ABSTRACT

The combination of 5-fluorouracil (FUra) and cyclophosphamide (CP) was found to be therapeutically synergistic against the L1210 ascites tumor. Concurrent i.p. administration of FUra (50 mg/kg) plus CP (200 mg/kg) to C57BL/6 x DBA/2 F, mice bearing Day 4 tumors resulted in a significant increase in median life span compared to treatment with maximally tolerated doses of either FUra or CP alone.

A basis for the synergistic response was a greater than additive effect of the combination in decreasing tumor cell viability when compared to the sum of the log cell kill obtained with FUra (50 mg/kg) and CP (200 mg/kg) individually. In contrast to its effect on tumor, the combination showed less toxicity to normal tissues than did the maximally tolerated dose of CP alone as indicated by measurements of body weight and bone marrow nucleated cellularity.

Measurements of [3H]deoxyuridine incorporation into DNA suggested that FUra and CP acted in complementary fashion selectively to inhibit tumor DNA synthesis, while in upper small intestine and bone marrow the time course of inhibition and recovery of [3H]deoxyuridine incorporation was similar for the FUra, CP, and FUra plus CP treatment groups. No alterations in the distribution of either 5-fluoro-2'-deoxyuridine 5'-phosphate or alkylating metabolites of CP could be detected in tumor or normal tissues when FUra and CP were administered concurrently.

Data obtained on the proliferative states of tumor and normal tissues were used to predict improved combination scheduling. The administration of full doses of the combination on either Days 4 and 10, or 4 and 12, when [3H]deoxyuridine into DNA of upper small intestine and bone marrow nucleated cellularity had returned to pretreatment values, resulted in 3 of 10 and 4 of 10 long-term survivors, respectively.

INTRODUCTION

The use of drug combinations either as a primary form of therapy or as an adjuvant to surgery and radiation is now widespread. Preclinical and clinical studies have demonstrated that chemotherapy has the potential of eradicating metastatic disease foci not generally accessible to other treatment modalities (1, 34). By exploiting this potential, most effectively with drug combinations, it has been possible to increase survival in a significant number of individuals with either hematological or the more prevalent solid tumors, including breast, prostate, and uterus (10, 20).

The antimitabolite, FUra, and the alkylating agent, CP, have been used successfully as single agents and as components of multiple drug combinations (1, 6, 7, 17). Of particular interest were the results of Schabel (31) and Wodinsky, which demonstrated therapeutic synergism in the Riddway osteogenic sarcoma an L1210 tumor systems, respectively, following sequential treatment with CP and FUra. However, further studies were not done to characterize this effect. This report describes some biochemical and pharmacological effects of FUra and CP when used alone and in combination against the L1210 ascites tumor and how these data were used to rationally improve the efficacy of this combination.

MATERIALS AND METHODS

Animals. The murine L1210 leukemia carried i.p. in male DBA/2 mice was used as a tumor source and transplanted i.p. into male C57BL/6 x DBA/2 F, (hereafter called B6D2F,) mice for all experiments. All mice were obtained from Sprague-Dawley Co., Madison, Wis. Mice were fed standard laboratory chow and water ad libitum.

Chemicals. CP, obtained as a generous gift of Mead Johnson and Co., Evansville, Ind., was dissolved in 0.9% NaCl solution (w/v) just prior to i.p. injection. FUra, obtained as a generous gift of Hoffman LaRoche Inc., Nutley, N. J., was dissolved in 1.0% Na2CO3, (w/v) just prior to i.p. injection. [6-3H]dUrd (15 to 30 Ci/mmol) was obtained from New England Nuclear, Boston, Mass. NBP reagent consisted of 5% NBP (w/v) (Eastman Kodak Co., Rochester, N. Y.) in acetone. Dowex 1-X8 chloride resin (200 to 400 mesh) was obtained from Fisher Scientific Co., Silver Spring, Md. ScintiVerse liquid scintillation cocktail was purchased from Fisher Scientific Co. dL-Tetrahydrofolic acid,FdUMP,dUMP, and calf thymus DNA (sodium salt) were obtained from Sigma Chemical Co., St. Louis, Mo. Tris-mercaptoethanol-EDTA buffer consisted of 50 mM Tris-HCl, containing 10 mM 2-mercaptoethanol and 1.0 mM disodium EDTA, pH 7.4. N5,N10-Methylentetrahydrofolate cofactor solution consisted of 870 μM dl-Tetrahydrofolic acid, 60 mM formalde-
Flowing, 23 mM MgCl₂, and 400 mM 2-mercaptoethanol, adjusted to pH 7.4 with KOH. Thymidylate synthetase (EC 2.1.1.45) that had been partially purified from dichloromethotrexate-resistant Lactobacillus casei according to the method of Crusberg et al. (8) was obtained from New England Enzyme Center, Tufts University Medical School, Boston, Mass. The enzyme formed 5.5 nmol thymidylate per hr per mg protein at pH 7.0 and 30°.

Effect of Chemotherapy on Survival of Mice Bearing L1210 Ascites Cells. Chemotherapy was initiated 4 days following i.p. inoculation of 1 × 10⁶ L1210 cells. In the survival experiments, groups of 10 mice received either (a) no treatment (controls); (b) FUra (25, 50, 100, 200, or 400 mg/kg); (c) CP (100, 200, 300, or 350 mg/kg); or (d) CP (200 mg/kg) plus FUra (25, 50, 100 or 200 mg/kg). All drugs were injected i.p. at a volume of 0.01 ml/g of body weight. In the drug-scheduling experiment, groups of mice bearing Day 4 tumors were given i.p. injections of FUra (50 mg/kg) plus CP (200 mg/kg) either on Day 4 only or on Days 4 and 6, 4 and 8, 4 and 10, or 4 and 12. In both the survival and drug-scheduling experiments, the median survival time of a treatment groups of animals surviving was halfway between N/2 and (N/2 − 1), where N is the number of animals per groups at the start of the experiment (15). Statistical analysis was performed using the Wilcoxon signed-rank test.

Biosassay of Tumor Cell Viability. The procedure was a modification of the method of Skipper et al. (38). On Day 4 after i.p. inoculation of 1 × 10⁶ L1210 cells, mice received either: (a) no drug treatment (4 mice); (b) FUra (50 mg/kg) (4 groups of 2 mice each); (c) CP (200 mg/kg) (4 groups of 4 mice each); or (d) FUra (50 mg/kg) plus CP (200 mg/kg) (4 groups of 8 mice each). Twenty-four hr later, the tumor cells from the mice within each group were harvested in ice-coldEarle's solution (11), pooled, centrifuged, and reinoculated i.p. into 4 groups of 8 mice each per drug treatment. On Day 4, the ascites cells from the control mice were counted electronically and pooled, and inocula of either 1 × 10⁶, 1 × 10⁵, 1 × 10⁴, or 1 × 10³ cells were implanted into groups of 8 mice each in order to generate a standard curve relating the size of the i.p. tumor inoculum to the median host life span. A linear relationship existed between the size of the i.p. tumor inoculum and the median host life span over the range of 1 × 10⁶ to 1 × 10³ cells/mouse (r = 0.996, p < 0.005). The viability of untreated cells throughout the harvesting procedure remained greater than 95% as estimated by trypan blue exclusion. The Student t test was used to determine whether the mean log cell kill produced by the FUra plus CP combination was either additive, less than additive, or greater than additive. In this procedure, the mean log cell kill and associated variance of combination-treated animals were compared to the theoretically additive mean log cell kill and its associated variance that was calculated from the pooled variance of the FUra and CP treatment groups.

Measurement of Drug Toxicity to Normal Tissues. Body weights and bone marrow nucleated cell numbers were determined in control (untreated), FUra (50 mg/kg), CP (200 mg/kg), CP (300 mg/kg), and FUra (50 mg/kg) plus CP (200 mg/kg)-treated mice. For measurement of body weight, groups of 10 non-tumor-bearing mice per drug treatment were weighed daily for 10 days after drug administration. Statistical significance between mean body weights of CP (300 mg/kg) and combination-treated animals was determined using the Student t test. The number of nucleated cells per femur in groups of 4 mice bearing Day 4 ascites tumors was determined for 7 days after drug administration by the method of Fruhman (13). Statistical significance among treatment groups was determined by analysis of variance.

Assay of FdUMP, dUMP, and [³H]dUrd Incorporation into DNA. Mice bearing Day 4 ascites tumors were given i.p. injections of either FUra (50 mg/kg), CP (200 mg/kg), or FUra (50 mg/kg) plus CP (200 mg/kg). At specific time intervals, the animals received 50 μCi of [6-³H]dUrd s.c. (carrier-free), and 30 min later they were sacrificed when the incorporation of radioactivity into DNA was still linear and near maximal with respect to time. Levels of FdUMP and dUMP in the pooled ascites tumors, upper small intestine, and femoral bone marrow from 6 mice per time point were determined by the method of Myers et al. (25) with the following modification. Instead of isolating small intestinal mucosa for analysis, a 5-cm segment of upper small intestine beginning approximately 2 cm from the pylorus was isolated and homogenized in 5 ml of ice-cold 1 M acetic acid prior to freeze-thawing. The acetic acid extracts obtained by freeze-thawing of ascites cells, intestinal homogenates, or bone marrow were lyophilized to remove acetic acid, and the residues were taken up in 2 ml of Tris-2-mercaptoethanol-EDTA buffer, pH 7.4. Extracts from small intestine or bone marrow often produced interference in the spectrophotometric enzyme assay, resulting in an apparent stimulation or inhibition of the reaction. To remove interfering substances the reconstituted extract of either tumor, small intestine, or bone marrow was routinely purified by the anion-exchange column chromatography procedure of Deutscher (9). The eluant was lyophilized, and the residue was reconstituted in 1 ml of Tris-2-mercaptoethanol-EDTA buffer and assayed for FdUMP and dUMP. Recovery of exogenous FdUMP during the combined extraction and chromatography procedures averaged 91 and 60% from tumor and small intestine, respectively. DNA present in the acid-insoluble material was extracted by a modified Schmidt-Thannhauser-Schneider procedure (33), which includes hydrolysis of RNA in 0.5 N KOH at 37° for 1 hr. The DNA content of an aliquot of the hot 5% trichloroacetic acid extract was measured by the method of Burton (4) using calf thymus DNA as a standard, and the radioactivity was counted in ScintiVerse with a Beckman DPM-100 liquid scintillation spectrometer. Counting efficiency was determined by the external standard method, and the results were expressed as total dpm/tissue sample. Statistical significance between treatment groups was determined using the paired t test.

Assay of CP-alkylating Activity. Groups of mice bearing Day 4 ascites tumors received a single i.p. injection of either CP (200 mg/kg) or CP (200 mg/kg) plus FUra (50 mg/kg) and 4 mice/treatment group were sacrificed by decapitation at 10, 20, 60, and 160 min after drug administration. Blood was drained into a heparinized centrifuge tube, and the appropriate volume of ice-cold 10 mM phosphate buffer, pH 7.4, was added to yield a final volume of 3 ml. Tumor cells and a 10-cm segment of upper small intestine from each
mouse were homogenized in 5 ml of 10 mM phosphate buffer with 2 to 4 strokes in a loosely fitting Teflon-glass homogenizer. Alkylating activity in 3 ml of blood or tissue homogenate was determined as previously described (21). The precipitates from tumor and intestine were analyzed for DNA content as described above. Results were expressed as either μmol of acid-hydrolyzed CP equivalents (1 N HCl for 30 min in boiling water) per ml of blood or per mg of DNA. Color formation in extracts of serum, tumor, and small intestine of control mice that received no drug treatment was subtracted from all experimental values.

RESULTS

Effect of Chemotherapy on the Survival of Tumor-bearing Mice. Dose-response (survival time) curves were established for FUra and CP when used individually and in combination in order to determine if the combination produced therapeutic synergism (16, 37) against the ascitic L1210 leukemia.

The superiority of the combination of FUra plus CP over an optimal dose of either FUra or CP alone in enhancing survival is shown in Chart 1. Plotted in this figure are the results obtained when CP at a fixed dose of 200 mg/kg was combined with single doses of FUra ranging from 25 to 200 mg/kg. When administered to mice bearing the Day 4 L1210 ascites tumors, the combination of either CP (200 mg/kg) plus FUra (50 mg/kg) or CP (200 mg/kg) plus FUra (100 mg/kg) produced a statistically significant ILS of 150% compared to optimal single doses of either FUra (100 mg/kg, 60% ILS) or CP (300 mg/kg, 110% ILS, p < 0.05) alone. Since 50% (5 of 10) of the animals that received CP (200 mg/kg) plus FUra (100 mg/kg) died from drug toxicity (as indicated by a loss in body weight greater than 15%, atrophy of the intestinal mucosa, and the absence of tumor cells in peritoneal cavity and viscera), this regimen was not used in experiments. The dosage schedule of FUra (50 mg/kg) plus CP (200 mg/kg) on Day 4, however, fulfilled the definition of therapeutic synergism in that the combination was superior in enhancing survival when compared to maximally tolerated doses of either drug alone. When the experiment was repeated, an enhanced response following combination treatment was again observed. In the second experiment (not shown), CP (200 mg/kg) plus FUra (50 mg/kg) produced a 186% ILS, which was significantly greater than the ILS obtained following CP (300 mg/kg) (129% ILS, p < 0.01), the optimal single drug treatment.

Effect of Chemotherapy on the Survival of i.p. L1210 Cells. Viable tumor cells within the peritoneal cavity of mice treated with either FUra, CP, or FUra plus CP were quantitated by host bioassay. In this experiment, the assumption was made that the maximum cytotoxic effects of each drug on leukemic cells were achieved within 24 hr after therapy. This assumption seemed justified since DNA synthesis was maximally suppressed in L1210 cells at 24 hr after either FUra (50 mg/kg), CP (200 mg/kg), or FUra (50 mg/kg) plus CP (200 mg/kg).

Table 1 shows that the mean percentage of tumor cells killed by treatment with either FUra (50 mg/kg), CP (200 mg/kg), or FUra (50 mg/kg) plus CP (200 mg/kg) was calculated to be 85.2, 99.97, and 99.9993%, respectively. Therefore, the FUra-plus-CP combination had a greater

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean no. of viable i.p. L1210 cells/mouse after drug treatment</th>
<th>Calculated mean % of i.p. L1210 cells killed by drug treatment</th>
<th>Calculated mean log cell kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>1.74 ± 0.02 × 10⁶</td>
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<td></td>
</tr>
<tr>
<td>FUra (50 mg/kg)</td>
<td>2.57 ± 0.04 × 10⁶</td>
<td>85.2</td>
<td>0.83</td>
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<tr>
<td>CP (200 mg/kg)</td>
<td>6.00 ± 1.17 × 10⁶</td>
<td>99.97</td>
<td>3.46</td>
</tr>
<tr>
<td>FUra (50 mg/kg) + CP (200 mg/kg)</td>
<td>1.17 ± 0.03 × 10⁶</td>
<td>99.9993</td>
<td>5.17⁹</td>
</tr>
</tbody>
</table>

⁹ The numbers of i.p. L1210 cells surviving drug treatment are expressed as the mean ± S.E. (N = 4).

b p < 0.025, compared to the theoretically additive log cell kill of 4.29.

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CHART 1. Animal model for demonstrating therapeutic synergism with FUra (FU) plus CP in murine ascitic L1210 leukemia. An inoculum of 1 x 10⁶ Li210 cells was implanted i.p. into groups of 10 B6D2F1 mice on Day 0. Male B6D2F1 mice were inoculated with 1 x 10⁶ L1210 cells by i.p. injection on Day 0. On Day 4, mice received one of the following: (a) no drug treatment (4 mice); (b) FUra (50 mg/kg) (4 groups of 2 mice each); (c) CP (200 mg/kg) (4 groups of 4 mice each); or (d)FUra (50 mg/kg) plus CP (200 mg/kg) (4 groups of 8 mice each). Twenty-four hr later, the tumor cells from the mice within each group were harvested, pooled, and reinoculated i.p. into 4 groups of 8 mice each per drug treatment; and the median life span of the groups of recipient mice was determined. On Day 4, the ascites cells from the control mice were counted electronically, and pooled, and inocula of either 1 x 10⁶, 1 x 10⁷, 1 x 10⁸, or 1 x 10⁹ cells were implanted i.p. into groups of 8 mice in order to generate a standard curve relating the size of the tumor inoculum to the median host life span.
than additive effect in reducing the i.p. tumor cell burden, since the 5.17-log reduction in tumor cell numbers produced by the combination was statistically greater ($p < 0.025$) than the sum of the log cell kill of 4.29 obtained with FUra (50 mg/kg) (0.83 log kill) plus CP (200 mg/kg) (3.46 log kill).

**Effects of Chemotherapy on the Normal Tissues of Tumor-bearing and Non-Tumor-bearing Mice.** To evaluate the host toxicity of the FUra-plus-CP combination, serial determinations of body weight and bone marrow nucleated cellularity were made following drug treatment. For body weight determinations, non-tumor-bearing mice were used rather than tumor-bearing animals so that any decreases in body weight would be attributable to drug toxicity and not to tumor regression.

It can be seen in Chart 2 that the mean body weight of non-tumor-bearing mice that had received CP (300 mg/kg), which was the most effective single drug treatment in enhancing survival, was significantly lower on Days 1, 2, 3, 4, 7, 9, and 10 after drug administration than the mean body weight of combination-treated animals. By Day 9, the mean body weight of FUra (50 mg/kg) plus CP (200 mg/kg)-treated mice had returned to pretreatment weight, while in the CP (300 mg/kg) group the mean body weight was still significantly depressed below pretreatment levels on Day 10.

Bone marrow, which normally has a large proportion of cells in cycle, is a host tissue that is particularly sensitive to antiproliferative drugs such as FUra or CP (5). As seen in Chart 3, the total number of nucleated cells per femur of mice treated with FUra (50 mg/kg) reached the nadir 2 days after drug administration. On Day 3 after drug treatment, when the nadir in marrow nucleated cell counts of the CP (200 mg/kg), CP (300 mg/kg), and FUra (50 mg/kg) plus CP (200 mg/kg) groups was reached, the number of nucleated cells averaged $2.34 \pm 0.17$ (S.E.), $1.48 \pm 0.17$, and $2.01 \pm 0.14 \times 10^6$ per femur, respectively. Analysis of variance indicated that the mean number of nucleated cells per femur of mice treated with the optimal dose of CP (300 mg/kg) alone was significantly less ($p < 0.05$) on Day 3 when compared to either the CP (200 mg/kg) or FUra (50 mg/kg) plus CP (200 mg/kg) groups. No differences were observed among these groups in the time course of recovery of marrow nucleated cell numbers to the pretreatment value.

Chart 2. The effects of drug treatment on the body weights of non-tumor-bearing mice. B6D2F, mice with an initial body weight (pre-drug treatment) of 23 to 25 g received either no drug treatment (control) (○) or a single i.p. injection on Day 0 of either FUra (50 mg/kg) (○), CP (200 mg/kg) (△), CP (300 mg/kg) (△), or FUra (50 mg/kg) plus CP (200 mg/kg) (●). Each point represents the mean of 10 mice. Bars, S.E. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ compared to FUra (50 mg/kg) plus CP (200 mg/kg).

Chart 3. Serial changes in the numbers of nucleated cells per femur of B6D2F, mice following chemotherapy. Four days after i.p. inoculation of $1 \times 10^5$ L1210 ascites cells, the mice received either no further treatment, time 0 control (○), or a single i.p. injection of either FUra (50 mg/kg) (○), CP (200 mg/kg) (△), CP (300 mg/kg) (△), or FUra (50 mg/kg) plus CP (200 mg/kg) (●). The values obtained of nucleated cells for the paired femora were averaged, and the results were expressed as the mean of 4 mice/treatment group unless otherwise indicated by the number in parentheses. Bars, S.E. * $p < 0.05$ compared to either CP (200 mg/kg) or CP (200 mg/kg) plus FUra (50 mg/kg).
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When taken together, the above results indicate that the combination of FUra (50 mg/kg) plus CP (200 mg/kg), which was superior to CP (300 mg/kg) in prolonging survival of tumor-bearing mice (Chart 1), was not as toxic as was CP (300 mg/kg) to normal tissues as measured by either losses in body weight (Chart 2) or bone marrow nucleated cellularity (Chart 3).

Effect of FUra on the Physiological Disposition of CP Alkylating Metabolites. It was of interest to determine if the simultaneous administration of FUra plus CP to tumor-bearing mice produced changes in the physiological disposition of alkylating metabolites of CP in tumor and normal tissues. Chart 4 shows the levels of CP-alkylating metabolites as assayed by NBP in the blood of mice bearing Day 4 tumors. No statistical differences were observed between the CP (200 mg/kg)- and FUra (50 mg/kg)-plus-CP (200 mg/kg)-treated groups at any time points in the levels of CP-alkylating metabolites in either blood, tumor (not shown), or small intestine (not shown). In each tissue for both the CP and FUra plus-CP treatment, peak levels of alkylating metabolites were attained within 30 min after drug administration and declined in a first-order process to very low levels by 160 min. The results indicate that FUra did not alter the rate of conversion of CP to alkylating metabolites or their persistence in serum, tumor, or small intestine.

Effect of CP in the Intracellular Distribution of FdUMP. In addition to examining the possible influence of FUra on the physiological disposition of CP-alkylating metabolites, it appeared equally important to examine the effect of CP on FUra metabolism in tumor and normal tissues. For example, if a selective alteration in the disposition of FdUMP was found, it could provide an explanation, at least in part, for the enhanced therapeutic effect observed with this combination.

Chart 5, which represents the combined results of 3 experiments, indicates that the accumulation of free intracellular FdUMP (i.e., FdUMP not tightly bound to thymidylate synthetase) in the tumor was more rapid in combination-treated animals, but no differences were observed between the FUra (50 mg/kg)- and FUra (50 mg/kg) plus-CP (200 mg/kg)-treated groups in the peak levels of the inhibitor. In both groups, FdUMP levels fell rapidly in the tumor to very low levels at 24 hr. Comparable results were obtained with upper small intestine (not shown). Thus, the combination did not enhance the peak levels or persistence of acid-soluble FdUMP.

Effect of Chemotherapy on the Incorporation of $[^3H]$dUrd into DNA of Tumor and Normal Tissues. It is generally thought that inhibition of DNA synthesis is a primary effect related to cell death induced by both FUra (26) and CP (24, 38). Accordingly, the magnitude and duration of the effect of FUra and CP, alone and in combination, on DNA synthesis rates in tumor and host sensitive tissues were examined in order further to understand therapeutic synergism and presumably form a basis for rationally improving the efficacy of this combination.

In the tumor (Chart 6A), maximal inhibition of $[^3H]$dUrd incorporation into DNA was seen between 24 and 48 hr in the FUra (50 mg/kg), CP (200 mg/kg), and FUra (50 mg/kg) plus CP (200 mg/kg) treatment groups. It is of interest that $[^3H]$dUrd incorporation into DNA, which was markedly inhibited at 6, 24, and 48 hr by either FUra or CP alone, was further suppressed by combination treatment at these time periods. Measurements of $[^3H]$dUrd incorporation into DNA of tumor beyond 48 hr after CP or FUra plus CP were not considered meaningful because the extensive cell loss
Chart 6. Effect of either FUra, CP or FUra + CP on the incorporation of $[^3H]$dUrd into DNA of the L1210 ascites tumor (A), upper small intestine (B), and bone marrow (C). On Day 4 after i.p. inoculation of $1 \times 10^6$ ascites cells, the mice received either no drug treatment (control) (x) or a single i.p. injection of either FUra (50 mg/kg) (○), CP (200 mg/kg) (△), or FUra (50 mg/kg) plus CP (200 mg/kg) (●). Thirty min before sacrificing, all mice received 50 μCi of $[^3H]$dUrd s.c. Each point represents the mean of 4 determinations, 3 mice/determination, *, $p < 0.05$; **, $p < 0.005$ compared to CP (200 mg/kg) by paired t test. Bars, S.E.

On the other hand, the incorporation of $[^3H]$dUrd into DNA of upper small intestine (Chart 6B) and bone marrow (Chart 6C) was inhibited to an equal extent by either FUra, CP, or FUra plus CP. The time course of the recovery of DNA synthesis in upper small intestine and bone marrow was also similar for all treatment groups with recovery of 100% of control occurring at about 48 hr, followed by equal periods of supranormal DNA synthesis.

Effect of Drug Scheduling on the Survival of Tumor-bearing Mice. Based on the results presented above, we attempted to predict the optimal time at which a full dose of the FUra-plus-CP combination could be repeated in order to maximize the therapeutic effect. It was reasoned that FUra plus CP on Days 4 and 6 after i.p. inoculation of $1 \times 10^6$ L1210 cells would be more toxic and therefore result in a smaller increase in median life span when compared to Day 4 treatment. This was because the rate of DNA synthesis on Day 6 was at either normal or supranormal levels in small intestine (Chart 6B) and bone marrow (Chart 6C), and thus the tissues were more sensitive at these times to antiproliferative drugs such as FUra and CP. Furthermore,
on Day 6 the bone marrow reserve was below normal as demonstrated by measurements of bone marrow nucleated cell numbers (Chart 3). Similar conditions applied to treatment with FURA plus CP on Days 4 and 8 with the exception that bone marrow functions had partially returned to normal by Day 8. By Days 9 and 10, DNA synthesis in small intestine and bone marrow nucleated cell numbers, respectively, had returned to control values. Therefore, treatment on Days 4 and 8 should have led to an increased toxicity compared to treatment on either Day 4, Days 4 and 10, or Days 4 and 12. It was assumed that the ascites tumor was equally susceptible to the cytotoxic effects of FURA and CP on Day 4 and Days 8 through 12, since the cells were in logarithmic phase of growth on all of these days (results not shown).

As reasoned when compared to treatment with FURA plus CP on Day 4 only, treatment on Days 4 and 6 was inferior due to drug-induced toxic deaths (Table 2). Treatment on Days 4 and 8 was also associated with significant toxicity since mice in this group began to die as early as Day 8. In contrast, the Days 4 and 10 and Days 4 and 12 schedules were increasingly more effective with the median life span being significantly enhanced compared to Day 4 only ($p < 0.01$ and $< 0.03$, respectively) and with all animals having an ILS of greater than 100%. In addition, while there were no long-term survivors (>45 days) with FURA plus CP given on Day 4 only, treatment on Days 4 and 8, 4 and 10, and 4 and 12 resulted in 2 of 10, 3 of 10, and 4 of 10 long-term survivors, respectively.

**DISCUSSION**

Therapeutic synergism has been demonstrated in mice bearing ascitic L1210 leukemia following simultaneous administration of FURA plus CP. The combination of FURA (50 mg/kg) plus CP (200 mg/kg) was more effective in prolonging survival than maximally tolerated doses of FURA or CP alone, thus providing a therapeutic advantage over the single agents. The synergistic response as measured by survival studies was the overall result of a greater than additive antitumor effect that was not accompanied by a proportional increase in toxicity to normal tissues. As determined by host biosay, the ascites tumor cell kill produced by FURA (50 mg/kg) plus CP (200 mg/kg) was greater than the sum of the corresponding effects obtained with FURA (50 mg/kg) and CP (200 mg/kg) individually (Table 1). In contrast to the effect on tumor cells, serial determinations of body weight and bone marrow nucleated cellularity indicated that the combination was less toxic to the host than CP (300 mg/kg), which was the optimal single drug treatment for enhancing survival. Therapeutic synergism with other drug combinations has also been characterized by a net increase in toxicity to the tumor relative to the host. For example, Schabel (31, 32) has shown that, for over 50 binary drug combinations, many of which produced therapeuticsynergism against one or more tumors, in almost all cases host toxicity was less than additive as determined by a combination toxicity index.

DNA synthesis was monitored in tumor, upper small intestine, and bone marrow in order to determine whether or not complementary inhibition (29) of DNA synthesis occurred after combination treatment. CP, through alkylation of DNA, and FURA, by decreasing the availability of thymylate for DNA synthesis and possibly DNA repair could produce a greater inhibition of DNA synthesis than either drug alone and consequently an augmented cell kill. If this effect occurred to a greater extent in tumor than in normal tissues, it would be manifested by an enhanced antitumor effect and a lack of cumulative host toxicity which together could result in therapeutic synergism.

The measurements of [H]dUrd were expressed as total dpm/tissue, as opposed to dpm/mg of DNA, in order to reflect changes in the rates of total DNA synthesis and total cell production. When expressed as dpm/mg of DNA, alterations in [H]dUrd incorporation rates do not necessarily reflect the effect of drug treatment on cell number, since cell kill and subsequent loss of DNA will tend to enhance the specific radioactivity of DNA. As shown in Chart 6A, the maximum depression of [H]dUrd incorporation into DNA of L1210 cells was greater at 6 and 24 hr after FURA-plus-CP treatment than after either FURA or CP alone. However, in the upper small intestine and bone marrow, the time course of inhibition and recovery of [H]dUrd incorporation into DNA was similar for all treatment groups. Thus, the data were consistent with the concept that complementary inhibition of DNA synthesis occurred selectively in the tumor as compared to normal tissue after combination treatment. The enhanced effect of the combination in inhibiting DNA synthesis was also consistent with its greater than additive effect of the combination in decreasing tumor cell viability.

Alterations in the physiological disposition of anticancer agents during combination therapy can affect the therapeutic outcome (28). The persistence of free intracellular FdUMP is probably an important determinant of the cytotoxic effect of FURA (22, 25). Since FdUMP is essentially an irreversible inhibitor of mammalian thymydylate synthetase (23), the synthesis of new enzyme is presumably necessary for resumption of DNA synthesis following therapeutic doses of FURA. A persistence of free intracellular FdUMP could inactivate enzyme synthesized at later time periods.

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**Table 2**

**Effects of drug scheduling with CP plus FURA on the survival of mice bearing ascitic L1210 leukemia**

An inoculum of $1 \times 10^6$ L1210 cells was implanted i.p. into groups of 10 B6D2F, mice on Day 0. Control animals received no further treatment. Treated animals were given i.p. injections of CP (200 mg/kg) plus FURA (50 mg/kg) either on Day 4 only or on Days 4 and 6, 4 and 8, 4 and 10, or 4 and 12 after tumor inoculation.

<table>
<thead>
<tr>
<th>CP (200 mg/kg) + FURA (50 mg/kg) (days after tumor inoculation)</th>
<th>Median survival time (days)</th>
<th>Range of individual survival time (days)</th>
<th>% of increase of median survival time</th>
<th>Long-term survivors (&gt;45 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>8.0</td>
<td>7-10</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>17.5</td>
<td>9-28</td>
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<td>0/10</td>
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<td>6</td>
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<td>7-17</td>
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<td>8</td>
<td>30.0</td>
<td>8-45</td>
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<tr>
<td>4, 10</td>
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<td>16-45</td>
<td>425</td>
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<td>4.12</td>
<td>35.0b</td>
<td>16-45</td>
<td>338</td>
<td>4/10</td>
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a $p < 0.01$ compared to CP plus FURA treatment on Day 4 only.
b $p < 0.03$ compared to CP plus FURA treatment on Day 4 only.
(25). Our study revealed that, although differences were noted in the rate of accumulation of FdUMP in tumor (Chart 5), no differences were observed between FUra-plus-CP-treated animals in either the peak levels or persistence of free intracellular FdUMP in tumor or small intestine.

Alterations in the hepatic metabolism of CP in mice by drugs and other chemicals have led to alterations in its toxicity (14, 18). Because several metabolites of CP are capable of alkylating NBP under the assay conditions (2, 3, 19), the NBP assay provided only an estimation of the amount of biologically active metabolite(s) present. Nevertheless, it appeared that FUra did not change the rate of conversion of CP to alkylating metabolites, nor their persistence in blood, tumor or small intestine. Since no differences in alkylating metabolites between CP- and FUra-plus-CP-treated animals were observed, the binding of CP metabolites to cellular macromolecules was not studied.

It was possible, based on measurements of bone marrow nucleated cellularity and DNA synthesis in normal tissues, to determine when a second full dose of the combination could be administered in order to maximize the antitumor effect without producing intolerable host toxicity. There were no long-term survivors (i.e., >45 days) following FUra plus CP treatment either on Day 4 only or on Days 4 and 6. However, treatment with FUra plus CP on either Days 4 and 8, 4 and 10, or 4 and 12 resulted in 2 of 10, 3 of 10, and 4 of 10 long-term survivors, respectively. Comparable drug-scheduling studies have been carried out in tumor-bearing mice with FUra (39) and CP (27) when used as single agents.

This study with L1210 tumor-bearing mice may have relevance to the clinical situation. Skipper et al. (35) have shown that on Day 4 after inoculation of 1 x 10^8 L1210 cells i.p., large numbers of cells could be detected within the spleens, livers, brains, and lungs of B6D2F1 mice. The ability of FUra plus CP when used on a favorable schedule to “cure” a significant number of animals having advanced disease is particularly noteworthy, since the majority of patients are also believed to have disseminated disease foci at the time chemotherapy is initiated (10, 30, 36). In like manner to what has been described for the L1210 system, data obtained on the proliferative states of human biopsy specimens of tumor and normal tissues could hopefully be used rationally to predict optimal drug scheduling during combination cancer chemotherapy.

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A Biochemical and Pharmacological Study of Therapeutic Synergism with 5-Fluorouracil plus Cyclophosphamide in Murine L1210 Leukemia

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