Improved Therapeutic Index with Sequential N-Phosphonacetyl-L-aspartate plus High-Dose Methotrexate plus High-Dose 5-Fluorouracil and Appropriate Rescue

Daniel S. Martin, Robert L. Stolfi, Robert C. Sawyer, Sol Spiegelman, and Charles W. Young


ABSTRACT

Although clinical trials of high-dose methotrexate (MTX) sequenced before 5-fluorouracil (FUra) with leucovorin (LV) rescue apparently have resulted in increased numbers of tumor responses, this increased antitumor activity often has been accompanied with toxicity. The present report describes an attempt to improve therapeutic results with this drug combination by appropriate metabolic modulation in the preclinical BALB/c × DBA/2 F1 murine breast tumor model. A LV rescue schedule consisting of 300 mg/kg administered at 4.5 and 19.5 hr after high-dose MTX (300 mg/kg/week for 3 weeks) prevented MTX toxicity. When FUra was administered 2.5 hr after MTX (with LV rescue), the dose of FUra had to be decreased, and we could not obtain convincing evidence for a differential cytotoxic effect on tumor versus normal host tissue. However, when a delayed uridine rescue schedule was added to protect the host from the toxic activity of FUra, the FUra dose could be increased even in the presence of high-dose MTX, and the therapeutic result was enhanced significantly without an increase in host toxicity. Finally, it was possible to add N-phosphonacetyl-L-aspartate to this drug combination (in the appropriate sequence: N-phosphonacetyl-L-aspartate before high-dose MTX-before high-dose FUra, followed by double rescue with LV and uridine) without producing increased toxicity to yield a significant increase in partial tumor regression rate. The biochemical rationale for the selection and sequence of administration of these agents is discussed.

INTRODUCTION

In vivo schedule-dependent antitumor synergy when MTX preceded FUra was reported in our laboratories in 1976 (43) and subsequently confirmed in both in vitro (3, 4, 6, 16, 17, 23, 25, 50, 66) and in vivo (8, 13, 28, 40, 45, 53) preclinical studies. Less than additive, or even antagonistic, interactions may be obtained when the 2 drugs are given simultaneously or if FUra precedes MTX (11, 12, 32, 63, 64, 66, 68).

The mechanism for the synergistic interaction has not been established. Two FUra mechanisms need to be considered: (a) inactivation of thymidylate synthetase; and/or (b) the formation of (FUra)RNA. Inactivation of thymidylate synthetase by FdUMP requires the formation of a ternary complex among FdUMP, 5,10-methylenetetrahydrofolate, and thymidylate synthetase, leading to the covalent binding of FdUMP to this enzyme (58). On the basis of data from Santl et al. (58), Bertino (7, 8) has postulated that treatment with MTX would result in high intracellular levels of MTX which would act as an analogue of the tetrahydrofolate cofactor (5,10-methylenetetrahydrofolate) for thymidylate biosynthesis; as a result, FdUMP would bind irreversibly in ternary complex with MTX to thymidylate synthetase. More recently, Fernandes et al. (25) have proposed that MTX inhibition of dihydrofolate reductase results in a trapping of intracellular folates as dihydrofolate polyglutamates that could increase the extent of FdUMP binding to thymidylate synthetase. A different mechanism of synergism, involving the greater formation of (FUra)RNA, is proposed by Cadman et al. (16, 17) and Benz et al. (3, 4). This group finds that pretreatment by MTX inhibits de novo purine synthesis causing an accumulation of PRPP. Since supplies of PRPP are rate-limiting for the ribosylphosphorylation of FUra into its active ribonucleotides (56), the MTX-induced increased intracellular pools of PRPP result in greater intracellular conversion of FUra into its ribonucleotides, enhanced incorporation of FUra in RNA, and a greater antitumor effect.

The above preclinical reports have stimulated a number of pilot clinical trials of sequential MTX-FUra therapy in the treatment of advanced head and neck cancers (33, 54, 55, 57), breast cancer (1, 5, 26, 31, 65), colorectal cancers (9, 19, 24, 30, 35, 60, 65, 69) and gastric (36) and miscellaneous tumors (14, 71). Although these preliminary clinical trials are all noncontrolled, nonrandomized pilot probes, it is significant that an apparently greater number of antitumor responses (compared to historical controls) were noted in most trials (Table 1). However, of concern in Table 1 is the observation that patient toxicity, sometimes severe, was also reported (33, 60, 69, 71). Mulder et al. (53) have warned, "As the toxicity of this sequential treatment schedule should lead to drug dose reductions, the physician may wind up with a less toxic but at the same time also less effective antitumor treatment schedule... More preclinical research is needed...".

Wieman et al. (71) noted that, "the incidence and severity of toxicity... were best related to the 5-FUra dose rather than to MTX pharmacokinetics." Of pertinence to this toxicity, we (49), Johnson et al. (34), Klubes et al. (38), and Wickramanayake and Klein (70) have reported that a uridine rescue schedule can control FUra-induced murine host toxicity. Thus, since the toxicity of the MTX-FUra sequential combination is "best related to
the 5-FUra dose* (71), we have added uridine rescue to a high-
dose MTX → FUra → LV sequence. This publication now reports
that the sequential combination of high-dose MTX followed by
high-dose FUra-uridine-LV rescue in a preclinical solid breast
tumor murine model achieves enhanced antitumor efficacy with
control of host toxicity.

The effect of adding PALA to this MTX-FUra combination was
also studied. PALA, an inhibitor of de novo pyrimidine synthesis,
(20, 34, 51, 52) can increase the entry of FUra into RNA (2, 39,
59, 61) by lowering the levels of the normal pyrimidine nucleotide
that competes with FUra for incorporation into RNA. Perhaps
less significantly, PALA also can produce a modest elevation of
PRPP levels by its inhibition of pyrimidine biosynthesis (3, 39,
41). PALA was added to the combination of MTX with FUra
based upon our previous in vivo findings with the combination
of PALA, FUra, and 6-methylmercaptopurine riboside. The latter
agent, like MTX, is an inhibitor of de novo purine synthesis and
also elevates PRPP levels as MTX does, and the sequential
PALA-6-methylmercaptopurine riboside-FUra combination was
shown to modulate the metabolism of FUra, to enhance the
formation of (FUra)RNA, and to increase antitumor activity of
FUra in vivo (44, 45, 47, 49). Others have reported similar findings
(3, 15, 39, 41) in in vitro studies. In accord with these previously
reported preclinical studies, we now report that the prior admin-
istration of PALA to the sequential combination of MTX-FUra
followed by LV-Urd rescue in vivo further enhances antitumor
efficacy. It seems particularly important to stress that these are
in vivo studies, and the improved results are accomplished
without untoward toxicity.

MATERIALS AND METHODS

Murine Tumor System. For each experiment, BALB/c × DBA/8 F,1
(hereafter called CD8F1) mice bearing a first-generation spontaneous
syngeneic tumor transplant were distributed among experimental groups
so that mice bearing tumors of approximately equal weight were repre-
sented in each group. The mice were weighed before each of 3 weekly
courses of treatment (described in text) and again at 5 days after each
course of treatment. Tumor measurements were recorded at 5 days after each weekly course of treatment.

Tumor Measurements. Two axes of the tumor (the longest axis, L,
and the shortest axis, W) were measured with the aid of a vernier caliper.
Tumor weight was estimated according to:

\[
\text{Tumor wt (mg)} = \frac{L (\text{mm}) \times W (\text{mm})^2}{2}
\]

Chemotherapeutic Agents. FUra, MTX, LV, and uridine were ob-
tained from Sigma Chemical Co., St. Louis, Mo. Each of these agents
was dissolved in 0.85% NaCl solution immediately before use. PALA
was obtained from the Department of Health, Education, and Welfare,
USPHS of the National Cancer Institute, Bethesda, Md. PALA was
dissolved in 0.85% NaCl solution, and the pH was adjusted to 7.2 to 7.5
with 1 N NaOH before adjustment to final volume. All agents were
administered i.p. so that the desired dose was contained in 0.1 ml/10 g
of mouse weight. A 0.85% NaCl solution was administered to controls.

Statistical Evaluation. Student's t test was used for statistical eval-
uation of differences in tumor size between groups. Differences in partial
tumor regression rate between groups were compared for statistical
significance by \(\chi^2\) analysis. For all statistical evaluations, differences
between groups with \(p < 0.05\) were considered significant.

Determination of PR. The initial size of each animal's tumor prior to
initiation of therapy was recorded. Tumor size was recorded weekly,
and final tumor size was determined 5 days after the third weekly course
treatment by the same observer of the initial tumor size. By convention,
PR is defined as at least a 50% shrinkage in the volume of the initially
measured tumor lesion, i.e.:

\[
\text{Final tumor size} \times 100\% = \frac{\text{Initial tumor size}}{\text{No. of PR/group}} \times 100\% \\
\text{Total no. of animals/group}
\]

The PR rate per treatment group is expressed as a percentage, i.e.

The same degree of caliper error is usually made by a single observer in
repeated measurements. Hence, the same observer always performs
both the initial and the final tumor measurements.

RESULTS

High-Dose MTX → LV Rescue. Table 2 records 8 (4 male
and 4 female) experiments in CD8F1 mice bearing well-advanced

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**Table 1**

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Response rate (%)</th>
<th>MTX dose* (mg/sq m)</th>
<th>MTX-FUra interval (hr)</th>
<th>FUra dose (mg/sq m)</th>
<th>Schedule</th>
<th>Toxicity</th>
</tr>
</thead>
</table>
| Head and neck (54, 55) | 71 | 90-250 | 1 | 600 | Every 1-2 wk | ++
| Head and neck (57) | 64 | 200 | 2 | 600 | Every 1-2 wk | +
| Head and neck (33) | 16 | 250 | 1 | 600 | Every 1-2 wk | ++
| Gastric (36) | 63 | 1500 | 1 | 1500 | Every 28 days* | +
| Colorectal (24) | 33 | 200-600 | 7 | 300-600 | Every 2 wk | +
| Colorectal (30) | 60 | 200-300 | 3 | 900 | Every 2-3 wk | ++
| Colorectal (69) | 42 | 250 | 4 | 600 | Every 3-4 wk | ++
| Colorectal (19) | 6 | 250 | 1 | 600 | Every 2-3 wk | +
| Colorectal (65) | 28 | 1500 | 1 | 1500 | Every 3-4 wk | +
| Colorectal (35) | 34 | 40 | 24 | 600 | Twice/mo. | +
| Colorectal (9) | 0 | 200 | 1 | 600 | Twice/mo. | +
| Colorectal (30) | 38 | 200 | 1 | 600 | Twice/mo. | +
| Breast (69) | 23 | 1500 | 1 | 1500 | Every 1 wk | ++
| Breast (65) | 23 | 1500 | 1 | 1500 | Every 1 wk | ++
| Breast (5) | 50 | 50 every 6 hr for 5 doses | 24 | 600 | Every 23 days | +
| Breast (31) | 50 | 300 | 7 | 900 | Every 2-3 wk | +
| Breast (25) | 55 | 200 | 1 | 600 | Twice/mo. | +
| Miscellaneous (14) | 160 | 1.5 | 600 | Every 1-2 wk | ++
| Miscellaneous (71) | 1000 | 20 | 800 | Every 28 days | ++

* All trials received high-dose MTX followed by LV rescue except for Ref. 35.
+++, very severe; ++, severe; +, moderate; +++, less significant.
Patients in Ref. 58 received Adriamycin at 14 days after MTX-FUra; those in Ref. 60 received tamoxifen followed by premarin.

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The same degree of caliper error is usually made by a single observer in
repeated measurements. Hence, the same observer always performs
both the initial and the final tumor measurements.

**RESULTS**

High-Dose MTX → LV Rescue. Table 2 records 8 (4 male
and 4 female) experiments in CD8F1 mice bearing well-advanced
Control of antitumor effect and host toxicity of weekly high-dose MTX by LV rescue

Shown are advanced (24, 25, 20, 26, 17, 17, 24, and 19 days old, and averaging 120, 160, 95, 160, 225, 160, 170, and 170 mg, respectively, in Experiments 1044, 1050, 1069, 1075, 1077, 1081, 1095, and 1100 when treatment initiated) first-generation CD8F, breast tumor transplants in CD8F, mice. All injections were given i.p. at indicated times once per week 3 times. Observations were recorded 5 days after third weekly injection.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Sex</th>
<th>NaCl solution</th>
<th>MTX</th>
<th>LV</th>
<th>Host toxicity</th>
<th>Antitumor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1044</td>
<td>1</td>
<td>M</td>
<td>0.9%</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+19 4/10</td>
<td>7976</td>
</tr>
<tr>
<td>1044</td>
<td>2</td>
<td>M</td>
<td>MTX LV</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+32 4/10</td>
<td>9237</td>
</tr>
<tr>
<td>1050</td>
<td>1</td>
<td>M</td>
<td>0.9%</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+16 2/10</td>
<td>6170</td>
</tr>
<tr>
<td>1050</td>
<td>2</td>
<td>M</td>
<td>MTX LV</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+12 0/10</td>
<td>4760</td>
</tr>
<tr>
<td>1069</td>
<td>1</td>
<td>F</td>
<td>0.9%</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+26 0/10</td>
<td>5109</td>
</tr>
<tr>
<td>1069</td>
<td>2</td>
<td>F</td>
<td>MTX LV</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+13 0/10</td>
<td>4541</td>
</tr>
<tr>
<td>1075</td>
<td>1</td>
<td>F</td>
<td>0.9%</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+14 2/10</td>
<td>8708</td>
</tr>
<tr>
<td>1075</td>
<td>2</td>
<td>F</td>
<td>MTX LV</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+4 3/10</td>
<td>5649</td>
</tr>
<tr>
<td>1077</td>
<td>1</td>
<td>M</td>
<td>0.9%</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+12 2/10</td>
<td>9231</td>
</tr>
<tr>
<td>1077</td>
<td>2</td>
<td>M</td>
<td>MTX LV</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>0 2/10</td>
<td>7224</td>
</tr>
<tr>
<td>1081</td>
<td>1</td>
<td>M</td>
<td>0.9%</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>−1 5/10</td>
<td>6773</td>
</tr>
<tr>
<td>1081</td>
<td>2</td>
<td>M</td>
<td>MTX LV</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+3 3/10</td>
<td>6821</td>
</tr>
<tr>
<td>1095</td>
<td>1</td>
<td>F</td>
<td>0.9%</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+13 3/10</td>
<td>6644</td>
</tr>
<tr>
<td>1095</td>
<td>2</td>
<td>F</td>
<td>MTX LV</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+8 2/10</td>
<td>4038</td>
</tr>
<tr>
<td>1100</td>
<td>1</td>
<td>F</td>
<td>0.9%</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+19 1/10</td>
<td>8388</td>
</tr>
<tr>
<td>1100</td>
<td>2</td>
<td>F</td>
<td>MTX LV</td>
<td>4.5 hr</td>
<td>19.5 hr</td>
<td>+15 1/10</td>
<td>4987</td>
</tr>
</tbody>
</table>

a MTX at 300 mg/kg; LV at 300 mg/kg.

b Student's t test; p ≤ 0.05.

NS, not significant.

CD8F, mammary tumor first-generation transplants. Therapy consisted of weekly doses of MTX at 300 mg/kg followed by a LV rescue schedule consisting of the administration of LV at 300 mg/kg at 4.5 and 19.5 hr after the administration of MTX. Without LV rescue, the same high dose of MTX alone results in 100% mortality (data not shown). However, Table 2 shows that mortality rates were either negligible or comparable (due to large tumor growth) in both 0.85% NaCl solution- and MTX-LV-treated groups, and since weight changes also were comparable in both groups, this relatively simple LV rescue schedule clearly protected the CD8F, host from HDMTX toxicity. Further, 6 of the 8 experiments in Table 2 demonstrated that LV rescue blocked the potential for a MTX-induced antitumor effect in this otherwise MTX-sensitive murine tumor (27). In 3 of the 4 female experiments, there is evidence for a degree of MTX-induced antitumor effect breaking through despite the LV rescue: Experiments 1075 and 1100 are significant, Experiment 1095 is almost significant (p = 0.06), and only Experiment 1069 is clearly nonsignificant. Collectively, however, the data demonstrate that the MTX-LV regimen used is devoid of antitumor activity in tumor-bearing male mice and displays only weak to insignificant activity in tumor-bearing female mice.

High-Dose FUra preceded by High-Dose MTX with “Double” Rescue By LV and Undine. Table 4 records the results of 3 male and 2 female tumor-bearing murine experiments with groups receiving the above weekly HDMTX-LV regimen with MTX sequenced 2.5 hr prior to weekly FUra (75 to 88 mg/kg) compared to groups receiving FUra alone at its MTD of 100 mg/kg. Three male experiments reveal that HDMTX-LV with FUra at 80 to 88 mg/kg either causes increased toxicity (Experiment 1077, Group 3 versus Group 2), or fails to significantly improve the antitumor effect over FUra alone (Experiment 1081, Group 3 versus Group 2; Experiment 1093, Group 2 versus Group 1). A slight lowering of the FUra dose in the presence of the HDMTX-LV regimen may ameliorate host toxicity (Experiment 1081, Group 4), but there is no increase in antitumor effect beyond what can be achieved with FUra alone (Experiment 1082, Group 2). In the 2 female experiments of Table 3, one (Experiment 1075) displays a gain in antitumor effect with the sequential combination in comparison to FUra alone, but the other (Experiment 1095) does not. Collectively, while the data do indicate a tendency for greater antitumor activity with the sequential combination, there is not good evidence for a differential cytotoxic effect on tumor versus host normal tissue.

High-Dose FUra preceded by High-Dose MTX with “Double” Rescue By LV and Undine. Table 4 records the results of 3 male and 2 female tumor-bearing murine experiments with groups receiving the above weekly HDMTX-LV regimen with MTX sequenced 2.5 hr prior to weekly FUra (75 to 88 mg/kg) compared to groups receiving FUra alone at its MTD of 100 mg/kg. Three male experiments reveal that HDMTX-LV with FUra at 80 to 88 mg/kg either causes increased toxicity (Experiment 1077, Group 3 versus Group 2), or fails to significantly improve the antitumor effect over FUra alone (Experiment 1081, Group 3 versus Group 2; Experiment 1093, Group 2 versus Group 1). A slight lowering of the FUra dose in the presence of the HDMTX-LV regimen may ameliorate host toxicity (Experiment 1081, Group 4), but there is no increase in antitumor effect beyond what can be achieved with FUra alone (Experiment 1082, Group 2). In the 2 female experiments of Table 3, one (Experiment 1075) displays a gain in antitumor effect with the sequential combination in comparison to FUra alone, but the other (Experiment 1095) does not. Collectively, while the data do indicate a tendency for greater antitumor activity with the sequential combination, there is not good evidence for a differential cytotoxic effect on tumor versus host normal tissue.
Tabte3
Sequential high-dose MTX followed by FUra and LV rescue: failure to improve therapeutic index over FUra alone

Shown are advanced (26, 17, 17, 27, and 24 days old, and averaging 160, 225, 160, 75, and 170 mg, respectively in Experiments 1075, 1077, 1081, 1093, and 1095 when treatment initiated) first-generation CD8F breast tumor transplants in CD8F mice. All injections were given i.p. at indicated times once per week for 3 doses. Observations were recorded 5 days after third weekly course.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Sex</th>
<th>Treatment</th>
<th>% of wt change</th>
<th>Dead/total</th>
<th>Tumor wt (mg)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1075</td>
<td>1</td>
<td>F</td>
<td>0.9% NaCl solution FUra (100 mg/kg)</td>
<td>+14</td>
<td>2/10</td>
<td>8708</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>F</td>
<td>FUra (88 mg/kg)</td>
<td>-15</td>
<td>1/9</td>
<td>818</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>M</td>
<td>FUra (100 mg/kg)</td>
<td>+12</td>
<td>2/10</td>
<td>9231</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>M</td>
<td>LV rescue</td>
<td>-4</td>
<td>0/10</td>
<td>2967</td>
<td></td>
</tr>
<tr>
<td>1077</td>
<td>1</td>
<td>M</td>
<td>0.9% NaCl solution FUra (100 mg/kg)</td>
<td>+14</td>
<td>2/10</td>
<td>9231</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>M</td>
<td>FUra (88 mg/kg)</td>
<td>-15</td>
<td>1/9</td>
<td>818</td>
<td>0.03</td>
</tr>
<tr>
<td>1081</td>
<td>1</td>
<td>M</td>
<td>0.9% NaCl solution FUra (100 mg/kg)</td>
<td>+14</td>
<td>2/10</td>
<td>9231</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>M</td>
<td>FUra (88 mg/kg)</td>
<td>-15</td>
<td>1/9</td>
<td>818</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>M</td>
<td>FXra (100 mg/kg)</td>
<td>+14</td>
<td>2/10</td>
<td>9231</td>
<td></td>
</tr>
<tr>
<td>1093</td>
<td>1</td>
<td>M</td>
<td>0.9% NaCl solution FUra (100 mg/kg)</td>
<td>-15</td>
<td>1/9</td>
<td>818</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>M</td>
<td>FUra (88 mg/kg)</td>
<td>-15</td>
<td>1/9</td>
<td>818</td>
<td>0.03</td>
</tr>
<tr>
<td>1095</td>
<td>1</td>
<td>F</td>
<td>0.9% NaCl solution FUra (100 mg/kg)</td>
<td>-15</td>
<td>1/9</td>
<td>818</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>F</td>
<td>LV rescue</td>
<td>-15</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>F</td>
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<td>-15</td>
<td>1/9</td>
<td>818</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Student’s t test; p ≤ 0.05.
Numbers in parentheses, group.
LV rescue, 300 mg/kg at 4.5 hr after MTX once per week, and again at 19.5 hr later. Note that the LV rescue is not begun until 2 hr after FUra.
NS, not significant.

Addition of PALA prior to High-Dose MTX prior to High-Dose FUra followed by LV and Uridine Rescue. Table 5 records 4 experiments affording comparison of this PALA-containing sequential combination to one without PALA (Experiment 1080, Group 2 versus Group 3; Experiment 1081, Group 5 versus Group 7; Experiment 1100, Group 6 versus Group 8; and Experiment 1158, Group 5 versus Group 7). The difference in the antitumor effects of the aforementioned 2 groups of Experiment 1100 is not significant, perhaps because there is only a 2-hr interval between MTX and FUra administration as compared to the 2.5 hr in all of the other experiments. The differential antitumor results in Experiments 1080 and 1081 are statistically significant, and are almost so in Experiment 1158. These 4 experiments, along with those in Table 4, provide a total of 7 separate experiments, 4 male and 3 female, for comparative evaluation of sequential addition of HDMTX prior to HDFUra to the MTD of FUra as a single agent. The overall results demonstrate significantly enhanced (6 of 7 experiments) and safe (average mortality, 6%) antitumor efficacy with the sequential combination of HDMTX prior to HDFUra followed by both LV and uridine rescue.

Addition of PALA prior to High-Dose MTX prior to High-Dose FUra followed by LV and Uridine Rescue. Table 5 records 4 experiments affording comparison of this PALA-containing sequential combination to one without PALA (Experiment 1080, Group 2 versus Group 3; Experiment 1081, Group 5 versus Group 7; Experiment 1100, Group 6 versus Group 8; and Experiment 1158, Group 5 versus Group 7). The difference in antitumor activity between the aforementioned 2 groups in Experiment 1080 is not statistically significant, but it is in the other 3 experiments (Experiments 1081, 1100, and 1158). Tumor regression data from each of these 2 groups in all 4 experiments (Experiments 1080, 1081, 1100, and 1158) have been pooled in...
Sequential PALA + MTX + FUrA + Leucovorin + Uridine

Table 4
Sequential combination chemotherapy: enhancement of the anticancer activity of weekly "high-dose" (130 mg/kg) FUrA by 2.5-hr prior "high-dose" MTX, with "double" rescue by LV and uridine

Shown are advanced (25, 20, and 19 days old, respectively) first-generation CD8F, breast tumor transplants in CD8F, mice, averaging 160, 95, and 160 mg, respectively, in experiments 1050, 1069, and 1070 when treatment initiated. All injections were given i.p. at indicated times once per week for 3 doses. Observations were recorded 5 days after third weekly course.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
<th>Sex</th>
<th>Treatment</th>
<th>% of wt change</th>
<th>Dead/total</th>
<th>Tumor wt (mg)</th>
<th>p*</th>
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<td>-19 1/10</td>
<td>998</td>
<td>NS</td>
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* p ≤ 0.05.
Numbers in parentheses, group.
LV rescue, 300 mg/kg at 2 hr after MTX, and again at 19.5 hr later. Note that LV rescue is not begun until 2 hr after high-dose FUrA.
NS, not significant.
Uridine rescue, 3000 mg/kg at 2 hr after FUrA, and 3000 mg/kg at 22.5 hr after FUrA.

Table 5 also affords comparison of the PALA-HDMTX-HDFUra-LV-uridine sequential combination to the same sequential combination without HDMTX in 4 experiments (Experiment 1081, Group 6 versus Group 7; Experiment 1085, Group 4 versus Group 5; Experiment 1100, Group 7 versus Group 8; and Experiment 1158, Group 6 versus Group 7). In 3 of these experiments (Experiment 1181, 1085, and 1100), the addition of HDMTX to the sequential PALA-FUrA-LV-uridine regimen significantly enhanced the antitumor effect. Also, although in one (Experiment 1085) of these 3 experiments there are no partial regressions, the other 2 experiments (Experiments 1081 and 1100) reveal a greater number of partial regressions with the addition of HDMTX to the PALA-FUrA-LV-uridine combination, and Group 7 of Experiment 1158 shows the appearance of partial tumor regressions as compared to that of Group 6, even though there is no difference between the average tumor weights of the 2 groups.

Table 6 contains pooled tumor regression data from each of the 2 groups treated with PALA-HDMTX-HDFUra-LV-uridine, or with the same sequential combination without HDMTX, in each of the 4 experiments (Experiments 1081, 1085, 1100, and 1158). The 3-drug combination with double rescue yielded a significantly higher number of partial tumor regressions (10 of 37; p < 0.01) than did the 2-drug combination with double rescue (1 of 38). Again, host toxicity was minimal with only modest weight loss and virtually no mortality.

DISCUSSION

The data of Table 3 indicate that, although a sequential HDMTX followed by FUrA and LV rescue sometimes may increase the antitumor effect over that of the MTD of FUrA as a single agent (as in Experiment 1075), more often (Experiments 1077 and 1081, Group 3) the sequential combination does not increase the therapeutic index. In Experiments 1081, 1093, and 1095 of Table 3, further FUrA dose reduction from 88 to between 75 and 80 mg/kg results in a nontoxic but also a less effective antitumor treatment schedule. In contrast, in experiments where the dose of FUrA is markedly raised (130 and 150 mg/kg; Tables...
Table 5

Sequential combination chemotherapy: enhancement of the anticancer activity of high-dose FUrA by prior PALA and by prior high-dose MTZ, with "double" rescue by LV and uridine

Shown are advanced (24, 17, 20, 19, and 25 days old, and averaging 215, 160, 85, 135, and 100 mg, respectively, in Experiments 1080, 1081, 1085, 1100, and 1158 when treatment initiated) first-generation CDFB, breast tumor transplants in CDFB, mice. All injections were given i.p. at indicated times once per week for 3 doses except for Experiment 1158 (5 doses). Observations were recorded 5 days after last weekly course.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Group</th>
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<th>Treatment</th>
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<th>Tumor wt (mg)</th>
<th>p</th>
<th>% of PR</th>
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<td>268</td>
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Sequential PALA + MTX + FUrA + Leucovorin + Uridine

4 and 5) and administered after (2.5 hr) the prior administration of HDMTX, but the host is subsequently protected by delayed uridine rescue as well as LV rescue, the result is a nontoxic but more effective antitumor treatment schedule.

And, since any antitumor activity of HDMTX is often obviated by LV rescue (e.g., always in male mice), the enhanced therapeutic result appears to be due to MTX biochemically modulating and thereby enhancing antitumor activity of FUrA rather than to any additive antitumor contribution of MTX per se.

The enhanced antitumor effects that importantly include partial tumor regressions with the addition of PALA to the sequential HDMTX-HDFUrA combination (Table 5) are produced by a low dose of PALA. We have previously demonstrated that this low dose of PALA, although devoid of antitumor activity, is nevertheless capable of lowering pyrimidine pools in this tumor, increasing (FUrA)RNA in the tumor, and augmenting the antitumor activity of FUrA (48).

The tumor model in those studies, the CD8F1 murine spontaneous mammary tumor, is used in the drug screening program of the National Cancer Institute (27). It is utilized (for practical economic reasons) as a first-generation (first-passage) transplant. First-generation transplants are not far removed, biologically, from the parent autochthonous tumor and closely mimic the growth pattern and drug response of the original tumor response (46, 67). Venditti (67) has “implicated differences among CD8F1 spontaneous tumors as an important factor... [contributing to the]... test-to-test variation....” Corbett et al. (21) have observed that “it is abundantly clear that no 2 tumors of even the same tissue of origin have exactly the same biological characteristics.” The reason for this variation among tumors of similar histology and origin is the extreme cellular heterogeneity of each spontaneous tumor, whether in mouse or human, and it is this heterogeneity that accounts for the differing susceptibilities and degree of response to specific therapeutic agents (18, 29). Taking into account the marked cellular heterogeneity and test-to-test variation, investigators found that the CD8F1 tumor model identified as positive (active) all drugs reported clinically active against human breast cancer. (27, 46)

Such correlations augur well for the translation of the CD8F1 findings reported in this publication into successful clinical trial.

The combination of low PALA-HDMTX-HDFUrA with LV-uridine rescue in the appropriate sequential schedule warrants consideration for clinical trial for additional reasons. As noted in Table 1, there are a number of preliminary positive clinical reports of augmented therapeutic effects with sequential MTX and FUrA in advanced breast cancer, and there is also a preliminary positive clinical report with PALA-FUrA in advanced breast cancer (42). Moreover, MTX and FUrA, widely used with CMF as postmastectomy adjuvant CMF chemotherapy (10), reportedly give a clear dose-response effect (i.e., CMF is therapeutically useful only when administered at full or nearly full doses), but often must have CMF dosage reduction due to toxicity (10). Putting all of these clinical findings together, it seems reasonable to investigate the sequential combination of low PALA with HDMTX and HDFUrA along with the utilization of LV-uridine rescue to ameliorate undue toxicity and facilitate full dose courses. MTX and FUrA are anticancer agents of proven clinical worth, and the utilization of techniques (i.e., biochemical modulation, appropriate sequence and scheduling intervals, high dosage, and rescue agents) to diminish toxicity while optimizing the anticancer effect should yield therapeutic advance.
ACKNOWLEDGMENTS

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REFERENCES


Sequential PALA + MTX + FUra + Leucovorin + Uridine
Improved Therapeutic Index with Sequential N-
-Phosphonacetyl-l-aspartate plus High-Dose Methotrexate plus
High-Dose 5-Fluorouracil and Appropriate Rescue


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