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Report

Cytotoxicity of Three Novel Fluoropyrimidines in Cultured L1210 Murine Lymphocytic Leukemia Cells

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Cultured L1210 murine lymphocytic leukemia cells were used to compare metabolic activation and cytotoxicity of 5-fluorouracil (FU), Ftorafur (FT), and three novel FU-sulfur analogues. These analogues, 1-(2'-tetrahydrothienyl)-5-fluorouracil (FUS), 1-(2'-tetrahydrothienyl)-5-fluorouracil-1'-oxide (FUSO), and 1-(2'-tetrahydrothienyl)-5-fluorouracil-1'-1'-dioxide (FUSO2), have yet to be fully evaluated for potential therapeutic value based on *in vitro* cytotoxicity. The role of these FU analogues as prodrugs was evaluated by comparing metabolism of normal pyrimidine pathways and activation by hepatic mixed function oxidases (MFO). Significant differences in biochemical activity and cytotoxicity were measured between FU and FU analogues. FU and FU analogues were cytotoxic to L1210 cells (63–92% growth inhibition of 100 μM concentrations after 72 hr of incubation). However, at equimolar concentrations cytotoxicity of the FU analogues after MFO activation (56–66% growth inhibition) was greater than FU (47% growth inhibition). Hypoxanthine, a purine precursor, did not significantly alter fluoropyrimidine cytotoxicity with or without MFO. Thymidine and uridine, pyrimidine precursors, reduced FT and FUS cytotoxicities in the presence (27, 40%) and absence (25, 15%) of MFO but did not modify FU, FUSO, or FUSO2 cytotoxicities.

KEY WORDS: fluoropyrimidine; cytotoxicity; L1210 cells; 5-fluorouracil.

INTRODUCTION

Enhanced understanding of 5-fluorouracil (FU) metabolism and toxicity has stimulated interest in synthesis and clinical testing of N-substituted FU analogues with expanded antitumor spectra, enhanced absorption and distribution, and higher therapeutic indices (1). Therefore, analogues of FU that exhibit less host toxicity have been synthesized and tested (2–4). One N-substituted analogue, Ftorafur, [FT; *R,S*,-1-tetrahydro-2-(furyl)-5-fluorouracil], has been extensively studied and has undergone clinical trials (5). FT and other N-substituted analogues are presumed to behave as prodrugs or depot forms which are metabolically activated *in vivo* to FU and miscellaneous metabolites (6). Free FU then exerts its cytotoxicity by inhibition of DNA and RNA synthesis (7). The mechanism of FT conversion to FU is not completely known. FT is metabolized primarily by the hepatic cytochrome P-450 mixed-function oxidase (MFO) system (8). MFO activation and non-MFO activation may occur at different sites of the furanyl moiety releasing different metabolites (9–11).

Several newer analogues—1-(2-tetrahydrothienyl)-5-fluorouracil (FUS), 1-(2'-tetrahydrothienyl)-5-fluorouracil-1'-oxide (FUSO), and 1-(2'-tetrahydrothienyl)-5-fluorouracil-1'-1'-dioxide (FUSO2)—have been synthesized (12) and are yet to be fully evaluated for antitumor effect. These compounds are a series of FT derivatives (Fig. 1) with varying electronegativity at the 1' position of the furanyl ring. It has been proposed that increasing electronegativity would facilitate cleavage of the analogue to free FU, thereby providing an effective prodrug or depot form of FU.

This study was conducted to evaluate these three new FU analogues based on *in vitro* cytotoxicity to L1210 cells since FU and FU analogues have been studied extensively with L1210 cells (13,14). The role of these FU analogues as prodrugs was evaluated by comparing *in vitro* cytotoxicity as influenced by altering normal pyrimidine pathways in the presence or absence of activation by hepatic mixed-function oxidases.

MATERIALS AND METHODS

L1210 Cells. L1210 cells were maintained as stationary suspension cultures in a growth medium (GM) containing Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (Gibco, Grand Island, NY). The fetal calf serum and the penicillin/streptomycin solution were added to RPMI-1640 prior to use of the GM. Cultures were incubated in 5 ml GM/tissue culture flask (Corning Plastics, Corning, NY) in a carbon dioxide incubator (Forma Scientific, Marietta, OH) at 37°C, 98% humidity, and 5% carbon dioxide. Cell viability in all experiments was determined by trypan blue exclusion.

Fluoropyrimidines. A 5 mM suspension of each fluoro-

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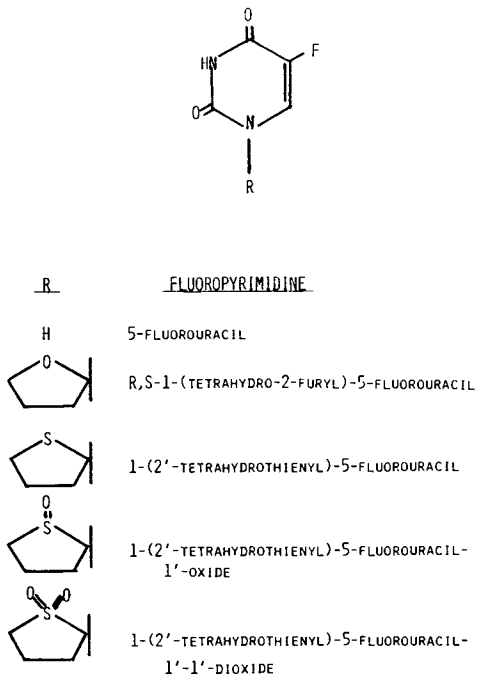

Fig. 1. Structures of 5-fluorouracil and selected N-substituted fluorouracil analogues.

pyrimidine was prepared in 5.0 ml sterile phosphate buffer (0.22 *M* $Na_2HPO_4 \cdot 7H_2O$, 0.04 *M* $NaH_2PO_4 \cdot H_2O$, pH 7.4, in glass-distilled water). FU was purchased from Sigma Chemical Company (St. Louis, MO). FUS, FUSO, and FUSO2 were synthesized according to Holshouser *et al.* (12). Each drug suspension was serially diluted in sterile phosphate buffer so that 0.1 ml, when added to 5.0 ml GM, produced final drug concentrations of 1, 10, or 100 μ*M*. Prior to use, each solution was tested by thin-layer chromatography for purity and spontaneous conversion to free FU.

L1210 Cytotoxicity. The susceptibility of L1210 cells to FU analogues was evaluated using a modification of the procedure of Bhuyon *et al.* (15). Exponentially growing L1210 cell suspensions (4–5 × 10^5 cells/ml) were incubated with 0.1 ml of either FU, FT, FUS, FUSO, or FUSO2 at final concentrations of 1, 10, or 100 μ*M*. Controls were incubated with 0.1 ml of sterile phosphate buffer. At 24, 48, and 72 hr after initial inoculation, 200 μl was removed from each flask for measurement of cell viability. Cytotoxicity of each compound was measured as the percentage of L1210 growth inhibition in comparison to the control.

Hepatic Microsomal Preparations. Male Sprague-Dawley rats (300–350 g) were lightly anesthetized with ether and killed by cervical dislocation. Livers were removed and placed in ice-cold 1.15% KCl. A liver homogenate was prepared by adding 1.15% KCl (1:3, w/v) then homogenizing using a Polytron (Brinkmann Instruments, Westbury, NY). The microsomal S-9 fraction was separated by centrifugation (9000*g*) at 4°C in a Beckman L575 ultracentrifuge. After centrifugation, the supernatant was collected. A fresh 2.5% (v/v) solution of the S-9 supernatant with cofactor mix was used as the MFO source. The cofactor mix contained 8 m*M* magnesium chloride, 33 m*M* potassium chloride, 5 m*M* glucose-6-phosphate, and 4 m*M* NADP in phosphate buffer (0.22 *M* $Na_2HPO_4 \cdot 7H_2O$, 0.04 *M* $NaH_2PO_4 \cdot H_2O$, pH 7.6). Exponentially growing L1210 cells (4–5 × 10^5 cells/ml) were incubated with 0.1 ml of either FU, FT, or FUS, at a final drug concentration of 100 μ*M*, and 0.5 ml of the 2.5% S-9 preparation. Corresponding controls were prepared with 0.1 ml phosphate buffer with and without an exogenous MFO source. At the end of a 3-hr incubation, 200 μl was removed for measurement of cell viability. Triplicate samples were tested in each of three separate experiments using freshly prepared enzyme preparations.

Purine and Pyrimidine Precursors. Hypoxanthine (50 m*M*), thymidine (2.5 m*M*), and uridine (2.5 m*M*) were prepared in phosphate buffer. Thymidine and uridine were readily soluble in aqueous phosphate buffer. The hypoxanthine solution was sonicated for 5–7 min, producing a finely dispersed suspension, and was thoroughly mixed before addition to each flask. Exponentially growing L1210 cell suspensions (4–5 × 10^5 cells/ml) were preincubated (37°C, 90 min) with 0.1 ml of either 1 m*M* hypoxanthine, 50 μ*M* thymidine, 50 μ*M* uridine, or phosphate buffer. FU was added at concentrations ranging from 0.1 to 100 μM. Incubation continued for 3 hr (37°C), after which 200 μl was removed from each flask for determination of cell viability (16,17).

The effects of purine and pyrimidine precursors on MFO activation of fluoropyrimidines were also evaluated. Exponentially growing L1210 cells (4–5 × 10^5 cells/ml) were preincubated with either 1 m*M* hypoxanthine, 50 μ*M* thymidine, or 50 μ*M* uridine at 37°C for 90 min. One-tenth milliliter of FU, FT FUS, FUSO, or FUSO2 (100 μ*M*) was added with or without 0.5 ml of a fresh 2.5% S-9 fraction. Controls were prepared with 0.1 ml phosphate buffer. Cell viability was determined after an additional 3-hr incubation. Samples were tested in triplicate in each of three experiments.

Statistics. Data were initially evaluated by analysis of variance. Then Dunnett's test for multiple comparisons was used to compare data between the control and the various experimental groups or between the FU-treated and the other experimental groups (18).

RESULTS

Cytotoxicity. L1210 cells were sensitive to FU and FU analogues in the absence of an exogenous activating system such as MFO (Table I). Maximum cytotoxicity was achieved after 72 hr of incubation at 100 μ*M* drug concentrations. At 72 hr and 100 μ*M* concentration, FUSO2 exhibited the greatest cytotoxicity (92% growth inhibition) when compared to FU (90%), FUSO (85%), FT (82%), and FUS (63%). At 24, 48, and 72 hr, the FU dose–response relationships were relatively shallow, whereas the FT and FUS dose–response relationships at each time period were steep and characteristic of prodrugs. FUSO and FUSO2 dose–response relationships were also steep at 48 and 72 hr, however, at 24 hr, concentration-dependent increases in L1210 cell growth inhibition were not evident. The responses with FUSO and FUSO2 at 24 hr were not significantly different over the two-log range but remained at approximately 65% for FUSO and 60% for FUSO2. At the 48- and 72-hr incubation periods, cytotoxicity increased in a concentration-dependent manner for all analogues, with 100 μ*M* producing the greatest inhibition.

Table I. Fluoropyrimidine Cytotoxicity

Concentration (μM)	L1210 cell growth inhibition (%) at time after inoculation		
	24 hr	48 hr	72 hr
FU			
100	80 ± 1[a,b]	82 ± 1	90 ± 1
10	70 ± 1	74 ± 1	81 ± 1
1	50 ± 1	65 ± 1	79 ± 1
FT			
100	73 ± 1	79 ± 1	81 ± 1
10	36 ± 1	67 ± 1	58 ± 1
1	2 ± 1	4 ± 1	17 ± 1
FUS			
100	64 ± 1	68 ± 1	63 ± 1
10	29 ± 1	52 ± 1	14 ± 1
1	16 ± 1	4 ± 1	0 ± 1
FUSO			
100	66 ± 1	80 ± 1	84 ± 1
10	66 ± 1	81 ± 1	82 ± 1
1	64 ± 1	23 ± 1	19 ± 1
FUSO2			
100	59 ± 1	79 ± 1	92 ± 1
10	62 ± 1	75 ± 1	85 ± 1
1	59 ± 1	47 ± 1	22 ± 1

[a] Composite results, $n = 9$.
[b] Mean ± standard error of mean.

The effects of MFO on fluoropyrimidine cytotoxicity were evaluated (Table II). After incubation for 3 hr at 37°C in the absence of MFO, FU and the FU analogues at 100 μM were cytotoxic to L1210 cells. Cell growth was inhibited by FU (49.7%), FT (37.3%), FUS (28.8%), FUSO (29.6%), and FUSO2 (28.5%) when compared to untreated control cell growth. In the presence of MFO, cytotoxicities were significantly enhanced in cells treated with FT (56%), FUS (62%), FUSO (63%), and FUSO2 (66%) but not FU (47%) when compared to control. FU cytotoxicity was not changed. Therefore, MFO activation enhanced FT, FUS, FUSO, and FUSO2 cytotoxicity by 18, 33, 32, and 34%, respectively. In the absence of MFO, FU exhibited greater cytotoxicity than the analogues tested. In the presence of MFO, all analogues were more cytotoxic than FU. The sulfur analogues were more cytotoxic than FT in this study when activated by MFO.

Table II. Effects of MFO on Fluoropyrimidine Cytoxicity[a]

	Cell growth[b]		
	% inhibition		% increase in inhibition with S-9
	Without S-9	With S-9	
FU	49.7 ± 3[c]	47.0 ± 3	—
FT	37.3 ± 2	55.5 ± 1*	18
FUS	28.8 ± 3	62.1 ± 2*	33
FUSO	29.6 ± 3	62.1 ± 3*	32
FUSO2	28.5 ± 2	65.8 ± 2*	34

[a] Composite results of three experiments with $n = 9$.
[b] Compounds at 100 μM concentrations, 3-hr incubation.
[c] Mean ± standard error of mean.
* $P = 0.01$ (significantly different from FU with S-9 and corresponding analogue without S-9).

Purine and Pyrimidine Precursors. The ability of hypoxanthine, a purine precursor, and thymidine or uridine, pyrimidine precursors, to interfere with fluoropyrimidine metabolism to cytotoxic intermediates was evaluated (Table III).

Hypoxanthine did not significantly alter fluoropyrimidine cytotoxicity with or without MFO. Thymidine and uridine reduced FT and FUS cytotoxicities in the presence and absence of MFO but did not modify FU, FUSO, or FUSO2 cytotoxicities. Without MFO, thymidine decreased FT and FUS cytotoxicities by 25 and 15%, respectively; with MFO, thymidine reduced the cytotoxicity of FT and FUS by 27 and 40%. Uridine rescue for FT and FUS was 17 and 15% in the absence of MFO and 10 and 36% in the presence of MFO.

DISCUSSION

L1210 cells were sensitive to FU and FU analogues in the absence of an exogenous enzyme system such as MFO. With the exception of FUSO and FUSO2 at 24 hr of incubation, the cytotoxicity of these analogues to L1210 cells increased in a concentration-dependent manner, with 100 μM concentrations producing the greatest inhibition after 72 hr. At 24 hr of incubation, concentration-dependent increases in L1210 cell growth inhibition were not evident with FUSO and FUSO2. The response was not significantly different over the two-log range but remained at approximately 60% for FUSO and 65% for FUSO2, which was greater than FU at 51%. With the exception of FUS, FUSO, and FUSO2 at 1 μM concentration, cell growth inhibition for each analogue at each concentration increased with time.

The purpose for synthesis of FUS, FUSO, and FUSO2 was to produce a series of FT derivatives with highly electronegative substituents at the 1′ position of the furanyl ring (12). It was proposed that increased analogue electronegativity would facilitate cleavage of the N-1–C-2′ linkage, thereby releasing free FU more rapidly. FUSO and FUSO2 contain the sulfoxide and sulfone groups, respectively, which are highly electronegative. At 72 hr, cytotoxicity was correlated with increasing electronegativity at the 1′ position of the furanyl ring and may indicate increased FU release. However, cell growth after 24 hr with FUS, FUSO, and FUSO2 at 1 μM indicated that the sulfur analogues acted in a transient or reversible manner since inhibition was decreased at 48 and 72 hr. In contrast, FU cytotoxicity appears to be irreversible. Brief (1-hr) exposure of L5178Y murine lymphoma cells to low concentrations of FU (1.5 μM) also produced sustained inhibition of cell proliferation following removal of the drug from the culture medium (19). In the same study after 1 hr of exposure to 100 μM FU, recovery of cell proliferation was not observed for 15 days in drug-free medium.

MFO activation did not significantly alter FU cytotoxicity but enhanced that of FT (18%), FUS (33%), FUSO (32%), and FUSO2 (34%). However, at equimolar concentrations, cytotoxicity of the analogues after MFO activation was significantly greater than FU alone. If cytotoxicity was

Table III. Effects of Purine and Pyrimidine Precursors on Fluoropyrimidine Cytotoxicity[a]

	Cell growth, % inhibition							
	Without S-9				With S-9			
	None	Hx	TdR	Urd	None	Hx	TdR	Urd
FU	49.7 ± 3	54.1 ± 3	46.3 ± 5	52.9 ± 4	47.0 ± 3	48.7 ± 2	48.8 ± 2	49.9 ± 3
FT	37.3 ± 2	37.2 ± 3	12.7 ± 3*	19.8 ± 4*	55.5 ± 1	56.9 ± 2	28.3 ± 2*	45.9 ± 3*
FUS	28.8 ± 3	32.6 ± 2	13.5 ± 2*	13.3 ± 1*	63.2 ± 1	64.2 ± 1	22.4 ± 2*	26.9 ± 3*
FUSO	29.6 ± 3	37.0 ± 3	29.9 ± 3	35.4 ± 2	62.1 ± 2	62.5 ± 1	60.5 ± 1	62.3 ± 2
FUSO2	28.5 ± 2	40.6 ± 3	33.7 ± 3	32.2 ± 2	63.8 ± 2	65.1 ± 2	65.6 ± 3	67.0 ± 2

[a] Composite results, mean (n = 9) ± standard error of mean; 100 μM concentrations, 3-hr incubation.
* $P < 0.05$; significant difference from control (none).

related only to FU release, expected toxicity should have been equal to, but not greater than, that of FU. This observation may be related to microsomal or nonmicrosomal activation at different sites of the furanyl moiety (11).

Purine and pyrimidine precursors were used to manipulate the biochemical pathways by which FU is metabolized in order to identify additional mechanisms of active metabolites (20). Hx, known to reduce FU metabolism by uridine phosphoribosyl transferase, did not alter FU or FU analogue cytotoxicities with or without MFO. Allopurinol, which has the same mechanism of action as hypoxanthine, reversed cytotoxicity in L1210 cells after 15 hr of incubation with 1×10^{-7} M FU (20). Hx reversed FU (5×10^{-6} M) cytotoxicity after 48 hr but did not reduce FU (7.7×10^{-5} M) cytotoxicity after 3 hr of incubation (21). In each of these studies, FU concentrations were considerably lower than used in our study. Also, Hx may not have reduced cytotoxicity within the short incubation time of 3 hr.

FU cytotoxicity (100 μM) was not affected by thymidine or uridine, confirming reports that 50 μM thymidine would not reverse cytotoxicity at FU concentrations greater than 1 μM (16,21,22). FU and FU analogue concentrations used in L1210 cytotoxicity and biochemical studies were 100 μM. Thymidine and uridine did, however, reduce FT and FUS cytotoxicities in the presence and absence of MFO. Neither thymidine nor uridine alone completely restored cell growth to control levels.

Thymidine and uridine did not reduce cytotoxicity of FUSO and FUSO2. Thymidine and uridine rescue of L1210 cells from FUS-mediated cytotoxicity (but not that of FUSO and FUSO2) may reflect differences in analogue potency rather than only differences in mechanism of action. These data are consistent with the original hypothesis that increasing electronegativity at the 1′ position would be correlated with increased cytotoxicity in L1210 cells. Although the exact mechanism remains uncertain, the MFO and pyrimidine data suggest that FU release may not be the only mechanism of FU analogue activation and cytotoxicity.

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