Schedule-dependent Enhancement of the Cytotoxicity of Fluoropyrimidines to Human Carcinoma Cells in the Presence of Folinic Acid

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ABSTRACT

Previous studies from this laboratory indicated that the cytotoxic effects of the fluoropyrimidines on mouse leukemic cells are substantially augmented by folic acid but that these effects are underestimated in growth inhibition experiments. These results have now been extended to two human tumor cell lines, the WiDr colorectal and T-24 bladder carcinoma cells. In both cell lines, the presence of folic acid in the medium substantially enhanced the cytotoxicity of a 72-h exposure to either 5-fluorouracil (FUra) or 5-fluoro-2'-deoxyuridine. Folic acid concentration-response curves for enhancement of the cytotoxicity of FUra to WiDr cells were broad but indicated that response was not maximal until at least 10 μM. Likewise, increased length of exposure to 10 μM folic acid continuously enhanced the cytotoxicity of a 72-h treatment with FUra, but substantial enhancement was observed even after a 2-h exposure to folic acid, and there was a diminished increment of cytotoxicity after 24-h exposure to folic acid. Surprisingly, folic acid augmentation of the cytotoxicity of a brief exposure to FUra (4 h) was minimal but enhancement of FUra cytotoxicity became much more pronounced with intervals of exposure to FUra of ≥24 h. If these results can be mimicked in vivo without undue host toxicity, our experiments suggest that a substantial improvement in the therapeutic activity of FUra plus folic acid would result from prolonged exposure to both agents.

INTRODUCTION

A number of laboratory and clinical investigations have demonstrated that the activity of the fluoropyrimidines against at least some human gastrointestinal carcinomas can be substantially increased when a reduced folate such as folic acid [leucovorin; (6-R,S)-5-formyltetrahydrofolate] is coadministered. This combination is of considerable importance because it represents one of the very few significant advances in the chemotherapy of colorectal carcinoma in 30 years. Recent randomized clinical trials have indicated that the response rate of human metastatic colorectal carcinomas to FUra alone is on the range of 8–15% at the highest tolerated dose of drug, whereas the response rate to FUra plus folic acid is 18–45% (1–5). The validity of this conclusion continues to be under clinical scrutiny in an attempt to establish whether both protocols have been compared at equivalent levels of toxicity.

FUra and its deoxyribonucleoside, FdUrd, can be metabolized toFdUMP (6), a potent inhibitor of TS (7). Inhibition of TS by FdUMP is the result of the formation of a covalent complex between enzyme and inhibitor that occurs in the presence of the folate cofactor for the TS reaction, 5,10-methyl-
were purchased from Sigma Chemical Co. (St Louis, MO). All other chemicals were reagent grade. The concentration of solutions of these compounds were adjusted spectrophotometrically using published extinction coefficients. The folinic acid concentrations listed refer to the total concentration of (6-R,S)5-formyltetrahydrofolate used.

Cell Culture. WiDr and T-24 cells were maintained in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and were passaged twice a week. Cultures were not allowed to remain at confluence prior to passage. New cultures were established from Mycoplasma-free frozen stocks every 3 months, and cultures were tested for Mycoplasma prior to being replaced by newly thawed stocks.

Cytotoxicity of drug treatments was determined by colony formation following plating at low density in either 60-mm (WiDr) or 100-mm dishes (T-24). Cells were plated at the desired densities in 5 (WiDr) or 12 (T-24) ml of medium and allowed to attach for 24 h prior to drug treatment. Fresh medium (RPMI 1640 with 10% dialyzed fetal calf serum) that contained FUra, FdUrd, and/or folinic acid was added and drug treatment was ended by aspiration of the medium and washing the cells once with phosphate-buffered saline. Fresh medium was added twice a week thereafter and colonies were fixed, stained with Giemsa, and counted manually after 2–3 weeks of growth. In experiments in which growth inhibition by drugs was noted (a smaller size of colonies on drug-treated plates when control dishes had colonies optimal for counting), drug-treated plates were allowed to incubate for 3 days longer. All macroscopically visible colonies were scored as viable. All experiments were performed at least twice on separate weeks.

RESULTS

Cytotoxicity of Fluoropyrimidines to Human Carcinoma Cells in the Presence of Folinic Acid. When WiDr human colon carcinoma cells were exposed to increasing concentrations of FUra for 72 h, the ability of individual cells to form colonies diminished at concentrations ≥2.5 μM, whereas in the presence of 10 μM folinic acid, FUra concentrations ≥0.5 μM diminished survival (Fig. 1A). At 3 μM FUra alone, 44 ± 9% (SD) of exposed WiDr cells formed progeny, while the same concentration of FUra reduced cell survival to 0.2 ± 0.06% in the presence of 10 μM folinic acid. A similar enhanced cytotoxicity was found for WiDr cells exposed to FdUrd and folinic acid compared to those exposed to FdUrd alone (Fig. 1B), although the potency of FdUrd was ~500 times higher than that of FUra. Thus, at a concentration of FdUrd (3 nM) that reduced survival to 62 ± 4% by itself, survival was reduced to 1.7 ± 0.3% in the presence of 10 μM folinic acid. A distinct shoulder on the cell kill curve was repeatedly observed with FUra (Fig. 1A) that was not seen with FdUrd, as evidenced by the fact that the cell kill curve passed through the origin with FdUrd but not with FUra (Fig. 1).

Folinic acid also increased the cytotoxic potency of both FUra and FdUrd against the T-24 human bladder carcinoma cell line (data not shown). Little or no shoulder was seen for the T-24 cells with either FUra or FdUrd. A 1-log kill (i.e., the LD90) for WiDr cells was reached at 4.7 μM FUra alone and 1.6 μM FUra in the presence of folinic acid (ratio = 2.9); for T-24 cells, the LD90 for FUra was 17.3 μM without and 5.6 μM with folinic acid (ratio = 3.1). Likewise, there was little difference in the ratio of LD90 for FdUrd in the absence and presence of folinic acid between WiDr (8.4 nM/1.7 nM = 4.9) and T-24 cells (7.6 nM/1.2 nM = 6.3). These results are very similar to those of our previous experience with these combinations for mouse L1210 cells (19). Hence, it appeared that the enhanced cytotoxicity of the fluoropyrimidines previously found in L1210 cells extends to at least these two examples of human carcinoma cells.

Exposure to Folinic Acid Required to Enhance the Cytotoxicity of FUra in WiDr Cells. The concentration of and length of exposure to folinic acid required to elicit an enhancement of the cytotoxicity of a 72-h exposure to FUra were examined in WiDr cells. Exposure of WiDr cells to either a minimally cytotoxic concentration of FUra (1.5 μM) or a concentration that individually caused 50% cell kill (2.5 μM) and increasing concentrations of folinic acid resulted in enhancement of the effects of both test doses of FUra over the same folinic acid concentration range (Fig. 2). Measurable enhancement was observed in the presence of 0.03 μM folinate but the dose-response curve did not maximize at the highest concentration tested (32 μM). However, the slope of this curve was very shallow. Likewise, when WiDr cells were exposed to 2.5 μM FUra for 72 h and to 10 μM folinic acid for increasing periods of this FUra treatment, the cytotoxicity of the test dose of FUra continually increased (Fig. 3). However, the survival of FUra-treated tumor cells exposed to folinic acid for 48 h was not different from that of cells exposed for 72 h. In addition, exposure to folinic acid for as little as 30 min increased the cytotoxicity of 2.5 μM FUra by more than 2.5-fold (Fig. 3, inset). We conclude that the more extensive the exposure to folinic acid, the greater is the cytotoxicity of a continuously present concentration of FUra, but a point of diminishing returns is reached at concentrations of about 10 μM folinic acid or exposure times (to folinic acid) of about 48 h.

Effectiveness of Folinic Acid-induced Augmentation of the Cytotoxicity of Brief Exposures to FUra. Although the enhancement of FUra by folinic acid demonstrated in Figs. 1–3 would apply to the circumstance of a 3-day infusion of FUra, the most
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A common method of administration of FUra is repeated bolus doses. Hence, we evaluated the effectiveness of FUra and folinic acid resultant from a 4-h exposure to FUra, while holding the exposure to folinic acid constant at 72 h, beginning simultaneously with the addition of FUra. As expected from previous literature (16, 21), much higher concentrations of FUra (≥50 μM) (Fig. 4) were required for cytotoxicity of a 4-h exposure to drug than for cytotoxicity during continuous exposure (Fig. 1). An unexpected finding of these experiments was that folinic acid augmented the cytotoxicity of brief exposures to FUra (Fig. 4) to a much lower degree than for a 72-h exposure to FUra (Fig. 1). Not only was the shoulder of the dose-response curve smaller, but the slope of the FUra alone cell kill curve was not significantly different than that of the FUra plus folinate cell kill curve. The cytotoxicity of FUra was no more than 5-fold different for these two conditions at any concentration on the cell kill curves for a 4-h exposure (Fig. 4).

In order to confirm this major difference in the degree of folinate-induced enhancement of the cytotoxicity of brief (Fig. 4) and extended (Fig. 1) exposures to FUra, the effects of folinic acid on the cytotoxicity of FUra were determined following different periods of exposure to FUra. In order to make this comparison within the same experiment, concentrations of FUra were chosen for each time interval so that the level of cytotoxicity of FUra alone was maintained on the range of 75 to 95% cell kill. The concentration of FUra that caused this level of cytotoxicity ranged from 100 μM for a 4-h exposure to 3.5 μM for a 72-h exposure. The results of these experiments (Table 1) confirmed a major difference in the effectiveness of folinic acid at enhancing the cytotoxicity of brief and extended exposures to FUra when both treatments were compared in the same experiment. These experiments indicated that enhancement of FUra by folinic acid became more extensive after 24-h or more of exposure to FUra (Table 1; Fig. 5). It was found that the enhancement of FUra by folinic acid did not significantly change for lengths of exposure to FUra of up to about one generation time, then, with longer exposure to FUra, folinate-induced enhancement dramatically increased (Fig. 5). It should be noted that our data do not indicate a maximization of this effect at the longest time of FUra exposure (72 h) studied.

DISCUSSION

An enhanced therapeutic effect of FUra by folinic acid has been documented clinically (2-5) but has been difficult to reproduce in animal tumor systems (22-24). Prior to this study, the addition of folinic acid had not been shown to result in major potentiation of the cytotoxicity of the fluoropyrimidines in any human carcinoma cell line in culture. In the original report on this interaction, Ullman et al. (15) demonstrated a 3 to 4-fold augmentation of the growth-inhibitory potency of FdUrd against mouse leukemic cells in culture. More extensive studies by Evans et al. (16, 17) documented a 2 to 3-fold increase...
in the growth-inhibitory potency of FUra and FdUrd to mouse Sarcoma 180 and human Hep-2 carcinoma cells. Several later studies by us (14, 19) and others (25, 26) have concentrated on
coloncarcinoma cell lines after continuous exposure
to drugs for 3 days. This augmentation of the cytotoxicity of the fluoropyrimidines by folinic acid in the WiDr colon
and T-24 bladder carcinoma cell lines was very similar in magnitude
to that previously reported for the mouse LI210 leukemia cell
line (19). This interaction was markedly more extensive than
the interactions previously reported (20) for colon
carcinoma cell lines, using a rapid assay that depended on scoring of cell
growth using tetrazolium dye staining and a microtiter plate
reader. In that report, Park et al. (20) reported that a shift in
growth inhibition curves (16, 19, 20). However, it is also the case that
experiments that rely on scoring of the production of progeny
from individual treated cells allows a more definitive assessment
of cytotoxicity per se and, often, a more unambiguous interpre-
tation of data.
Perhaps one of the more important questions in this area at

**Table 1** Folinic modulation of FUra cytotoxicity: effect of prolonged exposure to FUra

<table>
<thead>
<tr>
<th>FUra exposure</th>
<th>Folinic acid (µM)</th>
<th>Cells plated</th>
<th>Colonies formed</th>
<th>% of survival</th>
<th>Ratio</th>
<th>% of survival</th>
<th>Ratio</th>
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<tr>
<td>0 h 0 µM</td>
<td>0</td>
<td>250</td>
<td>175 ± 18</td>
<td>100 ± 15</td>
<td>1.00</td>
<td>100 ± 13</td>
<td>1.05</td>
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<tr>
<td>0 h 10 µM</td>
<td>0</td>
<td>250</td>
<td>167 ± 8</td>
<td>95 ± 11</td>
<td>0.95</td>
<td>105 ± 17</td>
<td>1.05</td>
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<td>4 h 100 µM</td>
<td>0</td>
<td>250</td>
<td>39 ± 7</td>
<td>22 ± 4.6</td>
<td>4.2</td>
<td>24 ± 5</td>
<td>4.5</td>
</tr>
<tr>
<td>4 h 100 µM</td>
<td>100 µM</td>
<td>1250</td>
<td>46 ± 6</td>
<td>5.6 ± 0.79</td>
<td>4.2</td>
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<td>4.5</td>
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<tr>
<td>8 h 40 µM</td>
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<td>250</td>
<td>21.7 ± 2.5</td>
<td>12.6 ± 2.7</td>
<td>4.9</td>
<td>15.7 ± 4.1</td>
<td>3.3</td>
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<tr>
<td>8 h 40 µM</td>
<td>10 µM</td>
<td>1250</td>
<td>22.3 ± 1.5</td>
<td>2.6 ± 0.3</td>
<td>4.9</td>
<td>5.5 ± 2.3</td>
<td>4.9</td>
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<tr>
<td>8 h 60 µM</td>
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<td>250</td>
<td>18.7 ± 5</td>
<td>10.9 ± 3.1</td>
<td>4.3</td>
<td>6.8 ± 0.2</td>
<td>8.1</td>
</tr>
<tr>
<td>8 h 60 µM</td>
<td>10 µM</td>
<td>1250</td>
<td>21.5 ± 6.4</td>
<td>2.5 ± 0.8</td>
<td>4.3</td>
<td>12.9 ± 3.1</td>
<td>4.2</td>
</tr>
<tr>
<td>12 h 25 µM</td>
<td>0</td>
<td>250</td>
<td>42 ± 9</td>
<td>24 ± 6</td>
<td>4.9</td>
<td>10.3 ± 0.9</td>
<td>5.0</td>
</tr>
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<td>12 h 25 µM</td>
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<td>1250</td>
<td>49 ± 4</td>
<td>5.7 ± 0.7</td>
<td>5.0</td>
<td>3.1 ± 0.4</td>
<td>4.2</td>
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<td>5000</td>
<td>149 ± 12</td>
<td>4.3 ± 0.5</td>
<td>5.6</td>
<td>3.3 ± 0.4</td>
<td>3.9</td>
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<tr>
<td>12 h 35 µM</td>
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<td>250</td>
<td>18.7 ± 5</td>
<td>10.9 ± 3.1</td>
<td>4.3</td>
<td>3.3 ± 0.8</td>
<td>3.9</td>
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<tr>
<td>12 h 35 µM</td>
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<td>1250</td>
<td>21.5 ± 6.4</td>
<td>2.5 ± 0.8</td>
<td>4.3</td>
<td>6.8 ± 0.2</td>
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<td>5000</td>
<td>90 ± 9</td>
<td>2.6 ± 0.35</td>
<td>4.2</td>
<td>0.86 ± 0.13</td>
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<td>37 ± 8</td>
<td>21 ± 5</td>
<td>4.9</td>
<td>10.3 ± 0.9</td>
<td>5.0</td>
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<td>1250</td>
<td>18.7 ± 3.5</td>
<td>2.2 ± 0.45</td>
<td>9.8</td>
<td>0.9 ± 0.15</td>
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<td>5000</td>
<td>88 ± 9</td>
<td>2.6 ± 0.34</td>
<td>8.1</td>
<td>1.1 ± 0.15</td>
<td>9.6</td>
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<tr>
<td>24 h 24 µM</td>
<td>0</td>
<td>250</td>
<td>10.3 ± 4.5</td>
<td>6.0 ± 2.7</td>
<td>4.9</td>
<td>0.17 ± 0.07</td>
<td>5.0</td>
</tr>
<tr>
<td>24 h 24 µM</td>
<td>10 µM</td>
<td>1250</td>
<td>4.7 ± 1.2</td>
<td>0.55 ± 0.15</td>
<td>11.0</td>
<td>0.17 ± 0.07</td>
<td>5.0</td>
</tr>
<tr>
<td>48 h 6 µM</td>
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<td>250</td>
<td>11.5 ± 2.1</td>
<td>6.7 ± 1.4</td>
<td>11.4</td>
<td>1.4 ± 2.3</td>
<td>4.8</td>
</tr>
<tr>
<td>48 h 10 µM</td>
<td>0</td>
<td>5000</td>
<td>8.0 ± 4.0</td>
<td>0.047 ± 0.024</td>
<td>144</td>
<td>0.24 ± 0.08</td>
<td>48</td>
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<tr>
<td>48 h 10 µM</td>
<td>25000</td>
<td>8.0 ± 4.0</td>
<td>0.047 ± 0.024</td>
<td>144</td>
<td>0.19 ± 0.04</td>
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<td>48 h 8 µM</td>
<td>0</td>
<td>250</td>
<td>3.0 ± 1.0</td>
<td>1.74 ± 0.6</td>
<td>4.7</td>
<td>1.4 ± 0.40</td>
<td>4.4</td>
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<tr>
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<td>10 µM</td>
<td>5000</td>
<td>4.8 ± 9.6</td>
<td>0.048 ± 0.018</td>
<td>36</td>
<td>0.05 ± 0.02</td>
<td>49</td>
</tr>
<tr>
<td>48 h 8 µM</td>
<td>10 µM</td>
<td>25000</td>
<td>4.3 ± 6.0</td>
<td>0.025 ± 0.004</td>
<td>70</td>
<td>0.03 ± 0.01</td>
<td>49</td>
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<tr>
<td>72 h 3.5 µM</td>
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<td>250</td>
<td>18.0 ± 2.0</td>
<td>10.5 ± 1.5</td>
<td>4.9</td>
<td>15.2 ± 2.7</td>
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</tr>
<tr>
<td>72 h 3.5 µM</td>
<td>10 µM</td>
<td>25000</td>
<td>2.0 ± 1.0</td>
<td>0.012 ± 0.006</td>
<td>900</td>
<td>0.050 ± 0.017</td>
<td>300</td>
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</table>

*Mean ± SD. Each group contained triplicate plates except where denoted by footnote b.*

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Fig. 5. Effect of time of exposure to FUra on the enhancement of a test dose of FUra by 10 µM folinic acid. WI/Dr cells were exposed to a concentration of FUra that killed 75–95% of the cells by itself; these concentrations were: 4 h, 80–120 µM; 8 h, 40 and 60 µM; 12 h, 25 and 35 µM; 24 h, 12 and 18 µM; 48 h, 6 and 8 µM; 72 h, 3.5 µM. Other groups of cultures were exposed to the same concentrations of FUra for these times and to 10 µM folinic acid for 72 h. The enhancement of the cytotoxicity of the test dose of FUra is plotted as the ratio of the survival of the FUra-treated cells to the survival of the FUra and folinic acid-treated plates as a function of the length of exposure to FUra. Points, average values pooled from replicate experiments; bars, SD. Data from 9 (4 h), 3 (8 h), 7 (12 h), 5 (24 and 48 h), and 6 (72 h) individual experiments performed on different days were pooled for these estimates; triplicate plates were used in each group in each experiment.
the moment is the generality of the effect demonstrated in Fig. 1. One would predict that this interaction would not be universally the case, given that clinical response rates to FUra plus reduced folates among colonic carcinomas have not been 100%. Identification of cell lines that would represent responding and nonresponding phenotypes would be a major help in understanding the limitations of FUra/folinic acid chemotherapy (27).

The magnitude of the folinic acid-induced increased FUra cytotoxicity was strikingly affected by the length of exposure to FUra for WiDr cells (Fig. 5; Table 1). If this time dependency holds under clinical circumstances, one would suggest that a long-term (≥2 days) coinfusion of FUra and folinic acid (or infusion of FUra with frequent oral administration of folinic acid) might offer advantages over bolus or short-term infusion of FUra with folinic acid. However, it seems unlikely that the tremendous increment in interaction demonstrated in Table 1 with long-term exposure to FUra and folinic acid would be clinically achievable without substantial increases in host toxicity. It should be noted that Lokich et al. (28) and Leichman et al. (29) have reported response rates of colorectal carcinomas to long-term infusions of FUra by itself that were as significant as those reported in the FUra/folinic acid trials (2–5). In addition, Leichman et al. have indicated that the protracted infusion of FUra given with folinic acid was a very active protocol, resulting in response rates greater than 45% in a small series of heavily pretreated patients (29).

The much larger interaction seen between folinic acid and FUra upon prolonged exposure to FUra, relative to that observed at shorter exposure intervals, was unexpected. The mechanistic explanation of this effect is unknown. Berger and Hakala (18) have shown that short-term exposure of Hep-2 and S-180 cells to high concentrations of FUra resulted in the synthesis of very high levels ofFdUMP which quickly decayed back to zero when drug was removed from the medium. Long-term exposure to FUra would be expected to generate lower levels ofFdUMP, but the FdUMP would be continuously present in the cells. If the major mechanism of folinic acid stimulation of FUra effects is due to stabilization of ternary complex (14), it may be the case that this stabilization functions optimally only when some FdUMP is available to replace inhibitor occasion ally lost from the surface of TS (14) under circumstances of an increased dUMP pool (30–33). However, it may also be the case that the continued synthesis of TS in the absence of cell division which results in accumulation of the cellular content of TS (14) occurs fast enough to allow tumor cell survival in the absence of a continual supply of FdUMP.

The cytotoxic potentional documented in this study appears to be underlying the clinical results that are being obtained with this combination. However, the need exists to extend the encouraging clinical results of increased response rate of colonic carcinomas to FUra afforded by folinic acid (1–5) into clear improvements in survival. Given the large number of potential variables (other drugs, other additional biochemical modifying agents, schedule of drug administration, etc.), it seems prudent to answer the question of how these possible modifications would be acting as a means of choosing among the large number of answers to the question “What next?”

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REFERENCES


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