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

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Received February 27, 1990; accepted June 19, 1990

Cultured L1210 murine lymphocytic leukemia cells were used to compare metabolic activation and cytotoxicity of 5-fluorouracil (FU), Florafor (FT), and three novel FU-sulfur analogues. These analogues, 1-(2'-tetrahydrothiényl)-5-fluorouracil (FUS), 1-(2'-tetrahydrothiényl)-5-fluorouracil-1'-oxide (FUSO), and 1-(2'-tetrahydrothiényl)-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 produgs 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, Florafor, [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'-tetrahydrothiényl)-5-fluorouracil (FUS), 1-(2'-tetrahydrothiényl)-5-fluorouracil-1'-oxide (FUSO), and 1-(2'-tetrahydrothiényl)-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 fluor-
pyrimidine was prepared in 5.0 ml sterile phosphate buffer (0.22 M Na₂HPO₄ · 7H₂O, 0.04 M NaH₂PO₄ · H₂O, 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 μM. 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 Bhuyan et al. (15). Exponentially growing L1210 cell suspensions (4.5 × 10⁶ cells/ml) were incubated with 0.1 ml of either FU, FT, FUS, FUSO, or FUSO2 at final concentrations of 1, 10, or 100 μM. 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 (9000g) 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 mM magnesium chloride, 33 mM potassium chloride, 5 mM glucose-6-phosphate, and 4 mM NADP in phosphate buffer (0.22 M Na₂HPO₄ · 7H₂O, 0.04 M NaH₂PO₄ · H₂O, pH 7.6). Exponentially growing L1210 cells (4–5 × 10⁵ cells/ml) were incubated with 0.1 ml of either FU, FT, or FUS, at a final drug concentration of 100 μM, 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 the 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 mM), thymidine (2.5 mM), and uridine (2.5 mM) 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 × 10⁵ cells/ml) were preincubated (37°C, 90 min) with 0.1 ml of either 1 mM hypoxanthine, 50 μM thymidine, 50 μM 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 × 10⁵ cells/ml) were preincubated with either 1 mM hypoxanthine, 50 μM thymidine, or 50 μM uridine at 37°C for 90 min. One-tenth milliliter of FU, FT FUS, FUSO, or FUSO2 (100 μM) 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 μM drug concentrations. At 72 hr and 100 μM 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 μM producing the greatest inhibition.