Combination Therapy of Mouse Leukemia L1210 by 1-β-D-Arabinofuranosylcytosine and 6-[(4-Nitrobenzyl)thio]-9-β-D-ribofuranosylpurine

Carol E. Cass, Huong Muzik, and Alan R. P. Paterson


SUMMARY

Nitrobenzylthioinosine (NBMPR), an inhibitor of nucleoside transport, was tested in combination with 1-β-D-arabinofuranosylcytosine (ara-C) for therapeutic activity against mouse leukemia L1210. NBMPR alone had no activity, whereas therapy with NBMPR and ara-C in combination was significantly better than with ara-C alone. The therapeutic potentiation resulting from the combination of NBMPR and ara-C appeared to be host mediated since NBMPR alone was not toxic to cultured L1210 cells. NBMPR treatment of normal mice increased the plasma half-time of ara-C and decreased rates of urinary excretion of ara-C and 2'-deoxycytidine; however, these effects were not large enough to explain the therapeutic potentiation. Because the drug combination appeared to be no more effective than ara-C alone in therapy of mouse leukemia L1210/TG (a thiopurine-resistant L1210 subline lacking hypoxanthine-guanine phosphoribosyltransferase), the host-mediated therapeutic potentiation was attributed in vivo breakdown of NBMPR to 6-mercaptopurine.

INTRODUCTION

In humans and in mice, the therapeutic effectiveness of ara-C and certain other nucleoside drugs is limited by rapid degradation to inactive products (1, 21, 25, 38). In liver and spleen of humans (7, 8, 19) and in kidneys of mice (7, 8, 20), ara-C is converted by cytidine deaminase to ara-U, a nontoxic compound which is subsequently excreted. In mice and humans, ara-C is rapidly inactivated and excreted; the half-time of the drug in the plasma of mice is approximately 20 min (1, 4, 28) and in humans it is 12 and 111 min, respectively, for the fast and slow phases of disappearance of the drug from plasma (20, 26, 38).

One aim of this work was to determine whether inhibitors of nucleoside transport, such as NBMPR, would influence the pharmacokinetics of a rapidly inactivated nucleoside drug such as ara-C. Previous studies with human erythrocytes have shown that transport of purine and pyrimidine nucleosides is inhibited by 6-thiopurine ribonucleosides with a variety of substituent groups on the sulfur atom (10, 11, 29). NBMPR, one of the most effective of these compounds, inhibits nucleoside transport in the human erythrocyte by binding with high affinity (Kd, 10^-9 M) to transport-specific sites in the plasma membrane (9). NBMPR also inhibits transport of various nucleosides and nucleoside drugs in mouse lymphoma cells (39), mouse erythrocytes, and cultured HeLa cells (C. E. Cass and A. R. P. Paterson, unpublished data).

MATERIALS AND METHODS

Mouse leukemia L1210 was obtained through the courtesy of Dr. W. R. Laster, Jr., Southern Research Institute, Birmingham, Ala. L1210/TG and L1210/ara-C, sublines of L1210 leukemia resistant to TG and ara-C, respectively, were provided by Dr. G. A. LePage of this laboratory. The mice used, DBA/2 or C57BL × DBA/2 (hereafter called BD2F1), were obtained from the Health Sciences Animal Breeding Unit, University of Alberta, Edmonton, Alberta, Canada. Leukemia L1210 lines were maintained by in vivo passage (10^5 cells/mouse i.p. weekly), L1210 in DBA/2 mice and the resistant sublines in BD2F1 mice. In pharmacokinetic, chemotherapeutic, and drug toxicity studies, mice of the same sex and weight (±0.5 g) were assigned randomly to treatment groups, each member of which received identical doses (i.p.) of ara-C dissolved or of suspensions of finely ground NBMPR (or MP) in 0.9% NaCl solution; for all injections, volumes were 0.01 ml/g body weight. Drug treatments were initiated 24 hr after i.p. implantation of 10^5 leukemic cells.

To determine the effect of NBMPR on the half-time of ara-C in plasma, BD2F1 females received NBMPR (100 mg/kg) 1 hr before [3H]ara-C (15 mg/kg) and, after anesthesia with Penthrane (Abbott Laboratories, North Chicago, Ill.), they were decapitated; blood from each was heparinized and centrifuged (1700 × g, 5 min) to obtain plasma. From each plasma sample, 100-μl portions were oxidized in a Packard Model 305 sample oxidizer (22) for determination of total radioactivity by liquid scintillation counting, added to 1.0 ml cold 5% trichloroacetic acid, and
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centrifuged. After 4 extractions of the centrifugation product with diethyl ether, supernatant fractions were dried in vacuum over P₂O₅. The residues were taken up in 20 μl water and applied to Whatman No. 3MM paper for chromatography in isobutyric acid:ammonium hydroxide:water (66:5:29) using unlabeled ara-C, ara-U, cytosine, and uracil as carriers. Chromatograms were cut into 1-cm strips and oxidized for determination of radioactivity (22).

For evaluation of the effect of NBMPR on excretion of ara-C and dCyd, groups of 5 BD2F₁ mice were treated with NBMPR (100 mg/kg) 1 hr before injection of [³H]ara-C or [³H]dCyd (15 mg/kg); control mice received 0.9% NaCl solution instead of NBMPR. Each treatment group was placed in a single metabolism cage, and urine collections were removed at 1-hr intervals. At the time of collection urine samples were analyzed for content of [³H]2O (22) and for metabolites of ara-C or dCyd by paper chromatography as above. The solvent system used in experiments with dCyd was isopropyl alcohol:H₂O:concentrated HCl (65:18.4:16).

L1210 cells were cultured at 37° in Fischer's medium (Grand Island Biological Co., Grand Island, N. Y.) without antibiotics and supplemented with 10% horse serum. Cultures were diluted daily to 8 × 10⁴ cells/ml with fresh growth medium. From a culture initiated with cells from ascitic fluid obtained from a mouse 5 days after implantation with in vivo-passaged leukemia, a stock of ampuls containing 10⁶ cells/ml in growth medium plus 10% dimethyl sulfoxide was frozen and stored in liquid nitrogen. Cultures were started from the frozen stock and were passaged for no more than 1 month. Cell cultures were consistently free of Mycoplasma.

The effect of ara-C and NBMPR on proliferation of L1210 cells in vitro was evaluated as follows. Duplicate 25-ml cultures containing 8 × 10⁴ cells/ml in media with and without drugs were incubated at 37° for 4 to 5 days with sampling at regular intervals for determination of cell numbers with an electronic particle counter. In some experiments, after exposure to ara-C and NBMPR for 18 hr, cells were washed once in warmed, drug-free medium.

NBMPR effects on ara-C and dCyd uptake by L1210 cells were appraised as follows: (a) on the 6th day after implantation of 10⁵ L1210 cells, mice were given injections i.p. of 0.9% NaCl solution or NBMPR (100 mg/kg), and 1 hr later cells were removed from ascitic fluid and incubated (37°) at 10⁶ cells/ml in growth medium containing 20 mM HEPES buffer (pH 7.4) and [³H]ara-C (or [³H]dCyd); or (b) L1210 cells obtained from untreated mice were incubated (37°) in HEPES-buffered growth medium containing NBMPR for 10 min before addition of [³H]ara-C (or [³H]dCyd). At timed intervals, 4-ml portions from the incubation mixtures of a and b were added to 35 ml cold HEPES-buffered growth medium, and cells were collected by centrifugation (2000 × g, 4°, 3 min). After a single wash in 30 ml cold medium, cell pellets were mixed with 70 μl 0.4 M cold perchloric acid; after 15 min at 4°, the mixtures were centrifuged, the extracts were reserved, and the residues were reextracted with 50 μl 0.4 M cold perchloric acid. The combined extracts were adjusted to pH 5 to 6, and portions were added directly to Bray's counting solution (5) or analyzed by descending paper chromatography as described above.

[G-³H]Ara-C (8.19 Ci/mmol) and [5-³H]dCyd (26.5 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. NBMPR and NBTGR were purchased from Raylo Chemicals, Edmonton, Canada.

RESULTS

The toxicities of NBMPR and ara-C were examined separately and in combination in normal mice (Table 1). Toxicities of NBMPR and ara-C administered in combination were significantly greater than those of either drug alone. NBMPR toxicity became apparent when treatment intervals, tolerated in the experiments of Table 1, were reduced from 24 to 8 hr (Table 2). The toxic effects of high doses of NBMPR were largely eliminated when the drug was administered in combination with ara-C (Table 2).

To estimate the effect of NBMPR on ara-C therapy of L1210 leukemia, ara-C treatment schedules that were only moderately effective (33) were selected. Results in Table 3 indicate that therapeutic effects of treatments that included both drugs were better than would be expected from

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### Table 1

**Toxicity of NBMPR and ara-C in normal mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survivors</th>
<th>MST*(days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl solution</td>
<td>12/12</td>
<td>17.0 ± 2.2</td>
</tr>
<tr>
<td>0.9% NaCl solution</td>
<td>12/12</td>
<td>29.7 ± 4.4</td>
</tr>
<tr>
<td>ara-C</td>
<td>8/12</td>
<td>17.0 ± 2.2</td>
</tr>
<tr>
<td>0.9% NaCl solution</td>
<td>15/18</td>
<td>29.7 ± 4.4</td>
</tr>
<tr>
<td>NBMPR</td>
<td>8/12</td>
<td>29.7 ± 4.4</td>
</tr>
<tr>
<td>0.9% NaCl solution</td>
<td>15/18</td>
<td>29.7 ± 4.4</td>
</tr>
</tbody>
</table>

* MST, mean survival time.
* Mean ± S.D.

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### Table 2

**ara-C protection against NBMPR toxicity**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survivors</th>
<th>MST*(days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl solution</td>
<td>18/18</td>
<td>17.0 ± 2.2</td>
</tr>
<tr>
<td>NBMPR</td>
<td>0.9% NaCl solution</td>
<td>18/18</td>
</tr>
<tr>
<td>ara-C</td>
<td>0.9% NaCl solution</td>
<td>15/18</td>
</tr>
<tr>
<td>NBMPR</td>
<td>ara-C</td>
<td>15/18</td>
</tr>
</tbody>
</table>

* MST, mean survival time.
* Mean ± S.D.
Combination Therapy by ara-C and NBMPR

addition of treatment effects with either drug alone. When the combination of NBMPR (67 mg/kg) and ara-C (15 mg/kg) was given at 8-hr intervals, 10 of 12 mice became long-term survivors, as compared with none when ara-C alone was used.

Since ara-C is a schedule-dependent drug (27, 33), the intervals between combination treatments were varied (Table 4). Treatment with NBMPR alone had no significant therapeutic effect in any of the 3 schedules, but ara-C alone was somewhat more effective when given every 8 or 12 hr. The NBMPR:ara-C combination exhibited marked schedule dependency. Intervals of 24, 12, and 8 hr between treatments resulted in 0, 50, and 78% long-term survivors, respectively.

The interval between administration of ara-C and NBMPR was varied. Mice were treated daily for 15 days with ara-C and NBMPR given simultaneously, or with NBMPR given 1, 2, or 3 hr before ara-C, or with NBMPR alone.

Table 3
Treatment of L1210 leukemia with combinations of NBMPR and ara-C

Groups of female BD2F1 mice, 24 hr after implantation with L1210 leukemia, were treated i.p. with 15 injections at 8-hr intervals with NBMPR (or 0.9% NaCl solution) followed 1 hr later by ara-C (or 0.9% NaCl solution). Mice that received only NBMPR (19 to 100 mg/kg) died within 1 day of mice treated with 0.9% NaCl solution (data not shown).

<table>
<thead>
<tr>
<th>NBMPR (mg/kg)</th>
<th>ara-C (mg/kg)</th>
<th>Survivors</th>
<th>MST* (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0/12</td>
<td>8.5 ± 0.5*</td>
</tr>
<tr>
<td>0</td>
<td>15</td>
<td>0/12</td>
<td>20.9 ± 2.6</td>
</tr>
<tr>
<td>19</td>
<td>15</td>
<td>2/12</td>
<td>20.7 ± 2.1</td>
</tr>
<tr>
<td>29</td>
<td>15</td>
<td>1/12</td>
<td>22.2 ± 2.5</td>
</tr>
<tr>
<td>44</td>
<td>15</td>
<td>2/12</td>
<td>23.9 ± 5.5</td>
</tr>
<tr>
<td>67</td>
<td>15</td>
<td>10/12</td>
<td>28.0 ± 7.1</td>
</tr>
<tr>
<td>100</td>
<td>15</td>
<td>7/12</td>
<td>29.0 ± 10.4</td>
</tr>
</tbody>
</table>

* MST, mean survival time.
* Mean ± S.D.

Table 4
Effect of varying the interval between treatments with NBMPR plus ara-C

Groups of female BD2F1 mice, 24 hr after implantation with L1210 leukemia, were treated i.p. as indicated with NBMPR (or 0.9% NaCl solution) followed 1 hr later by ara-C (or 0.9% NaCl solution).

<table>
<thead>
<tr>
<th>Schedule</th>
<th>NBMPR (mg/kg)</th>
<th>ara-C (mg/kg)</th>
<th>Survivors</th>
<th>MST* (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Every 24 hr × 15</td>
<td>0</td>
<td>0</td>
<td>0/24</td>
<td>8.4 ± 0.6*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
<td>0/24</td>
<td>9.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15</td>
<td>0/24</td>
<td>17.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15</td>
<td>0/24</td>
<td>24.2 ± 3.7</td>
</tr>
<tr>
<td>Every 12 hr × 15</td>
<td>0</td>
<td>0</td>
<td>0/41</td>
<td>8.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
<td>0/41</td>
<td>9.2 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>15</td>
<td>0/42</td>
<td>19.4 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15</td>
<td>21/42</td>
<td>28.1 ± 8.2</td>
</tr>
<tr>
<td>Every 8 hr × 15</td>
<td>0</td>
<td>0</td>
<td>0/18</td>
<td>9.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
<td>0/18</td>
<td>9.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>15</td>
<td>0/18</td>
<td>16.4 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15</td>
<td>14/18</td>
<td>21.0 ± 13.7</td>
</tr>
</tbody>
</table>

* MST, mean survival time.
* Mean ± S.D.
Chart 1. Effects of NBMPR and ara-C on proliferation of L1210 cells in culture. Cells were cultured in medium containing no drug ( ), 0.1 μM ara-C ( ), 0.5 μM ara-C ( ), 20 μM NBMPR ( ), 0.1 μM ara-C and 20 μM NBMPR ( ), and 0.5 μM ara-C and 20 μM NBMPR ( ).

Chart 2. Inhibition by NBMPR of ara-C uptake in L1210 cells in vitro. L1210 cells obtained from mice were suspended at 10^6 cells/ml in HEPES-buffered growth medium with and without 50 μM NBMPR and incubated for 10 min at 37° prior to addition of [3H]ara-C (final concentration, 20 μM). After incubation for the indicated periods, uptake of radioactivity was determined as described in “Materials and Methods.” Radioactivity present in acid-soluble fractions is plotted against incubation time. Chromatographic analysis of the incubation medium at 0 and 24 hr indicated a decrease in extracellular ara-C from 20 μM to 16.5 μM. When portions of cell extracts were analyzed chromatographically, most of the radioactivity remained near the origin as expected for ara-C nucleotides.

L1210 cells were incubated at 37° for 18 hr in culture medium containing 50 μM NBMPR, or 20 μM ara-C, or both drugs together, and the variously treated cells were then implanted in the mice. A 3-log kill of L1210 cells was observed with exposure to both drugs, whereas a 4-log kill was observed with ara-C alone; thus, NBMPR appeared to protect the tumor cells somewhat against ara-C toxicity.

Since NBMPR appeared to reduce rather than enhance ara-C toxicity to cultured cells, the effect of NBMPR on uptake of ara-C was examined. In the experiments of Chart 2, L1210 cells harvested from mice were exposed in vitro to NBMPR and then assayed for uptake of [3H]ara-C; NBMPR treatment reduced the rate of ara-C uptake by the L1210 cells, suggesting that the slight protective effect observed in cultured cells is due to a partial inhibition of ara-C uptake. Similar results were obtained when cells were exposed to NBMPR in vivo (by treating leukemic mice with NBMPR, 100 mg/kg, 1 hr before harvesting) and then assayed in vitro for uptake of 20 μM [3H]ara-C (data not shown).

The effect of NBMPR on plasma levels of ara-C was examined using previously untreated mice (Chart 3) or mice that had been treated daily for 7 days with NBMPR, 100 mg/kg (data not shown). In both the untreated and treated mice, treatment with NBMPR increased the plasma half-time of ara-C from 15 to 19 min. It is unlikely that this small increase in half-time was responsible for the observed therapeutic potentiation.

The effect of NBMPR treatment on urinary excretion of ara-C and ara-U by normal mice was investigated in the experiments of Chart 4. During the 1st hr after administration of [3H]ara-C, excretion of ara-C and ara-U by NBMPR-treated mice was significantly reduced. The increased plasma levels of ara-C observed after treatment with NBMPR (Chart 3) may result from the reduced rate of excretion during the 1st hr after injection of ara-C. In a similar experiment with [3H]dCyd (15 mg/kg, i.p.), NBMPR effects on the time courses of dCyd and 2'-deoxyuridine excretion were much the same as those observed for ara-C and ara-U.

Addition of dCyd to the growth medium of L1210 cells in culture reduced the toxicity of ara-C (data not shown). Since dCyd levels in rat blood plasma of 30 to 40 μM have been reported (19, 31), the possibility was considered that NBMPR enhancement of ara-C therapeutic effects might be due to reduced availability of dCyd in the host animal. The effect of NBMPR on uptake of dCyd by in vivo-grown L1210 cells was examined under conditions similar to those of the experiment of Chart 2. NBMPR reduced the rate of entry of dCyd into L1210 cells as it did that of ara-C (see Chart 2).

Since it was possible that NBMPR might break down in vivo to MP (We are grateful to Dr. G. B. Elion for this suggestion.), combination therapy with NBMPR and ara-C

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3 Plasma levels of ara-C shortly after injection of 15 mg per kg ara-C were approximately 20 μM.
Combination Therapy by ara-C and NBMPR

was tested against L1210/TG, a subline of L1210 that is resistant to TG and MP and lacks hypoxanthine-guanine phosphoribosyltransferase (G. A. LePage, personal communication). The absence of NBMPR:ara-C potentiation in treatment of the L1210/TG leukemia (Table 5) indicates that phosphoribosyltransferase activity is required for potentiation and implies that formation of MP from NBMPR is important in the therapeutic action of the NBMPR:ara-C combination against leukemia L1210.

NBMPR also synergized with ara-C in therapy of leukemia L1210, but leukemia L1210/TG was resistant to the drug combination. Increases in survival times similar to those reported in Table 4 resulted when mice bearing leukemia L1210 were treated with NBMPR (100 mg/kg) or 0.9% NaCl solution followed 1 hr later by ara-C (15 mg/kg) every 8 or 24 hr. When mice with leukemia L1210/TG were treated with NBMPR and ara-C, the mean survival time of mice receiving both drugs was less than that of mice treated with ara-C alone.

DISCUSSION

The data presented demonstrate synergism between NBMPR and ara-C in the therapy of L1210 leukemia in mice. As a single agent, NBMPR had only minor effects on growth of L1210 cells in vivo and in vitro. NBMPR failed to increase the toxicity of ara-C toward cultured L1210 cells, indicating that the therapeutic potentiation arising from the combined use of these 2 compounds was host mediated. Three possible mechanisms for the host effect were investigated.

NBMPR altered to some extent the pharmacokinetics of ara-C in normal BDF1 mice. Minor increases in plasma half-times of ara-C and apparently related changes in rates of ara-C excretion resulted from treatment with NBMPR. However, these relatively small effects did not appear sufficient to account for the therapeutic potentiation.

The growth-inhibitory activity of ara-C has been ascribed to inhibition of DNA synthesis by 1-β-d-arabinofuranosyl-CTP. Furth and Cohen (16) demonstrated that 1-β-d-arabinofuranosyl-CTP inhibited mammalian DNA polymerase through competition with dCTP. Several in vivo (30) and in vitro (13, 17, 18) studies indicated that dCyd protected sensitive cell populations against ara-C, apparently through expansion of cellular pools of dCTP. The experiments presented here showed that NBMPR inhibited, but did not prevent, uptake of dCyd by L1210 cells and reduced uptake of ara-C by L1210 cells, an effect counterproductive to therapy. Although suggestive, these experiments did not provide an adequate test for the possibility that reduced availability to L1210 cells of exogenous dCyd might be a factor in the NBMPR:ara-C potentiation.

A more likely basis for potentiation was conversion of NBMPR to MP in the host. Administered as a fine suspension, NBMPR appeared to be a slow delivery source of MP. Consistent with this postulate is the fact that therapeutic potentiation did not depend upon the sequence of administration of the 2 drugs. The immunosuppressive activity of MP was significantly increased when administered as azathioprine (Imuran), a compound from which MP is derived in vivo slowly by thiolyis (14).

Burchenal and Dollinger (6) have reported synergism between ara-C and MP in therapy of L1210 leukemia. ara-C and MP were also potentiating in combination therapy of Sarcoma 180 (15). Because ara-C synchronizes cell populations in vitro (3, 17, 23) and in vivo (2, 27, 36, 37) by preventing DNA synthesis, it is possible that ara-C might increase sensitivity to MP by increasing the fraction of cells in the thioptorine-sensitive S phase during periods of MP exposure. Previous data from this laboratory (34, 35) have indicated that MP is lethal to cells that convert MP to TG nucleotides and incorporate the latter into DNA.

NBMPR, a potent inhibitor of nucleoside transport (39), also synergized with ara-C in therapy of leukemia L1210 but not of leukemia L1210/TG. This result is consistent.
with the foregoing because NBTGR may serve as an in vivo source of TG which is known to potentiate ara-C therapy. Normal mice, treated simultaneously with ara-C and TG, were protected from lethal effects of TG because ara-C inhibited DNA synthesis in the bone marrow (24, 25, 32) apparently during the period of TG exposure; the observation of ara-C protection against NBMPR toxicity to normal mice may have a similar basis.

ACKNOWLEDGMENTS

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