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, and there was a diminished increment of cytotoxicity after 24-h exposure to folic acid. Surprisingly, folic acid enhancement 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 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 only to a small extent (2 to 4-fold). However, when the combination was used using a soft agar plating assay as an index of cytotoxicity, the activity of both FUra and FdUrd was found to be increased substantially in L1210 cells, with minimally cytotoxic concentrations of either drug killing >99.9% of these cells in the presence of folic acid (19). Clearly, the effects of the combination on tumor cells were the same whether examined by either growth inhibition or cytotoxicity assays, but an enhancement of activity with the combination was much more easily detected when tumor cell survival was followed. It could also be argued that tumor cell kill is more relevant to therapeutics than growth inhibition.

In this study, we reexamined the effects of FUra and FdUrd in the presence of folic acid on the ability of cultured cells to produce progeny, in an effort to determine whether the cytotoxic enhancement we previously observed in mouse leukemic cells extends to human carcinoma cells in culture. We have also examined the effects of changes in dose of fluoropyrimidines and of folic acid, time of exposure to folic acid, and length of exposure to Fura on the enhancement of the drug effects by the reduced folate. Our results suggest that the extent of the cytotoxic potentiation achievable with this combination is substantially greater when tumor cells are continuously exposed to FUra for several days.

MATERIALS AND METHODS

Materials. WiDr and T-24 cells were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media, reagents, and serum were from Gibco (Grand Island, NY). Folic acid [the calcium salt of (6- R,S)-5-formyltetrahydrofolate], FUra, and FdUrd were obtained from Sigma Chemical Co. (St. Louis, MO).

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1. The abbreviations used are: 5-FUra, 5-fluorouracil; 5-FdUrd, 5-fluoro-2'-deoxyuridine; LDM, concentration of drug that reduces cell survival to 10% of control; TS, thymidylate synthase; FdUMP, 5-fluoro-2'-deoxyribonucleoside-5'-monophosphate.
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 (6R,5S)-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 colonic 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 FUratreated 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
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 FUra addition. After the indicated periods of time, medium was removed, cells were washed with phosphate-buffered saline, and fresh medium containing FUra was added. After a total of 72-h exposure to FUra, fresh medium was added. Points, average survival of triplicate dishes from each of two experiments, expressed as a percentage of the plating efficiency of untreated controls; bars, SD. The inset replots early time points from the main figure on a linear scale.

**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 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.

**FLUOROURACIL AND FOLINATE CYTOTOXICITY**

**Fig. 2.** Augmentation of the effect of FUra on WiDr cells after exposure to increasing concentrations of folinic acid. WiDr cells (200–25,000/plate) were exposed to either 1.5 μM or 2.5 μM FUra for 72 h beginning 24 h after plating. The indicated concentrations of folinic acid [6-[(R,S)-5-formyltetrahydrofolate]] were added with the culture medium and were present throughout the exposure to FUra. The symbols denote the survival of cells relative to phosphate-buffered saline-treated cells, which had a plating efficiency of 143 ± 11 (SD) colonies for 200 cells plated (Experiment 1) and 206 ± 7 colonies for 300 cells plated (Experiment 2).

**Fig. 3.** Survival of WiDr cells treated with FUra for 72 h and folinic acid for increasing intervals. WiDr cells were treated with 2.5 μM FUra starting 24 h after plating and with 10 μM folinic acid for increasing lengths of time beginning simultaneously with FUra addition. After the indicated periods of time, medium was removed, cells were washed with phosphate-buffered saline, and fresh medium containing FUra was added. After a total of 72-h exposure to FUra, fresh medium was added. Points, average survival of triplicate dishes from each of two experiments, expressed as a percentage of the plating efficiency of untreated controls; bars, SD. The inset replots early time points from the main figure on a linear scale.

**Fig. 4.** Survival of WiDr cells after a brief exposure to FUra with (●) and without (○) folinic acid. WiDr cells (250–25,000 cells) were exposed to the indicated concentrations of FUra and/or 10 μM folinic acid starting 24 h after plating. FUra was removed after 4 h but folinic acid was present in the medium for 72 h. Points, mean survival of triplicate plates from 2–4 experiments; bars, SD.

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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 leukemic cells. In this study, we have demonstrated a marked cytotoxic potentiation of either FUra or FdUrd by folinic acid for two human carcinoma cell lines after continuous exposure to drugs for 3 days. This augmentation of the cytotoxicity of the fluoropyrimidines by folinic acid in the WiDr colonic and T-24 bladder carcinoma cell lines was very similar in magnitude to that previously reported for the mouse L1210 leukemia cell line (19). This interaction was markedly more extensive than the interactions previously reported (20) for colonic 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 the growth inhibitory potency of FUra of 2-fold was found in only 1 of the 11 colonic carcinoma cell lines screened, and that the growth inhibition by FUra was either affected to a smaller extent or was unaffected by addition of folinic acid in the remaining 10 cell lines. We have previously concluded (19) that reliance on growth inhibition as an index underestimates the extent or was unaffected by addition of folinic acid in the remainder of FUra exposure of 2-fold was found in

Table 1. Folinate 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>
</tr>
</thead>
<tbody>
<tr>
<td>0 h 0 µM 0 µM</td>
<td>250</td>
<td>175 ± 18*</td>
<td>100 ± 15</td>
<td>0.95</td>
<td></td>
<td>100 ± 13</td>
<td>1.05</td>
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<tr>
<td>0 h 10 µM 0 µM</td>
<td>250</td>
<td>167 ± 8</td>
<td>95 ± 11</td>
<td></td>
<td>0.91</td>
<td></td>
<td></td>
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<tr>
<td>100 µM 0 µM</td>
<td>250</td>
<td>39 ± 7</td>
<td>22 ± 4.6</td>
<td></td>
<td>0.90</td>
<td></td>
<td></td>
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<tr>
<td>100 µM 1250 µM</td>
<td>46 ± 6</td>
<td>5.4 ± 0.79</td>
<td>4.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>4 h 0 µM 0 µM</td>
<td>250</td>
<td>21.7 ± 4.2</td>
<td>12.6 ± 2.7</td>
<td>4.9</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>8 h 0 µM 0 µM</td>
<td>250</td>
<td>22.3 ± 1.5</td>
<td>2.6 ± 0.3</td>
<td></td>
<td>0.20</td>
<td></td>
<td></td>
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<tr>
<td>12 h 0 µM 0 µM</td>
<td>250</td>
<td>42 ± 9</td>
<td>24 ± 6</td>
<td></td>
<td>1.29</td>
<td></td>
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<tr>
<td>24 h 0 µM 0 µM</td>
<td>250</td>
<td>37 ± 8</td>
<td>21 ± 5</td>
<td></td>
<td>1.03</td>
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<tr>
<td>4 h 0 µM 10 µM</td>
<td>1250 µM</td>
<td>18.7 ± 3.5</td>
<td>2.2 ± 0.45</td>
<td>9.8</td>
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<tr>
<td>8 h 0 µM 10 µM</td>
<td>1250 µM</td>
<td>88 ± 9</td>
<td>2.6 ± 0.34</td>
<td>8.1</td>
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<tr>
<td>12 h 0 µM 10 µM</td>
<td>1250 µM</td>
<td>149 ± 12</td>
<td>4.3 ± 0.5</td>
<td>5.6</td>
<td></td>
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<tr>
<td>24 h 0 µM 10 µM</td>
<td>1250 µM</td>
<td>18.7 ± 5</td>
<td>10.9 ± 3.1</td>
<td>4.3 ± 0.86</td>
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<td>1250 µM</td>
<td>21.5 ± 6.4</td>
<td>2.5 ± 0.8</td>
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<td>24 h 10 µM 0 µM</td>
<td>1250 µM</td>
<td>90 ± 9</td>
<td>2.6 ± 0.35</td>
<td>4.2</td>
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<tr>
<td>48 h 6 µM 0 µM</td>
<td>250</td>
<td>11.5 ± 2.1</td>
<td>6.7 ± 1.4</td>
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<td>1.14</td>
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<tr>
<td>48 h 6 µM 10 µM</td>
<td>5000 µM</td>
<td>88 ± 9</td>
<td>2.6 ± 0.34</td>
<td>8.1</td>
<td></td>
<td></td>
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<tr>
<td>48 h 6 µM 25000 µM</td>
<td>1250 µM</td>
<td>8.0 ± 4.0</td>
<td>0.047 ± 0.024</td>
<td>144</td>
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<td>48 h 8 µM 0 µM</td>
<td>250</td>
<td>3.0 ± 1.0</td>
<td>1.74 ± 0.60</td>
<td>1.48 ± 0.40</td>
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<td>5000 µM</td>
<td>1.67 ± 0.6</td>
<td>0.048 ± 0.018</td>
<td>36</td>
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<tr>
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<td>1250 µM</td>
<td>4.3 ± 0.6</td>
<td>0.025 ± 0.004</td>
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<td>18.0 ± 2.0</td>
<td>10.5 ± 1.5</td>
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<td>15.2</td>
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<tr>
<td>72 h 3.5 µM 10 µM</td>
<td>25000 µM</td>
<td>2.0 ± 1.0</td>
<td>0.012 ± 0.006</td>
<td>900</td>
<td></td>
<td>0.050 ± 0.017</td>
<td>300</td>
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* Mean ± SD. Each group contained triplicate plates except where denoted by footnote b.

from individual treated cells allows a more definitive assessment of cytotoxicity per se and, often, a more unambiguous interpretation of data.

Perhaps one of the more important questions in this area at
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 folic acid-induced increased FUra cytotoxicity was strikingly affected by the length of exposure to FUra for WiDr cells (Fig; Table 1). If this time dependency holds under clinical circumstances, one would suggest that a long-term (>2 days) coinfusion of FUra and folic acid (or infusion of FUra with frequent oral administration of folic acid) might offer advantages over bolus or short-term infusion of FUra with folic acid. However, it seems unlikely that the tremendous increment in interaction demonstrated in Table 1 with long-term exposure to FUra and folic 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 colonic 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 folic 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 folic 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 of FdUMP 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 of FdUMP, but the FdUMP would be continuously present in the cells. If the major mechanism of folic 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 occasionally lost from the surface of TS (14) under circumstances of an expanded 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 potentiation 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 folic 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?’

ACKNOWLEDGMENTS

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REFERENCES


* This query was raised as the central point of a symposium held on chemotherapy of colorectal carcinoma in February of 1990 in Miami, FL, by the organizer of that meeting, Dr. Bach Ardalan.


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