Specificity, Schedule, and Proliferation Dependence of Infused L-Histidinol after 5-Fluorouracil in Mice

Mark B. Edelstein and Lance K. Heilbrun

INTRODUCTION

The general tendency of tumor cells to grow under conditions in which normal proliferative cells will not grow provides a possible key to preventing the toxicity of proliferation dependent anticancer agents against such normal cells. Should it be possible to take advantage of this fact by preventing the entry of bone marrow progenitor cells into active cell cycle, high dose or high frequency schedules for these drugs could be examined which might specifically affect the tumor cell population of interest without affecting the normal host tissues.

In vitro trials of this approach have been performed previously (1-9), most without in vivo confirmation. More recently a new drug known to inhibit protein synthesis, L-histidinol, has been tested both in vitro and in vivo (10, 11) with interesting and exciting results. Warrington et al., demonstrated that L-histidinol, given by repeated i.p. injections, was able to prevent the toxicity of high dose ara-C (25 mg/mouse, 1000 mg/kg) or very high dose FUra (30 mg/mouse, 1200 mg/kg) both in whole animal survival experiments and by in vitro assay of survival of marrow granulocyte precursors, the CFU-GM. At the same time, in vitro assayed L1210 leukemia cells were found to be more sensitive to L-histidinol plus ara-C or L-histidinol plus FUra injections than to injections of the cytostatic drug alone. These experiments were repeated with an in vivo line of L1210 cells; the i.p. injected L1210 leukemia cells given L-histidinol followed by either ara-C or FUra i.p. were also more effectively killed by the combination than by cytostatic drug alone. Survival was assayed using in vitro colony formation. While provocative, additional work was suggested, which is the subject of this paper. Specifically, confirmation of the possible protective effects of L-histidinol, utilizing continuous infusion of the drug, rather than the repeated intermittent schedule already reported was examined. Confirmation of whole animal protection, with the infused route at the highest possible L-histidinol dose, was one of the major end points used. FUra was selected as the test drug. Optimal dosage and sequence using 24-h infusion was tested followed by examination pre-or postcytostatic drug; again whole animal survival was the end point. Finally, having developed an optimal L-histidinol/FUra combination, studies were performed testing it on hematopoietic colony forming cells in various proliferative states in order to evaluate the proliferation dependence of the drug combination.

MATERIALS AND METHODS

Drugs. L-Histidinol was purchased from the Sigma Chemical Company and 5-fluorouracil was obtained from commercial sources. A Harvard infusion pump modified for the infusion of many animals is used to give infusions for up to 48 h of l-histidinol and/or other drugs. All drugs were given i.v.

Mice. Animals were those bred at the Michigan Cancer Foundation or purchased from The Jackson Laboratory. For all experiments, BALB/c × DBA/2 F1 (hereafter called CD2F1) mice were used. All mice were between 6 and 8 weeks old when utilized. Animals of both sexes were used and weighed between 20 and 25 g.

Survival Studies. In the initial series of experiments survival of the animals was evaluated with standard groups of 10 animals/point being used: FUra with or without L-histidinol (50 mg/24 h-100 mg/48 h); FUra (1, 5, 10 mg/mouse). Whole animal survival over 30 days was followed. Experiments were replicated twice and the tables represent the treatment of at least 20 animals/point.

The lowest effective dose in 24-h infusion was sought; therefore scheduling studies, i.e., infusions pre-or postcytostatic drug, were evaluated. An optimal schedule was sought consisting of either low dose 24-h simultaneous infusion, simultaneous with or following the cytotoxic drug.

In Vitro Bone Marrow Studies. For an assessment of CFU-GM, a modification of the method using a double agar system and L-cell derived colony stimulating factor proposed by Pike and Robinson (12) was used. After treatment, test animals were killed by cervical dislocation and their marrow was flushed by repeated aspiration with a 20-gauge needle into both the proximal and distal end of the femur with a standard tissue culture medium (Dulbecco's MEM) at 1 ml/femur. Appropriate dilutions were made, cells were counted and colonies formed per 5 × 10⁴ plated cells were determined. Normalization to untreated controls was used to control for interspecies variations in plating efficiencies. After collection of the marrow cells as described in MEM, 0.7 ml of medium containing 5 × 10⁴ cells, approximately 0.1 ml of L-cell conditioned media (10% v/v), 0.1 ml of newborn calf serum (10% v/v), and 0.1 ml of sterile 3% Bacto-agar (0.3% v/v) were combined, yielding a total volume of 1.0 ml, and plated in 35-mm Petri dishes. Plates for experimental point were used, and colony counts were performed after 14 days of growth in a humidified, CO₂ (5%), water jacketed incubator. Expected were approximately 50 colonies/5 × 10⁴ cells, enumerated 10–14 days after plating using an inverted microscope.
L-cells (transformed mouse fibroblasts) subline L60T (13) are carried in 250-ml spinner flasks as a suspension culture, cultured in αMEM (Flow Laboratories) supplemented with 10% fetal calf serum. Cultures are initiated with 5 × 10^6 cells/ml and grow exponentially within 6 days to densities of between 5 × 10^8 and 10^9 cells/ml, at which time the cells are subdivided and a new spinner flask is initiated. The remaining cells plus conditioned medium are separated by centrifugation, and the conditioned media are used as above.

In Vivo Marrow Studies. Normal CFU (CFU-S) the pluripotent hematopoietic stem cell, are assayed according to a modification of the technique of Till and McCulloch (14). The majority (>95%) of approximately 5 × 10^5 CFU-S/femur CFU-S are not actively in cell cycle. Recipient mice, immediately after whole body irradiation of 1200 rads, are given dilutions i.v. of the bone marrow removed from the treated animals in the same way as described for the in vitro assay. The recipient animals are assayed 10 days later; they are killed by cervical dislocation and the spleens are removed and fixed in Bouin's fixative. After fixation, macroscopic spleen colonies are counted and corrected for dilution; and by comparing to the number of colonies derived from control femurs, a fractional survival is calculated. In order to test CFU-S in their rapidly proliferating state, supralethally irradiated mice (1200 rads as above) are given 5 × 10^5 marrow cells i.v. and serve themselves as marrow donors 7 days after receiving this marrow. At this time, the transplanted CFU-S are rapidly proliferating in an attempt to repopulate the marrow of the primary recipient. Marrows from these donors are assayed in vivo, after appropriate treatment of the animals. Leukemia CFU-S are assayed similarly (15), using a modification of the method of Bruce and van der Gaag (16). Donors are given 10^4 L1210 cells i.v. on day 0; treatment is given on day 4, and assay is performed on day 5. Radiation to recipient mice is not necessary; macroscopic spleen colonies are counted on day 8. Colony counts are normalized to counts in untreated control mice, and a surviving fraction is calculated. Due to occasional early death, the number of animals per l-histidinol and FUra dose level varied somewhat from the intended sample size of 10. The L1210 leukemia was obtained from the National Cancer Institute 17 years ago and has been kept in serial passage in DBA/J mice since then.

Statistical Analysis. Four assay types were analyzed: CFU-S resting; CFU-GM; CFU-S proliferating; and leukemic CFU. Due to large variations in dilution fractions (or number of cells plated, for CFU-GM), the colony counts required scaling or adjustment to take this into account before examining drug effects. This was accomplished by multiplying the actual colony count by the inverse of the dilution fraction (e.g., 1/200 resulted in a multiplier of 200). For CFU-GM, the smallest number of cells plated, 10^3, was taken as 1, so that other multipliers were scaled relative to it (e.g., 10^3 resulted in a multiplier of 100). The dependent variable then reflected the relative differences in the dilutions (or number of cells plated) that were required to yield countable numbers of CFUs. This product could then be regarded as the colony count per dilution, or per 10^3 cells plated, depending on the assay type.

The colony counts per dilution ranged from 0 to 1,120,000. Due to the extreme skewness of this end point variable, rank transformations were applied before statistical analysis (17). This was very successful in achieving nearly normal distributions of the ranks of the product values. Means of colony counts per dilution are reported as summary statistics for presentation, although the analysis was carried out on the rank of the colony counts per dilution (17).

The simultaneous statistical effects of l-histidinol (coded 1 = present, 0 = absent) and dose of FUra (40, 200, or 400 mg/kg) were explored with two-way, unbalanced analysis of variance methods using a general linear models approach (18). A separate analysis of variance model was fit to the ranks of each of the four colony counts per dilution variables listed above. The statistical interaction effect of l-histidinol and FUra dose was tested by including a cross-product term of those two variables in preliminary analysis of variance models. Significant interactions were followed up with one-way analysis of variance models comparing the 6 combinations of levels of the two drugs so as to permit stratified inferences. In so doing, the Tukey multiple comparisons procedure was used to maintain the experimentwise type 1 error rate at 0.05 (18).

Results for each of the four dependent variables were replicated two or more times under identical experimental conditions, and the data are aggregated in order to provide adequate sample sizes in each of the resulting (2 × 3) experimental designs.

RESULTS

l-Histidinol Toxicity. An evaluation of l-histidinol given in a 24-h infusion at either 50 mg/mouse (2000 mg/kg) or 100 mg/mouse (4000 mg/kg) was the first series of experiments performed. Animals were confined in individual restraining cages and infusions were given through one of the two lateral tail veins (the tails were immobilized on a tongue blade) using a Harvard infusion pump modified to treat up to 12 animals at a time. Animals were given food ad libitum and provided the necessary liquids through the infused volume of approximately 2-3 ml over 24 h. The 50-mg/mouse (2000 mg/kg) dose was the same as the total dose given by Warrington et al. in divided doses and was completely acceptable to the animals; there were no toxic manifestations (weight loss, fur changes) or deaths over a 30-day period. The 100-mg dose of l-histidinol in 24 h killed one-half of the animals within 4 days after infusion. Then 75 mg/mouse for 24 h was evaluated. This proved nontoxic as did 100 mg infused over 48 h.

Whole Animal Protection. In order to confirm the results of Warrington et al., replicative experiments using the i.p. route of administration were performed. FUra at 4, 6, or 10 mg/mouse i.p. (approximately 160, 240, and 400 mg/kg, respectively) was given at time 0, with l-histidinol given i.p., 10 mg infection, at -2, 0, +2, +4, +6, and +8 h. In Table 1, the results of these experiments can be seen, with the two experiments combined in the table. l-Histidinol was particularly effective at the highest FUra dose, where survival in the FUra group alone was 0%, and all animals were dead by day 17.

Next, the i.v. route of administration for both drugs was examined, with l-histidinol given by 24 h infusion begun 24 h before FUra administration, given by i.v. bolus injection. Only the highest previous dose of FUra was used, i.e., 10 mg/mouse (400 mg/kg). One other modification was made; because considerable local toxicity was seen at the tail vein site of l-histidinol infusion in preliminary studies, buffering of the l-histidinol with citrate buffer (120 mmol) or phosphate buffer (100 or 200 mmol) was performed. Protection roughly comparable to that achieved by repeated i.p. injection was seen for the citrate buffered l-histidinol (Table 2) given by continuous infusion.

Finally, the schedule of the l-histidinol-FUra combination was changed, with the FUra given at t = 0 and the l-histidinol infusion buffered with citrate given immediately after the FUra for 24 h by infusion. Table 3 shows that, using this schedule, at the 50-mg/mouse (2000-mg/kg) dose of l-histidinol complete protection was confirmed. Despite buffering, no added protection was seen with higher l-histidinol dosage; in fact, frank toxicity was seen.

Table 1 Whole animal survival for normal CDF1 mice treated with bolus i.p. FUra ± bolus i.p. l-histidinol

<table>
<thead>
<tr>
<th>Bolus FUra time</th>
<th>l-histidinol (10 mg)</th>
<th>-2, 0, +2, +4, +6, +8 h i.p.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mg</td>
<td>Median 83% survival day 30</td>
<td></td>
</tr>
<tr>
<td>6 mg</td>
<td>Median 83% survival day 30</td>
<td></td>
</tr>
<tr>
<td>10 mg</td>
<td>Median day 10 0% survival day 17</td>
<td></td>
</tr>
<tr>
<td>FUra ± histidinol</td>
<td>Median 100% survival day 30</td>
<td></td>
</tr>
<tr>
<td>4 mg</td>
<td>Median 50% survival day 30</td>
<td></td>
</tr>
<tr>
<td>6 mg</td>
<td>Median day 13 50% survival day 30</td>
<td></td>
</tr>
<tr>
<td>10 mg</td>
<td>Median day 16 50% survival day 30</td>
<td></td>
</tr>
</tbody>
</table>

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EFFECT OF INFUSED L-HISTIDINOL AFTER FUra

Table 2 Whole animal survival for normal CD2F1 mice treated with FUra (i.v. bolus) after L-histidinol (24 h infusion) evaluation of L-histidinol buffering

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median Day</th>
<th>Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUra alone</td>
<td>Median day 9</td>
<td>0% survival day 11</td>
</tr>
<tr>
<td>50 mg histidinol before FUra (10 mg), citrate (120 mmol)</td>
<td>Median day 12</td>
<td>30% survival day 30</td>
</tr>
<tr>
<td>50 mg histidinol before FUra (10 mg), phosphate (100 mmol)</td>
<td>Median day 11</td>
<td>0% survival day 11</td>
</tr>
<tr>
<td>50 mg histidinol before FUra (10 mg), phosphate (200 mmol)</td>
<td>Median day 9</td>
<td>0% survival day 10</td>
</tr>
</tbody>
</table>

Table 3 Whole animal survival of normal CD2F1 mice treated with FUra (i.v. bolus) before L-histidinol (24-h infusion)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median Day</th>
<th>Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUra (10 mg alone)</td>
<td>Median day 9</td>
<td>0% survival day 10</td>
</tr>
<tr>
<td>FUra (10 mg) before histidinol (50 mg), citrate (120 mmol)</td>
<td>Median day 2</td>
<td>0% survival day 2</td>
</tr>
<tr>
<td>FUra (10 mg) before histidinol (100 mg), citrate (120 mmol)</td>
<td>Median day 9</td>
<td>50% survival day 30</td>
</tr>
<tr>
<td>FUra (10 mg) before histidinol (150 mg), citrate (120 mmol)</td>
<td>Median day 9</td>
<td>100% survival day 30</td>
</tr>
</tbody>
</table>

Hematopoietic Stem Cell Assays. Normal animals were treated with 5-fluorouracil at doses of 40, 200, and 400 mg/kg given by i.v. bolus injection followed immediately in some animals by L-histidinol at 2000 mg/kg by 24-h continuous infusion. This schedule had been found to provide optimal whole animal protection in previous experiments as shown. After the infusion mice were assayed for CFU-S and CFU-GM.

Descriptive statistics of the fractional survival data are presented in graphical form and are supportive of experimental hypotheses concerning L-histidinol and FUra effects. As can be seen in Fig. 1, FUra kills CFU-S in a dose dependent manner and no obvious protection was offered by additional L-histidinol treatment. Fig. 2 suggests that FUra kills CFU-GM in an exponential manner; however, protection seems to be offered to cells also treated with L-histidinol. More consistent protection is offered to proliferating CFU-S as seen in Fig. 3. Here through the whole range of FUra doses, but especially noted for the 200-mg/kg dose, protection is offered to these highly proliferative cells. Finally, in Fig. 4, the effects of L-histidinol cotreatment on L1210 leukemia is noted, with potentiation of FUra toxicity by L-histidinol apparent.

Inferential statistics and statistical tests of our hypotheses have been based on analysis of the colony count per dilution data, summarized in Table 4. A preliminary analysis of variance model showed a highly significant interaction effect ($P = 0.0013$) of the two drugs on colony count per dilution for resting CFU. This precluded reporting of the overall (i.e., main) effect of L-histidinol on resting CFU and required that L-histidinol effects be examined on a FUra dose specific basis. Multiple comparison analysis revealed that there were no sig-

Fig. 1. Fractional survival of resting CFU-S treated with FUra alone or FUra followed by L-histidinol infusion. Each symbol represents a different experiment; bars, SEM.

Fig. 2. Fractional survival of CFU-GM treated with FUra alone or FUra followed by L-histidinol infusion. Each symbol represents a different experiment; bars, SEM.
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significant effects (at the 0.05 level) of L-histidinol on resting CFU counts per dilution at any of the 3 FUra doses.

For the remaining three assay types, preliminary analysis of variance models showed no significant interaction effect of L-histidinol and FUra dose ($P \geq 0.126$ in each case). That permitted straightforward interpretation of the simultaneous effects of L-histidinol and FUra. As expected, the main effect of FUra dose was always statistically significant ($P < 0.0001$ for each these 3 assay types).

After adjusting for the FUra dose effect, there was a statistically significant protective effect of L-histidinol on proliferating CFU-S ($P < 0.0001$), with treated animals showing >2-fold higher mean colony counts per dilution across all 3 FUra dose levels. There was also a statistically significant results for CFU-GM ($P = 0.003$). Regardless of FUra dosage, L-histidinol treated animals had >2-fold higher mean CFU-GM colony counts per $10^4$ cells plated than did animals not receiving L-histidinol.

For L1210 leukemia CFU we observed the opposite effect of L-histidinol, which increased cell killing. There were significantly lower mean leukemic CFU counts per dilution from L-histidinol treated animals ($P = 0.015$), even after adjusting for the simultaneous effect of FUra dosage.

DISCUSSION

Hill and Baserga (19) were among the first to suggest that an increase in therapeutic ratio, due to a decrease of the toxicity of most anticancer agents for actively proliferating cells, could be achieved under conditions of differential cell cycle arrest. Such differential cell cycle arrest has generally been demonstrated in vitro by using a combination of drugs known to be active only in a single phase of the cell cycle whereby differential scheduling was believed to induce the differential cell cycle arrest on normal and malignant tissues respectively. In vivo efforts along these same lines have also been made, but they have generally been unsuccessful due to difficulty in synchro-
nizing the heterogeneous tumor cell population not allowing optimal cell kill as planned (20, 21). Alternatively, cycloheximide has been used repeatedly to demonstrate that through use of it (22, 23) or other drugs (24) classed as protein synthesis inhibitors, the effectiveness of S-phase specific agents could markedly be decreased. However, there has been no evidence that cycloheximide decreases cytotoxicity specifically for normal cells and no reason to believe that it should prove effective for non-S-phase specific drugs.

Attempts to translate the in vitro results to in vivo experiment have proved disappointing either because the drug used for modification of toxicity itself proves toxic or because it is unable to evoke differential cell cycle arrest. Warrington et al. (25) were the first to report that l-histidinol was a drug that in vivo apparently was capable of inducing arrest of the normal tissues at risk for proliferation specific agents while not protecting malignant cells treated concomitantly. L-Histidinol is known to be a competitive inhibitor of L-histidine t-RNA synthetase and therefore capable of inhibition of protein and RNA synthesis in eukaryotic cells (25). The inhibition of protein synthesis is known to have detrimental effects on the growth and therefore the proliferation of normal cells in vitro. L-Histidinol mediated histidine deprivation apparently maintains the normal cells in a noncycling G0 state, while allowing tumor cells to continue through the cell cycle. The corollary that l-histidinol could protect normal cells but not transformed cells from the toxic effects of proliferation dependent cancer drugs was tested and proved to be true for a number of cell lines by Warrington et al. More recently, the same group was able to demonstrate that l-histidinol could mediate improvement in the specificity for both ara-C and 5-fluorouracil in L1210 leukemia bearing mice. The limitations of these latter experiments have been pointed out, however, since the in vivo experiments were as close as possible to in vitro experimentation, i.e., the closed i.p. space, with addition of both drugs into the single area. A latter series of experiments by Warrington and Fang (26) did address part of the problem; drugs were given i.p. but the tumor for assay was obtained in the marrow.

The experiments described here have gone beyond these limitations, and, as shown particularly in Table 3, by choice of the correct schedule, marked protection by L-histidinol infusion could be achieved for FUra. This schedule and route of l-histidinol differs from that used by Warrington in his experiments. It was chosen on the basis of the whole animal survival experimentation and appears to be superior to other schedules of administration and to the reported i.p. injection technique used by Warrington in our hands. The total l-histidinol dose delivered in 24 h is 2000 mg/kg which is higher than the total of 1600 mg/kg given by the repeated i.p. injection regimen. This dose is also higher than that used in a negative study by Stolfi et al. (27) in which FUra plus l-histidinol did protect animals against FUra toxicity but also apparently protected tumor cells from FUra cytotoxicity. The i.p. use of FUra and l-histidinol may also have contributed to this negative result as has already been discussed.

We conclude that the relative protection of l-histidinol treated CFU-S, CFU-GM, and proliferating CFU-S is CFU-S < CFU-GM < proliferating CFU-S as suggested by Figs. 1, 2, and 3 and data in Table 4. This throws doubt on the mechanism of protection previously proposed. Protection by prevention of entry into cell cycle would be predicted in this order of protection: CFU-S (≤5% in cycle in the resting state; prevention of cycle entry would be very effective) > CFU-GM (90% of cells in cycle; protection thus could possibly accrue to the 10% of cells not in cell cycle at the time FUra/l-histidinol is given) > proliferating CFU-S (nearly all cells committed to the cell cycle). The predicted and observed protection occurs in the reverse order and must call into question the proposed mechanism. Warrington (28) has provided other evidence that a proliferative mechanism is not the whole explanation for the activity of l-histidinol. Using malignant cells differing in l-histidinol induced cell cycle effects from little to significant still demonstrated (28) similar increased killing by l-histidinol plus anticancer agents in combination. Further using P815 mastocytoma, Warrington (29) has most recently shown a dissociation between cell cycle effects of l-histidinol and cell killing for this neoplastic cell type, again questioning a purely cell kinetic mechanism.

Further work examining solid tumor:normal cell combinations, various l-histidinol administration schedules, l-histidinol plus other drugs from different classes (i.e., alkylating agents), and the pharmacokinetics of l-histidinol are in progress. The findings reported here do not in any way diminish interest in l-histidinol which, independent of exact mechanism, remains a fascinating and potentially important drug for cancer chemotherapy.

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REFERENCES


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