Antitumor Action of Pyran Copolymer and Tilorone against Lewis Lung Carcinoma and B-16 Melanoma

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SUMMARY

Pyran (copolymers of divinyl ether and maleic anhydride) and tilorone [2,7-bis(2-diethylaminoethoxy)fluoren-9-one dihydrochloride] are agents that stimulate host resistance-inducing agents that possess antitumor activity. The possible relationship of direct cytotoxicity to antitumor activity against the Lewis lung carcinoma and B-16 melanoma tumors was examined. Tilorone and tilorone congeners exhibited significant cytotoxicity in vitro for both tumor cells and normal cells. In general the 50% cytotoxic doses ranged from 1 to 20 µg/ml, similar to 50% cytotoxic doses observed with mechlorothamine. Despite this direct cytotoxic activity, tilorone was ineffective in vivo against the Lewis lung and B-16 melanoma tumors. In contrast, pyran showed minimal cytotoxic activity in vitro. Doses of 3,000 to 11,000 µg/ml were required for 50% cytotoxicity of tumor or normal cells. Yet pyran showed potent antitumor activity against both tumors in vivo. Even when drug therapy was delayed late in the course of disease, until 8 days after Lewis lung tumor implantation, treated mice showed a 123% increased lifespan. Similar late therapy in the B-16 melanoma disease resulted in a 162% increased life-span. These experiments support once more the ineffectiveness of tissue culture systems as in vivo predicting models for clinical antitumor activity. In addition, the effectiveness of late therapeutic administration of pyran lends promise to the usefulness of nontoxic synthetic polyanion adjuvants for surgical or radiation therapy for human neoplasia.

INTRODUCTION

Pyran (copolymer of divinyl ether and maleic anhydride) and tilorone [2,7-bis(2-diethylaminoethoxy)fluoren-9-one dihydrochloride] are agents that stimulate host resistance and exhibit potent activity against small growth fraction solid tumors (1, 10, 11, 18, 24).

Pyran is a polyanion that must be administered parenterally, while tilorone is effective when given p.o. or parenterally. The mechanism of antitumor action for these 2 drugs and for tilorone congeners is largely unknown. However, these agents possess certain similar effects on the host that may be responsible in part for the observed antitumor activity. Both pyran and tilorone are potent immunological adjuvants (2, 18). Both drugs induce interferon and exhibit a broad spectrum and antiviral activity; however, pyran induces much lower levels of interferon yet protects mice for a longer period than does tilorone (4, 7, 13, 17).

Pyran and tilorone also affect the reticuloendothelial system. Both drugs produce basophilic Feulgen-positive inclusions in white blood cells and phagocytic cells of liver and spleen (15, 23). Pyran produces in mice a marked biphasic phagocytic depression followed by stimulation (19). In contrast, tilorone does not depress phagocytosis but enhances liver phagocytosis after p.o. administration of the drug (18). Pyran and certain congeners of tilorone (but not tilorone) are also effective in protecting mice from lethal bacterial infection (18, 22).

The relationship of the drugs to the specific immune system is also complex. Both drugs enhance circulating antibody production to sheep erythrocytes (2, 18), yet pyran does not increase the cytotoxic antibody response to the allogeneic P-815 DBA/2J mastocytoma in C57BL/6J mice (A. G. Baird and A. M. Kaplan, unpublished observation). Both pyran and tilorone depress adjuvant-induced arthritis and do not accelerate skin graft rejection (Ref. 12; A. M. Kaplan and P. S. Morahan, unpublished observations).

However, both drugs act as adjuvants to chemotherapy in producing “cures” of a syngeneic leukemia (6). Paradoxically, pyran and tilorone can promote the growth of certain murine sarcoma virus-induced tumors as can polyriboinosinic-cytidyllic acid and Bacillus Calmette-Guérin (8). In other treatment regimens, however, these drugs can depress sarcoma formation (8). The exact cell populations that are affected by the drugs to cause these diverse effects have not been identified.

The relationship of any of these activities to the antitumor efficacy of pyran and tilorone remains uncertain. In addition to these pleiotropic effects on the host, tilorone is known to be cytotoxic in vitro for certain tumors (1). Tilorone has also been shown to form complexes with DNA and to inhibit DNA template functions and RNA-dependent DNA synthesis, observations which might explain the cellular toxicity (5). Pyran has been shown to inhibit RNA-dependent DNA polymerase of certain leukemia viruses (T. S. Papas, T. W. Pry, and M. A. Chirigos. Abstract of the Annual Meeting ASM-1973, p. 235). The cytotoxic action of pyran in vitro against tumor cells has not been measured.

These studies were undertaken to determine whether the antitumor activity of pyran or tilorone might be related to direct toxicity. The cytotoxic action of these drugs was

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assessed in vitro against Lewis lung carcinoma, B-16 melanoma, and certain normal cells in tissue culture. Cytotoxicity in vitro was not correlated with antitumor activity in vivo against the Lewis lung and B-16 melanoma tumors. Pyran was effective against both these tumors, while tilorone was inactive despite potent in vitro cytotoxicity.

MATERIALS AND METHODS

Drugs. Tilorone and congeners of tilorone were kindly supplied by the William S. Merrell Company, Cincinnati, Ohio. Pyran (preparation XA124-177) was kindly supplied by Hercules, Inc., Wilmington, Del. Pyran prepared for clinical use (NSC 46015) was used for some studies; mechlorethamine (nitrogen mustard, NSC 762) was used as a positive control for in vitro drug cytotoxicity.

For cytotoxicity assays, drugs were dissolved in appropriate growth media for the cell under study. For animal studies, drugs were dissolved in 0.15 N NaCl. Solutions were adjusted to pH 7.0 with 1 N NaOH if necessary.

Cells. Human WI-38 and primary mouse embryo cells (Swiss) were obtained from Flow Laboratories, Inc., Rockville, Md. Primary chicken embryo cells were prepared from 13-day-old embryos (Spafas, Inc., Biglersville, Pa.) by previously described procedures (16). WI-38, secondary mouse embryo, and secondary chicken embryo cells were grown and assayed in Eagle's minimal essential medium with Hanks' balanced salt solution, supplemented with 10% heat-inactivated fetal bovine serum, 100 units penicillin per ml, and 100 μg streptomycin per ml.

Cultures of carcinoma 755, Lewis lung carcinoma, and B-16 melanoma were initiated from tumors in C57BL/6J mice. The B-16 melanoma was obtained through the courtesy of Dr. J. Fidler, University of Pennsylvania, Philadelphia, Pa. The carcinoma 755 was grown and assayed in Eagle's minimal essential medium with Hanks' balanced salt solution, 20% fetal bovine serum, and antibiotics. The Lewis lung and B-16 melanoma tumors were grown and assayed in Eagle's minimal essential medium with 2 times amino acids and vitamins, Earle's balanced salt solution, 20% fetal bovine serum, and antibiotics. Carcinoma 755 was used during Passages 14 to 18, Lewis lung was used during Passages 3 and 4, and B-16 melanoma was used during Passages 2 to 4. At intervals, the oncogenicity of the cell lines was proven by observing 100% tumor incidence in mice inoculated with 10⁶ cultured cells.

A human melanoma cell strain (Tangney) was obtained through the courtesy of Dr. G. Heppner, Brown University, Providence, R. I. This tumor was grown and assayed in Waymouth 752/1 medium with 20% fetal bovine serum and antibiotics.

Cytotoxicity Assays. Cells were dispersed with 0.25% trypsin and 0.02% EDTA, and viable cells were determined by trypan blue exclusion. Approximately 50 viable cells per 0.2 ml were plated in microtest wells (Falcon 3040) and incubated at 35° in 5% CO₂ for 24 hr. Growth medium was then removed and new growth medium containing drugs was added. Drug concentrations used were 2.5 to 50 μg/ml for tilorone or its congeners and 250 to 10,000 μg/ml for pyran. Concentrations of mechlorethamine from 2.5 to 50 μg/ml were always used to provide a cytotoxic drug as a positive control for the test drugs. The cells were incubated with the drugs for an additional 24 hr, then washed with 0.15 N NaCl, and stained with crystal violet. A minimum of 8 wells per drug concentration was used, and the mean cell count and standard error were calculated. A dose-response curve was calculated by the method of least squares and the dose of drug required to kill 50% of the cells (CD₅₀/ml) was determined.

Tumor Testing in Vivo. The Lewis lung and B-16 melanoma tumors were maintained in C57BL/6J mice by s.c. implantation of 1-cu mm tumor fragments at 2- to 3-week intervals. All B-16 melanoma studies were also performed in C57BL/6J mice; Lewis lung carcinoma studies were performed in BDF₁ mice.

For tumor studies, all drugs were administered i.p.; tilorone or the 2,7-bis(2-dimethylaminoacetyl)fluorene dihydrochloride congener was given at 20 and 40 mg/kg, and pyran NSC 46015 was given at 25 and 75 mg/kg. Three drug treatment schedules were used. Drugs were administered on Days –2 and –1, on Days 1 through 8, or on Day 6 or 7 through Day 14. Tumors were implanted on Day 0. These drug doses and schedules caused no lethality. Deaths of mice were recorded daily, and MST and percentage survival were calculated. ILS of treated groups (T) over control groups (C) inoculated with tumors alone was calculated by T/C x 100. The MST's of drug-treated groups in comparison to those of groups receiving tumor alone were evaluated statistically by Student's t test.

RESULTS

Cytotoxicity of Tilorone and Pyran Compounds. The positive cytotoxic control drug, mechlorethamine, exhibited approximately the same cytotoxicity for normal cells and tumor cells (Table 1). The CD₅₀ values for normal cells were 0.5, 0.9, and 5.6 μg/ml and for neoplastic cells were 5.2, 2.0, 9.9, and 186.0 μg/ml. Mechlorethamine appeared to be less cytotoxic (CD₅₀ = 186 μg/ml) for the human melanoma than for any of the other cells tested.

Tilorone and the 5 congeners showed CD₅₀ values generally similar to those observed with mechlorethamine. There were no striking differences between CD₅₀ values for normal and neoplastic cells; the CD₅₀ values ranged from 1 to 20 μg/ml in most cases. In contrast to the cytotoxic effects of tilorone, pyran XA124-177 was essentially not cytotoxic at doses up to 250 μg/ml. Drug concentrations of 3,000 to 11,000 μg/ml were required to achieve 50% cytotoxicity. Studies with pyran (NSC 46015) indicated that this clinical preparation, similar to XA124-177, also showed minimal cytotoxicity for either normal or tumor cells.

Analysis of dose-response curves showed that the survival responses were linear (p < 0.05) (Chart 1). Mechlorethamine, tilorone, and pyran appeared to exhibit 1st-order kinetics toward the normal and tumor cell populations, at

The abbreviations used are: CD₅₀, 50% cytotoxic dose; MST, mean survival time; ILS, increased life-span.
Table 1

Direct cytoxicity of 8 antitumor drugs against normal and neoplastic cells in culture

<table>
<thead>
<tr>
<th>Drug</th>
<th>Normal cells</th>
<th>Neoplastic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chick embryo</td>
<td>Mouse embryo</td>
</tr>
<tr>
<td>Mechlorethamine</td>
<td>5.6</td>
<td>60.8</td>
</tr>
<tr>
<td>DEAE-fluorenone</td>
<td>14.2</td>
<td>4.6</td>
</tr>
<tr>
<td>DEAA-fluorene</td>
<td>96.9</td>
<td>ND</td>
</tr>
<tr>
<td>DMAE-fluorenone</td>
<td>16.1</td>
<td>ND</td>
</tr>
<tr>
<td>DMAA-dibenzofuran</td>
<td>7.3</td>
<td>ND</td>
</tr>
<tr>
<td>DMAE-xanthone</td>
<td>19.7</td>
<td>54.1</td>
</tr>
<tr>
<td>Pyran copolymer</td>
<td>6,000.0</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

* The abbreviations are: DEAE-fluorenone, 2,7-bis(2-diethylaminoethoxy)fluorene-9-one dihydrochloride; DEAA-fluorene, 2,7-bis(2-diethylaminoacetyl)fluorene dihydrochloride; DMAE-fluorenone, 2,7-bis(2-dimethylaminoethoxy)fluorene-9-one dihydrochloride; DMAA-dibenzofuran, 2,8-bis(2-dimethylaminoacetyl)dibenzofuran dihydrochloride; DMAA-dibenzothiophene, 2,8-bis(2-dimethylaminoacetyl)dibenzothiophene dihydrochloride monohydrate; DMAE-xanthone, 3,6-bis(2-dimethylaminoethoxy)-9-xanthone dihydrochloride.

ND, not done.

least to 1 to 2% of the tested cells which was the lowest number of cells which could be counted accurately in this assay.

Antitumor Activity In vivo. To determine whether the cytotoxicity observed in vitro with tilorone and the lack of cytotoxicity observed for pyran was correlated with antitumor action or lack of activity, respectively, the drugs were tested in vivo.

Pyran at 75 mg/kg significantly prolonged the MST in all treated groups of mice hosting the Lewis lung carcinoma (Chart 2). The most efficacious drug regimen was treatment on Days 1 to 8 after tumor implantation, producing an MST of 50.1 ± 0.9 days, a 135% increase over life-span of controls (p < 0.01). Drug treatment on Days -1 and -2 resulted in an MST of 44.7 ± 2.3 days (p < 0.025) and the delayed treatment resulted in an MST of 45.6 ± 1.2 days (p < 0.01). Groups of mice treated with lower doses of pyran (25 mg/kg) also showed a significant prolongation of
Mice treated on Days -2 and -1 showed a 124% ILS ($p < 0.01$), mice treated on Days 1 to 8 after tumor implantation showed a 127% ILS ($p < 0.01$), and mice treated late showed a 118% ILS ($p < 0.05$). In contrast to the protective effects of pyran, none of the 3 schedules of treatment with tilorone (40 mg/kg) significantly prolonged the life of the animals (Chart 2). The congener, 2,7-bis(2-dimethylaminoacetyl) fluorene dihydrochloride, also showed no antitumor activity against the Lewis lung carcinoma when tested in the same drug schedules of either 20 or 40 mg/kg.

Pyran was also effective against the B-16 melanoma, although not as active as against the Lewis lung carcinoma. Groups of mice treated with pyran at 75 mg/kg on Days -2 and -1 showed a 132% ILS ($p < 0.05$), mice treated on Days 1 to 8 following tumor implantation showed a 130% ILS ($0.10 > p < 0.05$), and mice treated on Days 7 to 14 following tumor implantation showed a 162% ILS ($p < 0.01$) (Chart 3). Treatment with pyran at the lower dose (25 mg/kg) was not effective in increasing survival time. Again, in contrast to the effectiveness of pyran, tilorone showed no antitumor activity with any of the 3 drug schedules. Indeed, treatment with tilorone on Days -1 and -2 significantly enhanced death due to B-16 melanoma. These results are similar to those of Gazdar et al. (8), showing enhancement of murine sarcoma virus with certain drug regimens.

Pyran showed a level of antitumor activity against the Lewis lung and B-16 melanoma tumors that was greater than the activity obtained with mechlorethamine and comparable to that obtained with cyclophosphamide, a liver-activated alkylating agent. When mice were implanted with Lewis lung and inoculated i.p. with cyclophosphamide (50 mg/kg) on Days 1 to 8, the life-span of the mice was increased >140% over nontreated mice. Comparable treatment with pyran resulted in 135% ILS. However, when the experiment was ended (Day 55), 4 of 10 mice treated with cyclophosphamide showed no evidence of tumor, while all mice treated with pyran eventually succumbed to tumor. Mechlorethamine (0.25 mg/kg, Days 1 to 9) showed only borderline activity against s.c. implanted Lewis lung carcinoma or B-16 melanoma (R. Geran and J. M. Venditti. National Cancer Institute Screening Data Summary, July 16, 1973).

**DISCUSSION**

Although pyran and tilorone are both agents that stimulate host resistance and exhibit antitumor activity, these 2 drugs demonstrated essential differences in *in vitro* cytotoxicity and in *in vivo* antitumor activity.

Tilorone and the 5 congeners studied were all cytotoxic for normal and neoplastic cells at doses similar to those seen for mechlorethamine. Most of the CD50's ranged from 1 to 20 μg/ml, similar to values of 1.2 to 4 μg/ml reported by Adamson (1) for tilorone against the leukemia L5178Y, Walker carcinoma 256, and Novikoff hepatoma. In the current study, cytotoxicity for various murine tumors and 1 human tumor was compared to cytotoxicity for normal murine, human, and chicken cells; no differential cytotoxicity for normal versus neoplastic cells was observed.

Despite effective *in vitro* cytotoxicity, tilorone showed no antitumor activity *in vivo* against either the B-16 melanoma or Lewis lung carcinoma. The drug was ineffective at any of 3 schedules of administration. In other studies, tilorone (30 to 50 mg/kg) has been shown to be effective against the Walker carcinoma 256 and reticulum cell sarcoma A-RCS, although not against a variety of other tumors (1). In the current study, a similar dose (40 mg/kg) of tilorone was used. This represents an *in vitro* cytotoxicity equivalent dose: sufficient drug concentration was administered (approximately 8000 μg/mouse) to parallel direct cytotoxicity seen *in vitro*. The ineffectiveness of tilorone against 2 slow-growing and metastasizing solid tumors, which provide models for human neoplasia (9, 14), is disappointing. The route of administration may be a factor. However, maximal tolerated doses were administered, and several schedules of administration were evaluated without success.

Tilorone has been given clinically to 41 patients with a variety of advanced cancers as part of a Phase 1 and Phase 2 study. Significant regression, 1 lasting 8 months, has been seen in 2 of 8 melanomas, with additional responses seen in single cases of breast, cylindroma, and undifferentiated carcinoma of the lung. In none of these patients has drug-related bone marrow depression or other side effects attributable to mitotic inhibition been seen (e.g., mucosal ulceration, hair loss, osteoporosis). In contrast, transient thrombocytosis and leukocytosis have been seen in patients with marrow depression (25).

While tilorone was cytotoxic *in vitro* and ineffective *in vivo* against Lewis lung and B-16 melanoma, pyran was not cytotoxic *in vitro* and was very active *in vivo* against tumors.
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Pyran was effective in vivo at 75 mg/kg on a treatment schedule which provided a cumulative dose of approximately 3,250 μg/mouse. However, doses of 3,000 to 11,000 μg/ml in vitro were required to destroy 50% of the normal or tumor cells in tissue culture. In addition, pyran has been shown largely to be localized in the phagocytic cells of the liver and spleen. 30% of injected drug remaining in these organs 5 weeks after a single injection (21). Thus, direct cytotoxicity for tumor cells is probably not a major mechanism of the antitumor activity of pyran, unless pyran may be metabolized to an active cytotoxic form in vivo or cause induction or activation of an antitumor agent in the host. However, it is more probable that pyran acts via modulation of host responses such as alteration of the immune response, effects on phagocytic activity or macrophage tumor-killing capacity. Peritoneal macrophages taken from mice inoculated with pyran exhibit the ability specifically to kill tumor cells, while not inhibiting normal cells. In addition, thymectomy of mice decreases the ability of pyran to protect mice from Lewis lung or B-16 melanoma tumors (unpublished observations). Thus, both the macrophages and thymus-derived cells appear to be involved in the mechanism of antitumor activity of pyran. Studies are currently underway to determine the interrelationships between macrophage activation, thymus function, and the antitumor activity of pyran.

Pyran was particularly active against the Lewis lung carcinoma. These results are similar to those obtained by Geran and Venditti, in studies in which the drug was administered on Days 1 to 8 after Lewis lung tumor implantation (National Cancer Institute Screening Data Summary, July 16, 1973). The current studies indicate that pyran was also effective when administered for 2 days before tumor implantation. Treatment was also effective when drug administration was begun as late as 8 days after tumor implantation, at a time when the Lewis lung carcinoma has metastasized widely (14). Pyran treatment late in the course of disease with the B-16 melanoma was also effective.

Pyran has previously been shown to be effective in maintaining remissions induced by standard chemotherapy against murine leukemia (6), although in the present experiments all mice eventually succumbed to tumor. Pyran itself has been used in Phase 1 studies in 67 patients with advanced cancer; toxic side effects of thrombocytopenia, pyrexia, and hypotension precluded further investigation (20). However, experimental pyran preparations have been prepared which maintain antitumor activity but are nontoxic for mice (3, 20). The effectiveness of late therapeutic administration of pyran in increasing life-span of tumor-bearing animals lends promise to the usefulness of the newer pyran preparations or other nontoxic synthetic polyanions as adjuvants to surgical or radiation therapy for human neoplasia.

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