Therapeutic Effects of Acivicin and \(N\)-(Phosphonomethyl)-L-aspartic Acid in a Biochemically Designed Trial against a \(N\)-(Phosphonomethyl)-L-aspartic Acid-resistant Variant of the Lewis Lung Carcinoma

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ABSTRACT

The \(N\)-(phosphonomethyl)-L-aspartic acid (PALA)-resistant variant of the Lewis lung carcinoma, LL/PALA-C, has a target enzyme (L-aspartate transcarbamylase) activity identical to that of its parent PALA-sensitive counterpart; however, the activity of the antecedent enzyme of de novo pyrimidine biosynthesis, carbamyl phosphate synthetase II (CPS II), is elevated two-fold in this resistant variant. Inasmuch as inhibition of L-aspartate transcarbamylase by PALA is competitive with respect to the substrate carbamyl phosphate, investigations were conducted to determine the effects of carbamyl phosphate depletion, provoked by inhibition of CPS II, on the biochemical and therapeutic activity of PALA in this tumor line. Towards this end, a spectrum of L-glutamine antagonists were screened in vitro as possible inhibitors of crude extracts of CPS II (a L-glutamine-utilizing amidotransferase) prepared from LL/PALA-C tumors. Acivicin was found to be the best of the CPS II inhibitors. The kinetics of Acivicin inhibition of CPS II activity was established. Doses greater than 10 mg Acivicin per kg inhibit CPS II activity in LL/PALA-C tumors up to 85% as assessed by direct enzyme assay and by measuring the inhibition of orotidine and orotidine accumulation in tumors of pyrazofurin-treated mice. Restitution of tumor CPS II following a single treatment with 10 mg Acivicin per kg was rapid (\(t_{1/2} = 24\) hr). Estimation of the impact on pyrimidine biosynthesis of Acivicin used singly and in combination with PALA were made by measuring the inhibition of the accumulation of orotidine and orotidine provoked by treatment with pyrazofurin. Combinations of 10 to 25 mg Acivicin per kg and 200 mg PALA per kg significantly inhibited inhibition of pyrimidine biosynthesis over single-drug activities. On the basis of these and collateral studies with PALA, a therapeutic trial was set up to evaluate the combination of PALA and Acivicin against LL/PALA-C tumors. The results of this trial show that, as single agents, neither drug was active against this line; however, combinations of 5 or 10 mg Acivicin per kg (every day, Days 1 to 9) and 200 mg PALA per kg (every other day, Days 1 to 9) showed >80% inhibition of tumor growth on Days 10 and 21. Long-term cures (3/10) and a 50% increase in life span were observed in the 10-mg Acivicin per kg plus 200-mg PALA per kg treatment group. Higher doses of Acivicin (e.g., 25 mg/kg) given with PALA were toxic. These experiments suggested that (a) carbamyl phosphate production is an important modulator of PALA therapeutic activity and (b) the rigorous biochemical definition of a rationally designed drug combination protocol can provide enhanced therapeutic results.

INTRODUCTION

PALA\(^2\) (NSC 224131), a potent inhibitor of de novo pyrimidine biosynthesis, has an unusual but limited spectrum of antitumor activity against transplantable murine tumors. This agent is curative against the generally refractory Lewis lung carcinoma (7) but is ineffective against several murine leukemias (5, 7). An expansion of the therapeutic activity of PALA has been attempted by using it in combination with other inhibitors of pyrimidine biosynthesis. Results have been mixed; for example, the combination of PALA and 5-fluorouracil is markedly synergistic in inhibiting the growth of human mammary carcinoma cells (1), whereas the combination of PALA and pyrazofurin is ineffective in a number of test systems (8).

We have extended this approach by using specific biochemical criteria to guide the selection both of the drug used in combination with PALA and the treatment protocol.

Resistance to PALA in murine tumors has, in general, been associated with an elevation in the target enzyme, ATCase (5). However, in one instance, the activity of the antecedent and rate-limiting enzyme, CPS II, has been invoked as a major determinant of resistance (11). In this latter case, a PALA-resistant variant of the Lewis lung carcinoma, LL/PALA-C, was shown to have an ATCase activity identical to that of the parent PALA-sensitive line, while having a CPS II activity that was elevated 2-fold. To further appraise the role that elevated CPS II activity may play in PALA resistance, we have chosen to use this line in the present study. Initially, a number of L-glutamine antagonists were screened in vitro as inhibitors of crude extracts of CPS II (a L-glutamine-utilizing amidotransferase) prepared from LL/PALA-C tumors. Subsequently, the best of these inhibitors, Acivicin [L-[\(\alpha\S,\alpha\S\)]-a-amino-3-chloro-4,5-di-dihydro-5-isoxazol-2-carboxylic acid (NSC 163501)], is shown to act in concert with PALA to materially impede flux through the de novo pyrimidine pathway in the LL/PALA-C tumors in vivo. Finally, a treatment schedule for Acivicin and PALA was established by investigating the time courses of inhibition of the respective target enzymes. Although, both Acivicin and PALA are completely ineffective as single agents against this tumor line, their use in combination provides a significant therapeutic advantage; under optimal dosing conditions, inhibition of tumor growth approached 100%. A case for the rational selection of drug combinations and their implementation is presented.

MATERIALS AND METHODS

Reagents. PALA, pyrazofurin (NSC 143095), and Acivicin as well as the other L-glutamine antagonists were procured...
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from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

\[ \gamma \text{-Thiocyano-\( \alpha \)-aminobutyric acid} \] was a gift of Dr. Charlotte Ressler, University of Connecticut, Farmington, Conn. Orotic acid and orotidine were purchased from Sigma Chemical Co. (St. Louis, Mo.) for use as chromatographic standards. Sodium \([^{14}\text{C}] \text{bicarbonate} \) (57.5 mCi/mmol) was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). Purified carbamyl phosphate synthetase (EC 2.7.9.2; specific activity, 1.1 unit/mg protein; 3.1 mg/ml) from \textit{Escherichia coli} was a gift of Dr. Joseph J. Villafranca, Department of Chemistry, Pennsylvania State University, University Park, Pa. All other chemicals were of the highest quality obtainable commercially.

\textbf{Assays.} C57BL/6J × DBA/2J F, (hereafter called B6D2F,) mice were killed by cervical dislocation; tumors were promptly excised and homogenized for 10 sec with a Polytron homogenizer in one volume of a buffer consisting of 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) and 2 mM dithiothreitol. After centrifugation for 3 min at 12,000 × g, supernatants were immediately used for assay. CPS II activity was measured by determination of the incorporation of NaHMCO3 into N-[^{14}\text{C}] \text{carbamyl-L-aspartate} as described previously (11).

Orotate and orotidine measurements were performed on acid-soluble extracts of tumors on a Waters Associates, Inc. (Milford, Mass.) high-performance liquid chromatographic system as described (11).

\textbf{Therapeutic Studies.} For chemotherapy studies, a PALA-resistant variant of the Lewis lung carcinoma, LL/PALA-C, was implanted (1 × 10⁶ viable cells/mouse) s.c. in groups of 10 male B6D2F, mice (25 to 30 g), and treatment with Acivicin and/or PALA on the indicated schedules was initiated 24 hr later. Tumors were measured on Days 10 and 21 after implantation, and mean tumor weight was estimated by the formula \( L \times W^2 / 2 \) (in mm); animals were observed for survival. The origin and biochemical characteristics of the LL/PALA-C tumor line are described elsewhere (11).

\textbf{RESULTS}

A number of chemotherapeutic antagonists of L-glutamine are known to inhibit carbamyl phosphate synthetase activity (4, 12), although the relative importance of inhibition of this particular amidotransferase in the oncotic activity of these drugs is not known. Table 1 summarizes the inhibitory potency of 1 mM concentrations of 15 drugs towards purified carbamyl phosphate synthetase from \textit{E. coli} and crude homogenates containing CPS II activity prepared from LL/PALA-C tumors. The relative potencies of the inhibitors against carbamyl phosphate synthetase from both sources of enzyme were comparable. Of the L-glutamine antagonists tested, Acivicin and 5-chloro-4-oxo-L-norvaline were by far the 2 most effective inhibitors. The thiol reagent, N-methylmaleimide was a better inhibitor than any of the L-glutamine antagonists, but because this compound can be presumed to share the property of delayed toxicity in common with other maleimides (3), its usefulness in therapeutic trials was adjudged to be compromised. From this panel of inhibitors, Acivicin emerged as the inhibitor of CPS II most appropriate for further evaluation \textit{in vivo} in combination with PALA.

Although it has been known for a number of years that Acivicin inhibits mammalian CPS II \textit{in vitro} (4), the magnitude and extent of this inhibition \textit{in vivo} remain undescribed. The effect of an inhibitor on enzyme activity \textit{in vivo} can be measured by both static and dynamic means. Both of these approaches have been utilized in describing the dose response of Acivicin inhibition of tumor CPS II \textit{in vivo}. As shown in Chart 1, inhibition of CPS II activity, as measured by direct assay of tumor homogenates prepared from drug-treated mice, is greater than

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>In vitro inhibition of carbamyl phosphate synthetase by antagonists of L-glutamine</td>
</tr>
<tr>
<td>Inhibitor (1 mM)</td>
</tr>
<tr>
<td>Azaserine</td>
</tr>
<tr>
<td>DON⁴</td>
</tr>
<tr>
<td>γ-Glutamyl hydrazide</td>
</tr>
<tr>
<td>S-Carbamyl-L-cysteine</td>
</tr>
<tr>
<td>DONV</td>
</tr>
<tr>
<td>CONV</td>
</tr>
<tr>
<td>O-Carboxamidase-L-serine</td>
</tr>
<tr>
<td>Acivicin</td>
</tr>
<tr>
<td>5-Hydroxy-L-lysine</td>
</tr>
<tr>
<td>Maleic hydrazide</td>
</tr>
<tr>
<td>L-Methionine-OX-sulfoximine</td>
</tr>
<tr>
<td>N-Methylmaleimide</td>
</tr>
<tr>
<td>Monomethylphosphonate</td>
</tr>
<tr>
<td>γ-Thiocyano-α-aminobutyric acid</td>
</tr>
</tbody>
</table>

⁴ DON, 6-diazo-5-oxo-L-norleucine; DONV, 5-diazo-4-oxo-L-norvaline; CONV, 5-chloro-4-oxo-L-norvaline.
85% 3 hr after the administration of doses of Acivicin larger than 10 mg/kg. In contrast to this static measurement, a dynamic evaluation of the perturbation of de novo pyrimidine biosynthesis was taken using the approach pioneered by Moyer and Handschumacher (14). In this instance, mice bearing s.c. LL/PALA-C tumors were treated with 100 mg pyrazofurin (a fraudulent nucleoside which, after its phosphorylation in vivo, inhibits orotidine-5'-monophosphate decarboxylase) per kg, and the accumulation of orotate and orotidine in tumors was measured 3 hr later. The effects of Acivicin inhibition of CPS II, an enzyme antecedent to that blocked by pyrazofurin, on pyrimidine flux were then evaluated by examining inhibition of orotate and orotidine accumulation in pyrazofurin-treated mice. The results of this approach are also included in Chart 1; the dose response of Acivicin-provoked inhibition of orotate and orotidine is quite similar to that described by direct assay of CPS II inhibition. Such a result could be anticipated when one is dealing with the rate-limiting enzyme for a pathway.

Chart 2 depicts the time course of Acivicin inhibition of orotate and orotidine accumulation induced by pyrazofurin treatment. Tumor-bearing mice were treated with 10 mg Acivicin per kg at Time 0. Beginning at the indicated times, mice were then treated with 100 mg pyrazofurin per kg; 3 hr later, they were sacrificed, and the concentrations of orotate and orotidine were measured in the tumors. Values are expressed relative to the orotate and orotidine accumulation induced by a 3-hr 0.9% NaCl solution and pyrazofurin treatment, which did not vary during the course of this experiment. Although a 90% inhibition of pyrimidine flux by Acivicin was seen when the drug was coadministered with pyrazofurin, inhibition was only 50% by 24 hr and essentially zero at 48 hr. This experiment suggested that Acivicin would have to be administered daily for maintenance of CPS II inhibition.

Correlative experiments defining the kinetics of PALA inhibition of its target enzyme, ATCase, in LL/PALA-C tumors have been described elsewhere (11). Briefly summarized, apparent inhibition of ATCase in excess of 80% cannot be achieved by therapeutic doses of PALA (100 to 400 mg/kg) in this PALA-resistant line. The ATCase inhibition achieved, however, is much more persistent than that observed for Acivicin against CPS II, the f, i7 for restitution of ATCase activity following a single 400-mg/kg dose of PALA being 4 to 5 days.

The potential for synergistic effects of PALA and Acivicin was first evaluated biochemically. Selecting doses of PALA (100 and 200 mg/kg) and Acivicin (10 and 25 mg/kg) that singly inhibit their respective target enzymes 70 to 90%, the effect of all possible drug combinations on tumor orotate and orotidine accumulation was measured in pyrazofurin-treated mice. As shown in Chart 3, treatment with 200 mg of PALA per kg inhibits accumulation by only 70% and 10 mg Acivicin per kg by about 85%. When PALA and Acivicin were administered together, however, inhibition of orotate and orotidine accumulation exceeded 95%. Experience with PALA in the PALA-sensitive parent Lewis lung carcinoma suggested that an inhibition of that magnitude would be sufficient to achieve therapeutic (11).

On the basis of the preceding results, the combination of PALA and Acivicin was put to therapeutic trial according to the following protocol. Twenty-four hr after implating mice s.c. with 1 x 10^6 LL/PALA-C tumor cells, treatment with 5, 10, or 25 mg Acivicin per kg i.p. was initiated. Animals were treated daily for 9 consecutive days. On Days 1, 3, 5, 7, and 9, mice were also given 200 mg PALA per kg. Appropriate control mice were also maintained, receiving either no treatment or single-drug treatments only. As summarized in Table 2, treatment either with any of the Acivicin doses or with PALA alone had no appreciable effect on tumor growth or animal survival when compared with tumor-bearing mice receiving no treatment. However, treatment with the 2 drugs in combination had pronounced effects. Mice receiving 25 mg Acivicin per kg and 200 mg PALA per kg all died of drug-induced toxicity 3 to 6 days after the cessation of treatment. By contrast, mice receiving either 5 or 10 mg Acivicin per kg and PALA showed significant inhibition of tumor growth at Days 10 and 21; in the later instance, 83 and 89% for the 2 treatments, respectively, when compared to untreated controls. Several long-term sur-
Effects of PALA and Acivicin on life span of mice inoculated s.c. with LL/PALA-C carcinoma

<table>
<thead>
<tr>
<th>Drug</th>
<th>Tumor wt inhibition</th>
<th>Median survival time (days)</th>
<th>Increase in life span (%)</th>
<th>Long-term survivors (&gt;60 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acivicin (5 mg/kg)</td>
<td>34 4 31</td>
<td>11</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Acivicin (10 mg/kg)</td>
<td>36 22 30</td>
<td>7</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Acivicin (25 mg/kg)</td>
<td>48 13 30</td>
<td>7</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>PALA (200 mg/kg)</td>
<td>18 15 31</td>
<td>11</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Acivicin (5 mg/kg) + PALA (200 mg/kg)</td>
<td>99 83 38</td>
<td>36</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Acivicin (10 mg/kg) + PALA (200 mg/kg)</td>
<td>99 80c 42c</td>
<td>50c</td>
<td>3/10</td>
<td></td>
</tr>
<tr>
<td>Acivicin (25 mg/kg) + PALA (200 mg/kg)</td>
<td>99 15 Toxic</td>
<td>0/10</td>
<td></td>
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</tr>
</tbody>
</table>

a Every day, Days 1 to 9.

b Every other day, Days 1 to 9.

c Excluding tumor-free survivors.

DISCUSSION

The first 3 enzymes of the de novo pyrimidine biosynthetic pathway, CPS II, ATCase, and dihydroorotase, exist as a cytoplasmic complex in mammalian cells (13, 16). Cells and tumors that are resistant to the cytostatic-toxic effects of PALA generally have elevated ATCase activities and coordinately elevated CPS II activity (5, 9). However, in at least 2 instances, PALA-resistant variants of the Lewis lung carcinoma have CPS II activities that are markedly elevated with respect to the CPS II:ATCase ratio seen in the parent PALA-sensitive line (11). An enhanced CPS II activity may play a prominent role in diminishing PALA inhibition, because the enzymic product, carbamyl phosphate, is a competitive substrate with respect to PALA for the ATCase binding site. An augmented carbamyl phosphate pool could serve to displace PALA from ATCase, thereby diminishing ATCase inhibition and the consequent perturbation of de novo pyrimidine flux. If this hypothesis were true, it should be possible to enhance PALA inhibition of ATCase by reducing carbamyl phosphate pools.

CPS II, a L-glutamine-utilizing amidotransferase, is subject to inhibition by L-glutamine antagonists (4, 12). Inhibition of CPS II should lead to a marked reduction in the carbamyl phosphate pool within the CPS II-ATCase-dihydroorotase complex and thus at the active site on ATCase. Consequently, a spectrum of potential inhibitors was evaluated for activity against CPS II activity in a PALA-resistant tumor line, LL/PALA-C. Acivicin was selected as the drug of choice because it powerfully inhibited tumor CPS II activity in vitro (Table 1) and did not produce unmanageable host toxicity. Next, the kinetics of Acivicin inhibition of CPS II were evaluated to define the dose levels needed to achieve appreciable enzyme inhibition in vivo and the duration of the inhibition that was achieved (Charts 1 and 2). With this information in hand, it was necessary to optimize the doses of Acivicin and PALA that were to be used in combination in the therapeutic trial. Minimization of the drug dosages used was essential because of the cumulative toxicity of Acivicin (15) and the likely presentation of synergistic toxicity arising from the use of 2 drugs with similar sites of dose-limiting toxicities, i.e., gastrointestinal. The lethality observed at the highest drug combination is a manifestation of this concern.

Dose optimization was accomplished by evaluating the impact of a number of possible PALA and Acivicin combinations on pyrimidine flux by measuring inhibition of pyrazofurin-induced accumulation of orotate and orotidine. This approach suggested that a combination of 10 mg Acivicin per kg and 200 mg PALA per kg would be maximally inhibitory (Chart 3). Inasmuch as Acivicin inhibition of CPS II is quite transient (restoration t1/2 is approximately 24 h) and plasma clearance of Acivicin in mice is very rapid (t1/2 = 27 min (6)), a daily dosing protocol was established. PALA, by contrast, is a very persistent inhibitor of its target enzyme, ATCase, even in this PALA-resistant tumor line (11). Furthermore, pharmacokinetic studies in mice show PALA to be present in plasma at concentrations in excess of its K, towards tissue ATCase for over 3 weeks after the single i.p. administration of 200 mg/kg (10). Thus, PALA was administered every other day during the treatment period. A less frequent treatment protocol might have been possible; however, the primary objective of this study was to demonstrate that the use of Acivicin could restore PALA therapeusis to the LL/PALA-C line rather than to exhaustively describe this therapeutic combination.

The attainment of a therapeutic effect with the combination of PALA and Acivicin in the LL/PALA-C variant of the Lewis lung carcinoma is of particular merit because the use of these 2 drugs runs contrary to 3 principles commonly applied for the selection of drug combinations used against human neoplasms (2). (a) Neither PALA nor Acivicin have any activity as single agents against the target tumor. (b) These 2 drugs share a common site of dose-limiting toxicity (gastrointestinal). And (c) these 2 drugs share a common mechanism of action: inhibition of de novo pyrimidine biosynthesis. These results clearly demonstrate that solely on the basis of monitoring biochemical end points that should result in therapeusis (i.e., virtual shutdown of the de novo pyrimidine pathway), a drug combination could be selected and a treatment protocol devised and optimized for therapeusis, even if drugs in the selected combination were without any therapeutic activity as single agents in the tumor system studied. The possibility is presented that a more judicious and informed use of presently available oncolytic drugs may provide valuable therapeutic advantages.

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REFERENCES


Combination Therapy with Acivicin and PALA


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