Therapeutic Synergism of Tiazofurin and Selected Antitumor Drugs against Sensitive and Resistant P388 Leukemia in Mice

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ABSTRACT

Tiazofurin is a synthetic "C" nucleoside analogue with a promising spectrum of experimental antitumor activity and a relatively novel mechanism of action. Previous work in our laboratories had revealed indications of collateral sensitivity and therapeutic synergism for selected murine tumor models treated with tiazofurin alone or in combination with an antimetabolite or an alkylating agent. Elucidation by others of biochemical indicators of tiazofurin activity provided the rationale for extending our studies to include the tiazofurin combinations reported here. Young, adult, female, BALB/c × DBA/2 F1, mice bearing body burdens of about 4 x 10^6 cells at the start of treatment were used. Cells were implanted either i.p. or s.c. Tiazofurin plus cisplatin or the 5'-palmitate of 1-β-D-arabinofuranosylcytosine (ara-C) was evaluated against the parent P388/D leukemia line. Tiazofurin plus 6-thioguanine was evaluated against the ara-C-resistant P388. All drug treatments were i.p. injections given daily for 9 days. The experimental design permitted comparison of optimal nontoxic single-agent and two-dose drug combination regimens on the basis of the estimated log_{10} change in tumor cell burden at the end of treatment. Concurrent untreated control mice bearing tumor burdens ranging from approximately one to 10^6 cells permitted estimates of cells surviving treatment. Optimal treatment with each of these combinations afforded tumor burden reductions that were greater by 1 to 7 orders of magnitude than the effects of the respective single agents. Optimal single-agent and combination dosages (mg per kg per dose) were as follows: tiazofurin, 500; cisplatin, 2.0; the 5'-palmitate of ara-C, 25; 6-thioguanine, 0.8; tiazofurin, 330 plus cisplatin, 0.58; tiazofurin, 220 plus the 5'-palmitate of ara-C, 20; tiazofurin, 100 plus 6-thioguanine, 0.8. The observed therapeutic synergism of these drugs with tiazofurin in animal models suggests the possibility that treatment with tiazofurin combinations may yield clinical results superior to those obtained with the single agents alone. Therapeutic synergism can be most readily maximized when biochemical markers of drug action are available to provide appropriate diagnosis and correlation of these approaches to the use of tiazofurin, for which biochemical markers and experimental combination chemotherapy leads are now available, would support the rational clinical development of tiazofurin combinations.

INTRODUCTION

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide; NSC 286193) as a single agent is the subject of several ongoing clinical evaluations for antitumor activity. Clinical study of tiazofurin seemed warranted because of the novel site of biochemical indicators of activity provided by the observation of Schabel et al. (7, 8) that P388/ARA-C is collateral sensitive to tiazofurin (8), and subsequent work, previously reported in part (9) and included in detail here, demonstrated that tiazofurin and PALA are therapeutically synergistic against P388/ARA-C. Early work in our laboratories included the observation that tiazofurin is therapeutically synergistic with the alkylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea against murine leukemia L1210 (10). These observations have been extended to include the additional combinations reported here. Experimental chemotherapy studies of tiazofurin in binary combinations with 6-thioguanine, palmO-ara-C (a depot form of ara-C), or cisplatin have been conducted. These drugs were selected as representative of purine and pyrimidine antimetabolites that inhibit DNA synthesis or are incorporated into DNA and of alkylating agents, respectively. Optimal treatment with each of these combinations afforded in vivo tumor burden reductions estimated to be greater by one to seven orders of magnitude than the effects of the respective single agents. A preliminary account of some of these results has been presented (11).

MATERIALS AND METHODS

Mice used were young, adult, BALB/c × DBA/2 F1 females obtained from various suppliers under contract with the Animal Genetics and Production Branch, National Cancer Institute. Mice were caged in stainless steel cages with hardwood bedding (Beta-Chip; Northeastern Products Corp.). Diet consisted of Wayne Rodent Blox (Continental Grain Co.) and tap water ad libitum. The experiments were conducted intermittently during 34 mo.

Antitumor drugs were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute. For injection into mice, tiazofurin (NSC 286193) was prepared as a solution in distilled water. PALA (NSC 224131), cisplatin (NSC 119875), and 6-thioguanine (NSC 752) were prepared as solutions in aqueous NaCl (0.9 g/100 ml). Slight alkalization was required to dissolve 6-thioguanine. PalmO-ara-C (NSC 135962) was prepared as a suspension in aqueous NaCl (0.9 g/100 ml).

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2 To whom requests for reprints should be addressed, at Southern Research Institute, P.O. Box 55305, Birmingham, AL 35255-5305.

3 The abbreviations used are: TAD, thiazole-4-carboxamide adenine dinucleotide; PALA, N-(phosphonacetyl)l-aspartate; ara-C, 1-β-D-arabinofuranosylcytosine; palmO-ara-C, the 5'-palmitate of 1-β-D-arabinofuranosylcytosine; P388/ARA-C, the 1-β-D-arabinofuranosylcytosine-resistant murine leukemia.

4 For a discussion of therapeutic synergism, see Ref. 6. For a definition of collateral sensitivity, see Ref. 8.
TIAZOFURIN COMBINATION CHEMOTHERAPY

Table 1 Design of a typical chemotherapy experiment with a drug combination incorporating tiazofurin

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumor site/treatment site</th>
<th>Drug dosage (mg/kg/injection)*</th>
<th>Titration† (no. of cells)</th>
<th>Additional controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>P388/ARA-C</td>
<td>i.p./i.p.</td>
<td>Tiazofurin, 500  330  6-Thioguanine, 1.8  1.2  0.8  Tiazofurin + 6-thioguanine  330  0.53  0.35  0.23  220  0.53  0.35  0.23  150  0.8  0.53  0.35  100  1.2  0.8  0.53  0.35</td>
<td>10 to approximately 1</td>
<td>PalmO-ara-C treated, tumored (10);*</td>
</tr>
</tbody>
</table>

* See Fig. 1, Experiment 1B.
† All dosages were administered daily for 9 days beginning with day 1 postimplant, except for the experiment designated 2B in Fig. 2 in which treatment began on day 3.
‡ Groups of untreated animals inoculated with serially diluted tumor cell suspensions to provide an internal standardization of median day of death as a function of body burden of tumor.
§ Toxic produced nonleukemic deaths based on the absence of ascites, solid tumor, or splenomegaly.
¶ To document resistance.

100 ml) plus Tween 80 (0.05%). All treatments were administered to groups of mice on the basis of their average body weight. Treatment consisted of daily i.p. injections for 9 days beginning on day 1 after tumor inoculation (day 0), except for the experiment designated 2B in Fig. 2 in which treatment began on day 3. Mice were observed daily, and survival was recorded through day 60.

Each experiment reported here included multiple dosages of each drug and combination ranging from nontoxic to frankly toxic dosages. A typical experiment is illustrated in Table 1. All other data presented in this report reflect optimal dosages only. Dying mice were necropsied, and if death was judged to be nonleukemic based on the absence of ascites, solid tumor, or splenomegaly, it was considered to be drug induced. Typically, each dosage group consisted of 10 mice. A tumored control group was treated with diluent only. In experiments with P388/ARA-C, a tumored group was treated with a standardized dose of palmO-ara-C (125 mg/kg, i.p., single dose, day 1) to confirm the drug resistance of the tumor.

Each of these internally controlled experiments included 5 to 8 groups of mice bearing initial tumor burdens ranging from 10 cells to about one cell (by serial dilution). This “titration” groups provided the basis for an assessment of therapeutic response in accordance with the principles and methods described by Schabel et al. (12). Therapeutic response was based on the median day of death of dying mice only. Long-term (45- to 60-day) survivors were excluded from calculations of the percentage of increased life span and from estimates of changes in tumor burden as a result of treatment (12). To assess tumor cell kill at the end of treatment, the survival time difference (T−C) between treated and control groups was adjusted to remove the ambiguity introduced when treatment, which may be cytostatic, spans multiple days. This adjustment consisted of subtracting the treatment duration from the life span increase, i.e., (T−C)−(t−t) where t = the time (in days) of the nth treatment (13).

The initial observation of apparent therapeutic synergism for each combination occurred as a result of i.p. treatment of an i.p. tumor, and earlier confirmatory experiments duplicated this design; however, later confirmatory experiments with cisplatin and with palmO-ara-C consisted of i.p. treatment of an s.c. tumor. This more-demanding experimental design seems to reflect more realistically the challenge of clinical cancer treatment. Presented in Table 1 are all dosages evaluated in a typical experiment (Fig. 1B) including an indication of which single drug or combination ranging from nontoxic to frankly toxic dosages. Subsequent data presentations focus on optimal dosages only. All experiments were repeated at least once for confirmation.

RESULTS

The initial observations of therapeutic synergism with tiazofurin in this series resulted from an early interest in improving the activity of PALA. In Fig. 1A are presented cumulative mortality data from a typical experiment for groups of mice reflecting optimal therapeutic activity at maximum tolerated dosages against P388/ARA-C leukemia. From the tumor cells surviving treatment, estimated as described in “Materials and Methods” and summarized in Table 2, it is apparent that the combination reduced the tumor cell burden 1 to 3 log10 units more than the best reduction achieved with tiazofurin alone and 4 to 6 log10 units more than with PALA alone. It is
Table 2  Estimated log_{10} change in P388/ARA-C cells after optimal nontoxic treatment in vivo

Estimates for each single agent or combination were based on the survival of concurrent groups of untreated control mice bearing tumor burdens ranging from approximately one to 10^7 cells as described by Schabel et al. (13). The tumor burden changes shown correspond to the optimal treatment regimens presented in Fig. 1. The log_{10} change is the net log change in the viable tumor cell population at the end of treatment as compared to the start of treatment; for example, a -4 indicates a 10,000-fold reduction in the tumor burden, and a +1 indicates a 10-fold increase in the tumor burden at the end of treatment.

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Tumor inoculum site</th>
<th>Tumor burden at time of first treatment</th>
<th>Tiazofurin</th>
<th>PALA</th>
<th>6-Thioguanine</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>i.p.</td>
<td>3 x 10^7</td>
<td>-4.1</td>
<td>-0.8</td>
<td>-7.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>i.p.</td>
<td>2 x 10^7</td>
<td>-2.7</td>
<td>+0.1</td>
<td>-4.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>i.p.</td>
<td>4 x 10^7</td>
<td>-4.2</td>
<td>+0.7</td>
<td>-5.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>i.p.</td>
<td>3 x 10^7</td>
<td>-2.6</td>
<td>+0.7</td>
<td>-5.4</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Survival of mice bearing either i.p. (IP) or s.c. (SC) P388/0 as indicated under IMPLANT and treated with the single agents and combinations indicated. Dosages (Rx; R_2) shown are the highest nontoxic dosages for each regimen unless otherwise indicated. Each part represents a single internally controlled experiment. PALMO-ARA-C, palmO-ara-C; CA, approximately; QD, daily.

important to note that one of the assumptions on which these estimates are based (13) is that cell killing is instantaneous at the time of treatment. For this reason, all observations of tumor cell burden refer to the time of last treatment, the time at which the tumor burden is assumed to be at its nadir. The second experiment, against more-advanced leukemia, confirmed the first (Table 2). With regard to the original objective of improving the activity of PALA, it is noteworthy that PALA alone achieved only cytostasis in these experiments (Table 2).

The purpose of extending this series to the other three combinations described here was to explore the potential of tiazofurin to contribute synergistically to the effects of agents with diverse mechanisms of antitumor activity. Combinations of tiazofurin with (a) a purine antimetabolite, 6-thioguanine, (b) a pyrimidine antimetabolite, palmO-ara-C, and (c) an alkylating agent, cisplatin, were studied. For tiazofurin plus 6-thioguanine, optimal data from two trials are shown in Table 2, and the results of one (Table 2, Experiment 4) are presented in Fig. 1B. Confirmed therapeutic synergism of tiazofurin and 6-thioguanine in mice bearing P388/ARA-C was clearly demonstrated. Similar results with another inhibitor of purine biosynthesis, L-alanosine, were obtained (9) and have been confirmed. The initial experiment with 6-thioguanine proved to consist primarily of toxic dosages. Lowering the dosages permitted observation of therapeutic synergism at six nontoxic dose combinations (data not shown) in addition to the combinations plotted in Fig. 1B and summarized in Table 2.

In Fig. 2 are presented the results of combination therapy of

5 Harrison and Trader, unpublished data.
TABLE 3

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Tumor inoculum site</th>
<th>Tumor burden at time of first treatment</th>
<th>Tiazofurin</th>
<th>Cisplatin</th>
<th>PalmO-ara-C</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>i.p.</td>
<td>5 x 10^7</td>
<td>-0.7</td>
<td>-3.1</td>
<td>-4.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>i.p.</td>
<td>4 x 10^7</td>
<td>-2.3</td>
<td>-2.3</td>
<td>-7.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>s.c.</td>
<td>3 x 10^7</td>
<td>-1.2</td>
<td>-0.9</td>
<td>-3.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>s.c.</td>
<td>1 x 10^7</td>
<td>-0.5</td>
<td>-0.9</td>
<td>-3.0</td>
<td>-8.1</td>
</tr>
</tbody>
</table>

the parent-sensitive P388/0 leukemia with tiazofurin plus palmO-ara-C (Fig. 2, A and B) or cisplatin (Fig. 2, C and D). Both combinations were clearly synergistic. Noteworthy in Fig. 2 is the observation that the therapeutic synergism achieved with combinations of tiazofurin with palmO-ara-C or cisplatin in an i.p./i.p. model (i.p. treatment of an i.p. tumor) was demonstrable against s.c. tumors as well. These combinations provided cell kill two to three orders of magnitude greater than that obtained with either of the single agents alone (Table 3). At the time of first treatment, the disease was advanced, consisting of tumor burdens in the range of 3 x 10^7 to 1 x 10^8 cells. Optimal nontoxic combinations reflected dosage fractions relative to optimal single agent dosages that ranged from 0.8 to 0.3. The effect of tiazofurin plus palmO-ara-C on i.p. P388/0 (Fig. 24) was dramatic: 8 of 9 tumor-free survivors ("cures") and a 28-day prolongation of survival in the one treatment failure. Overall, there were 28 of 59 cures among combination-treated mice receiving a range of dosages that were ≤LD_{10} in this experiment (all data not shown).

DISCUSSION

When it obtains, therapeutic synergism between one antitumor drug and selected other drugs may yield the important clinical advantages of increased antitumor activity and reduced host toxicity. Therapeutic synergism between tiazofurin and three clinically useful antitumor drugs was observed in the experiments reported here. This observation seems significant because the activity of tiazofurin as a single agent has not been established. Phase I trials have been completed (14, 15), but Phase II trials remain in progress. In contrast, drugs combined with tiazofurin in the present study provide established antitumor activity. These drugs have proven to be effective for the treatment of a variety of human neoplasms; moreover, the rationale for their use in these combinations has an attractive mechanistic basis. Tiazofurin should potenti ate cisplatin-induced DNA damage by interfering with NAD synthesis and by weak inhibition of poly(ADP-ribose) polymerase (16). Tiazofurin plus palmO-ara-C should inhibit both purine and pyrimidine metabolism and should inhibit repair of ara-C-induced DNA damage.

The choices of drugs and tumor models for this study were based on several additional considerations. Murine leukemia P388 seems to be broadly predictive for clinical activity of currently useful antitumor drugs (17). The generally drug-sensitive P388/0 line is variably sensitive to tiazofurin, cisplatin, and palmO-ara-C, but its sensitivity is sufficient to permit a demonstration of the effects of combinations of these drugs. Treatment of P388/0 with palmO-ara-C results in the relatively rapid emergence and overgrowth of ara-C-resistant P388 (7), a cell line deficient in deoxycytidine kinase and unable to activate ara-C (18). Tiazofurin is more active against P388/ARA-C than against P388/0 (8), and this differential activity was expected to contribute to therapeutic synergism with palmO-ara-C when the tumor burden was a mixed population. The P388/0 with emerging P388/ARA-C cells. The collateral sensitivity of P388/ARA-C to PALA and tiazofurin (8) suggested the choice of P388/ARA-C for evaluation of that combination. Because tiazofurin and 6-thioguanine are both inhibitors of purine biosynthesis, their activity might be expected to be at least additive in combination. The expectation of synergism was based on the observation that tiazofurin-resistant P388 cells are as sensitive to 6-thioguanine as are P388/0 cells. The tiazofurin and 6-thioguanine combination was evaluated against P388/ARA-C because this line was the most sensitive to tiazofurin (8), and the 6-thioguanine was expected to kill residual tiazofurin-resistant cells. These considerations all supported the choice of P388/ARA-C as a model with sufficient sensitivity to answer the question of therapeutic synergism; however, this model is also relevant to the common clinical situation of emergence of ara-C resistance in patients. The choice of cisplatin for combination with tiazofurin was based in part on the observation that 1,3-bis(2-chloroethyl)-1-nitrosourea, a nitrosourea alkylating and carbamoylating agent, exhibited therapeutic synergism with tiazofurin against L1210 leukemia (10).

In the main, the various expectations of therapeutic synergism just described have been realized experimentally as reported here; moreover, although available toxicity data on tiazofurin combinations are not sufficiently extensive to permit calculation of combination toxicity indices (6), 19), other combinations with cisplatin, ara-C, and 6-thioguanine exhibit less-than-additive toxicity in vivo (6). This suggests an additional possible advantage of tiazofurin combinations, namely, the ability to use nearly full dosages considered optimal for the single agents without additional dose-limiting toxicity. Selective dosage reduction may further reduce toxicity. Selected agents exhibit more troublesome target organ toxicities, for example, cisplatin nephrotoxicity, and careful dosage reduction in a combination may circumvent specific toxicity without loss of therapeutic activity.

Cisplatin, ara-C, and 6-thioguanine, the three drugs of primary interest in the tiazofurin combinations described here, were included in a series of 10 drugs evaluated in combination with tiazofurin for synergistic cytotoxicity in an in vitro model (20). The model comprised drug-induced inhibition of colony formation in human tumor cell lines. Neither cisplatin, ara-C, nor 6-thioguanine was synergistic with tiazofurin in vitro. Of the 10 drugs evaluated, only 5-fluorouracil exhibited any evidence of greater-than-additive cytotoxicity in combination with tiazofurin. This observation was confirmed independently (21) and extended to in vivo studies; however, the in vivo studies were not designed to permit an assessment of therapeutic synergism (21), and the effect of tiazofurin plus 5-fluorouracil...
predicted in vitro must await in vivo confirmation by additional work. With regard to the prediction of therapeutic synergism, the discrepancy between in vitro data (20) and the in vivo observations reported here for cisplatin, ara-C, and 6-thioguanine underscore the importance of caution in basing an expectation of clinical activity on any one of these approaches alone.

Studies of several synergistic combinations underscore the importance of dose and schedule in combining chemotherapeutic drugs, particularly in translating laboratory observations to clinical applications. Therapeutic synergism can be most readily maximized when biochemical markers of drug action are available to provide appropriate clinical-laboratory correlations; in that way, the translation of biochemical modulation (22) among in vitro and in vivo models and human patients will be most reliable. Application of such approaches to the use of tiazofurin, for which several biochemical markers may be examined, would support the rational clinical development of tiazofurin-containing combinations.

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