together with either dThd or dUrd. A concentration of 2 μM FdUMN prevented the growth of cultures, and at higher concentrations the agent lysed certain cells. The inhibition of cell division was reversed by supplementing the medium with 8.3 μM dThd. On the other hand, supplementing the medium with 8.3 μM dUrd did not reverse the inhibition of cell division by FdUMN, although slightly greater concentrations of FdUMN were required to produce equivalent inhibition (Fig. 5). In other studies, the inhibition of cell division was affected similarly when medium with FdUrd was also supplemented with either dThd or dUrd.

Effects on thymidylate synthetase by FdUMN activated by sonicated homogenate of L1210 cells. Since the observed cytotoxicity of FdUMN could not be attributed solely to contamination by FdUrd, FdUMN was either cytotoxic per se or was converted to an active metabolite. To test for this activation, FdUMN was incubated for 3 hr with a homogenate of L1210 ascites cells, and the activity of thymidylate synthetase was assayed. The activation of FdUMN by the homogenates was not discernible until ATP was supplemented in the mixture (Table 3).

DISCUSSION

Inhibition of thymidylate synthetase, one expression of the antineoplastic activities of FUra and other fluorinated pyrimidine derivatives, occurs only after intracellular conversion of these drugs to FdUMP (1, 5, 6). Cell

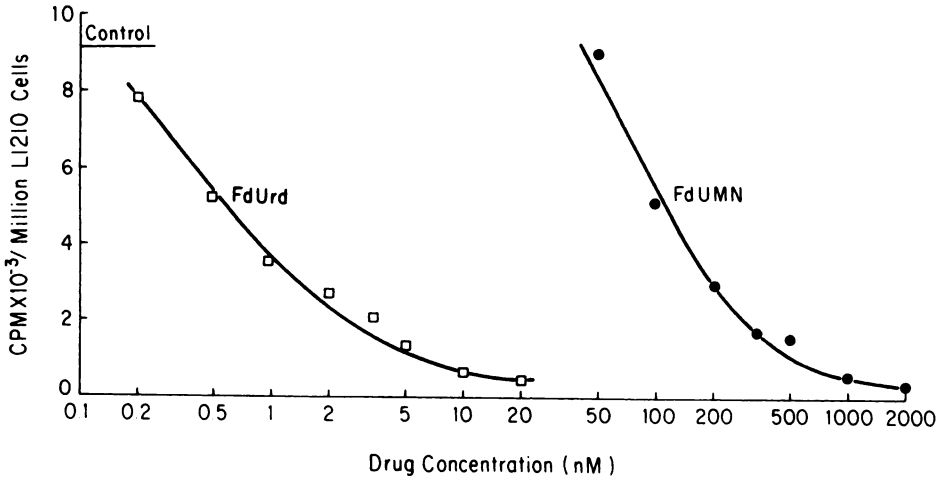

FIG. 3. *Inhibition of [6-^{3}H]dUrd incorporation into DNA of L1210 cells after 4 hr of drug exposure*
Cultures of L1210 cells were grown for 4 hr in the presence of various concentrations of either FdUMN or FdUrd. The incorporation of radioactivity into DNA was expressed as counts per minute × 10^{-3}/million L1210 cells.

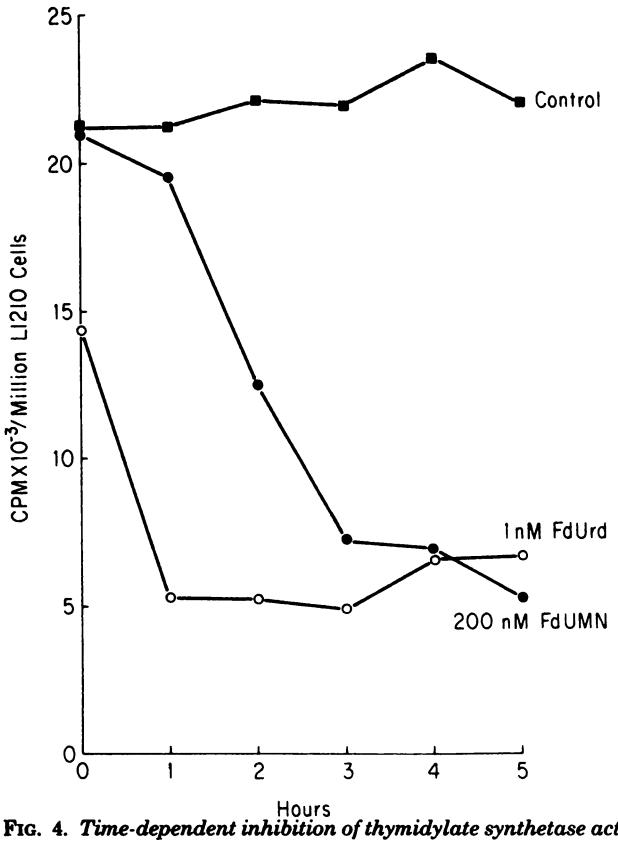

FIG. 4. *Time-dependent inhibition of thymidylate synthetase activity of L1210 cells*

Cultures were preincubated with either FdUrd or FdUMN for various periods of time, as indicated by the abscissa, before aliquots were incubated with [5-^{3}H]dUrd for 30 min at 37°. The appearance of tritiated water was expressed as counts per minute × 10^{-3}/million L1210 cells.

membranes are impermeable to negatively charged nucleotides, so that FdUMP cannot be transported across the plasma membrane, and hence is therapeutically ineffective. We sought to circumvent this limitation by introducing a neutral nitrate ester group at position 5′ of FdUrd. The resulting compound, FdUMN, can be considered a neutral isostere of FdUMP.

When evaluated 24 hr after treatment, the cytotoxicity of FdUMN for L1210 cells in culture was less than that of FdUrd. However, against L1210 cells localized i.p. in B6D2F$_1$J mice, the optimal dosage of FdUMN (≈800 mg/kg) reduced leukemia cell populations as effectively as did the maximally tolerated dosages of FdUrd or FUra. Furthermore, FdUMN was as effective as FdUrd in extending the life-spans of mice bearing L1210 leukemia.[2]

TABLE 1

Effects of FdUrd on thymidylate synthetase from L1210 cells in the presence of excess FdUMN

Enzyme activity was assayed by the release of tritium from [5-^{3}H]dUrd. FdUrd, FdUMN, or both agents were added to the cell suspension immediately before the cells were incubated for 30 min at 37°.

Agent added to L1210 cells	Tritium released[a]	Enzyme inhibition
	cpm/million cells	%
Control	18,000	None
FdUrd (5 nM)	6,300	65
FdUMN (1 μM)	18,300	None
FdUrd (5 nM) + FdUMN (1 μM)	6,700	63

[a] Values were corrected for nonspecific release of tritium by preincubating aliquots of the cell suspension with 3 μM methotrexate for 20 min at 37° before addition of tritiated dUrd; methotrexate blocks the activity of thymidylate synthetase by producing a deficiency in N^5,N^{10}-methylene tetrahydrofolate.

TABLE 2

Effects of FdUMN and FdUrd on thymidylate synthetase from a homogenate of L1210 cells

Enzyme activity was assayed by the release of tritium as tritiated water from [5-^{3}H]dUMP after 30 min at 37°.

Reagent added to cell homogenates	Tritium released	Enzyme inhibition
	cpm	%
Control	5059	None
ATP (0.68 mM)	5181	None
FdUMN (75 μM)	4565	10
ATP (0.68 mM) + FdUMN (75 μM)	2654	47
FdUrd (7.5 nM)	4990	None
ATP (0.68 mM) + FdUrd (7.5 nM)	2764	45

The cytotoxic effect of FdUMN in cultured L1210 cells cannot be attributed to contamination by the starting material, FdUrd. This toxicity was completely reversed by thymidine but poorly by dUrd, suggesting that the cytotoxicity of FdUMN could be related to an inhibition of thymidylate synthetase. The fact that the inhibition of cellular growth by FdUMN could be completely reversed by thymidine suggests that the primary inhibitory effect of FdUMN is on the synthesis of DNA; any effect on RNA synthesis would be of secondary importance. Like FdUrd, FdUMN stimulated the incorporation of exogenous thymidine into DNA of L1210 cells, presumably as a result of reduced *de novo* synthesis of thymidylic acid. However, unlike FdUrd, there was a delay before inhibition of dUrd incorporation into DNA was expressed by FdUMN. After the addition of FdUMN to the cells, about 3 hr elapsed before the incorporation of dUrd into DNA was maximally inhibited. Similarly, there was a 3-hr delay before the activity of thymidylate synthetase was fully depressed, as was evidenced by the reduced release of tritium from [5-^{3}H]dUrd. The change in cell count was very slight within the 3-hr period for the onset of inhibition; hence it was not possible to correlate the formation of inhibitory metabolite with the rate of cell growth during this period. Compared with FdUrd, FdUMN was a poor inhibitor of thymidylate synthetase in homogenates of L1210 ascites cells, but after incubation of FdUMN with homogenates of these cells, the agent generated an inhibitor of the enzyme in the presence of ATP—apparently through activation of FdUMN. Although the cytotoxicity of L1210 cells in culture appeared to be related to the inhibition of thymidylate synthetase, FdUMN per se did not inhibit the activity of thymidylate synthetase purified from *L. casei*.

In L1210 cells, FdUMN might be cleaved by a nucleoside phosphorylase to FUra, or it could be hydrolyzed by

[2] T. L. Chwang and T. L. Avery. Preliminary evaluation of the antineoplastic activity of 5-fluoro-5′-O-nitro-2′-deoxyuridine, a new fluorinated pyrimidine derivative. In preparation.

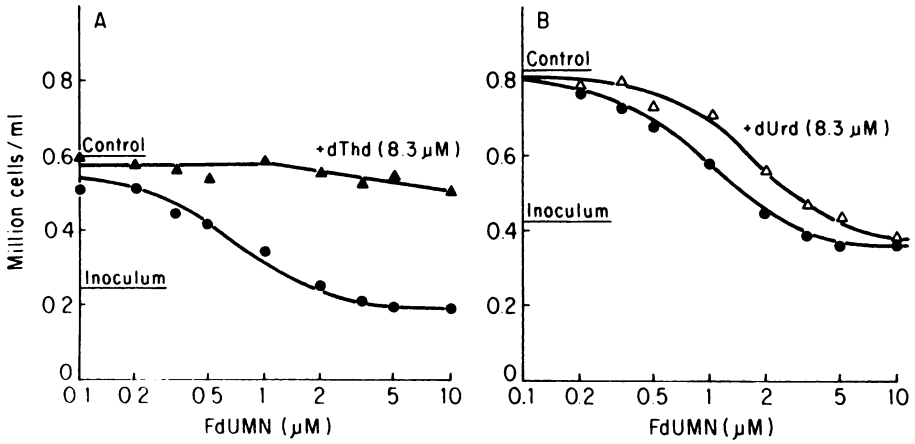

FIG. 5. *Effects of the simultaneous addition, at the time of initiating the cultures, of FdUMN and a normal metabolite (either dThd or dUrd) on the growth of L1210 cells after 24 hr*

an "organic nitrate hydrolase" to FdUrd. Either of these products could then be converted to FdUMP with resultant inhibition of thymidylate biosynthesis. That FdUMN was activated directly to an inhibitor of thymidylate synthetase is an alternative possibility. Inconsistent with these explanations, an analogous 5′-nitrate derivative of 5-fluorouridine, namely 5′-*O*-nitro-5-fluorouridine, is not cytotoxic to L1210 cells in culture at 10^{-4} M (25). Identification of intracellular metabolites of FdUMN will require the use of radiolabeled FdUMN, which is not currently available. Whatever mechanism ultimately emerges, our present working hypothesis is that FdUMN is transported across the membrane and subsequently converted to a metabolite that effectively inhibits thymidylate synthetase.

TABLE 3

Effects on thymidylate synthetase by FdUMN activated by a sonicated homogenate of L1210 ascites cells in the presence of ATP

Ascites L1210 cells were collected on Day 3 after the inoculation of 5 million tumor cells. After washing with phosphate-buffered saline (pH 7.2), the cells were disrupted by sonic oscillation in 5 volumes of 0.25 M sucrose solution that contained 0.1 M Tris-HCl at pH 7.5. Samples of the sonicated homogenate were diluted with an equal volume of 1 mM FdUMN or water and incubated for 3 hr in a shaking incubator at 37° before heating in boiling water for 3 min. Another equal volume of the drug solution was diluted in sucrose/Tris-buffer and treated similarly. Finally, an equal volume of the drug solution was diluted appropriately with water and used directly without incubation or heating. After centrifugation, inhibition of thymidylate synthetase by supernatant was assayed individually by the release of tritium as tritiated water from [5-^{3}H]dUMP, either in the absence or presence of ATP (0.9 M). In this assay, the final concentration FdUMN was 1.2 μM.

Component	Without ATP		With ATP	
	Tritium released	Enzyme inhibition	Tritium released	Enzyme inhibition
	cpm	%	*cpm*	%
Homogenate + water (37°, 3 hr)	5447	None	5072	None
Tris buffer + FdUMN (37°, 3 hr)	5566	None	4417	13
Homogenate + FdUMN (37°, 3 hr)	5667	None	1007	80
Water + FdUMN (25°)	5792	None	4940	3

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