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Inhibitory Effects of Interferon-inducing Pyrimidinones on the Growth of Transplantable Mouse Bladder Tumors[1]

Younan A. Sidky, Ernest C. Borden,[2] Wendell Wierenga, and George T. Bryan

Departments of Human Oncology [Y. A. S., E. C. B., G. T. B.] and Medicine [E. C. B.], Wisconsin Clinical Cancer Center, University of Wisconsin, Madison, Wisconsin 53792, and the Department of Cancer and Viral Diseases Research, Upjohn Company [W. W.], Kalamazoo, Michigan 49001

ABSTRACT

Pyrimidinones are low-molecular-weight compounds which are inducers of interferon in several animal species. They have established antiviral, immunomodulatory, and antitumor effects. Four pyrimidinones as well as another potent interferon inducer, polyriboinosinic-polyribocytidylic acid, and β-interferon were tested for effects on growth of the transplantable mouse bladder tumor (MBT-2). The pyrimidinones 2-amino-5-bromo-6-phenyl-4(3*H*)pyrimidinone (ABPP) and 2-amino-5-bromo-6-(3-fluorophenyl)-4(3*H*)pyrimidinone (ABMFPP) significantly inhibited MBT-2 growth in a dose-dependent manner and with equal potency when injected i.p. every 4 days starting 1 day after tumor cell inoculation. Administration of ABPP p.o. was as effective as i.p. injections. Direct intravesical application of ABPP to transplantable tumors growing in the bladder may be more effective in inhibiting MBT-2 growth than the same dose introduced p.o. Although ABPP (100 mg/kg) has an inhibitory effect comparable to 5000 units of β-interferon, both pyrimidinones even at 500 mg/kg were less inhibitory of tumor growth than 10 mg of polyriboinosinic-polyribocytidylic acid per kg. The pyrimidinones 2-amino-5-bromo-6-(2,5-difluorophenyl)pyrimidin-4(3*H*)one (ABDFPP) and 2-amino-5-iodo-6-(2,3-difluorophenyl)pyrimidin-4(3*H*)one (AIDFPP) were also of comparable potency in inhibiting MBT-2 growth and were more effective on mg/kg basis than both ABPP and ABMFPP. Treatment with ABDFPP or AIDFPP also resulted in long-term cures of up to 40% of mice. In this respect these latter two compounds were superior to treatment with 10 mg of polyriboinosinic-polyribocytidylic acid per kg, a treatment which reduced tumor size but had no effects on tumor incidence. The data suggest that tumors of bladder origin may be particularly sensitive to treatment with pyrimidinones.

INTRODUCTION

Aryl pyrimidinones have been reported to induce several biological activities including IFN[3] (1–5), antiviral activity (2, 3, 6, 7), and modulation of the immune response (8–11). As antitumor agents they have activity when applied alone or when combined with cytoreductive compounds.

Antitumor activities of pyrimidinones as single agents in experimental models in the mouse have varied (3, 9, 12, 13). ABPP or 2-amino-5-iodo-6-phenyl-4(3*H*)pyrimidinone injected i.p. at 250 mg/kg for 3 consecutive days before or after i.v. inoculation of fibrosarcoma NFSa, FSa, or mammary carcinoma MCa-K cells greatly reduced the number of tumor nodules in the lung as well as spontaneous metastasis of NFSa (13). In the rat, ABPP at 200 mg/kg 3 times per week reduced the size of 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinoma (14). Synergistic effects occurred when ABPP treatment was combined with injection of CY or other therapeutic agents (9, 14).

The effect of treatment with pyrimidinones on tumor growth may result from either their ability to induce IFNs or their immunomodulatory potency (9, 15). We have tested effects of four pyrimidinones which varied in their ability to induce IFNs (2) on the growth of transplantable FANFT-induced MBT-2 tumors in isologous C3H/He hosts and found inhibition of the growth of that tumor. Some pyrimidinones when used as a single modality even resulted in "cures" in inoculated mice.

MATERIALS AND METHODS

Agents and Vehicles. Pyrimidinones (Fig. 1) were prepared and provided by Upjohn Co., Kalamazoo, MI (2, 5, 7). These compounds were administered in fine suspension in a vehicle composed of carboxymethylcellulose (5 mg/100 ml), Polysorbate 80 (4 mg/100 ml), sodium chloride (9 mg/100 ml), and benzyl alcohol (9 mg/100 ml). Poly(I)·poly(C) (Lot 124723) was purchased from PL Biochemicals, Milwaukee, WI. It was allowed to dissolve in phosphate-buffered saline at 4°C overnight for a final concentration of 1 mg/ml. Poly(I)·poly(C) was reannealed by heating in a 70°C water bath for 10 min, the contents mixed, and then returned to a 70°C bath for 5 min. The water bath was then allowed to cool gradually overnight. The solution was then aliquoted and frozen. Murine IFN-β was purchased from Lee Biomolecular, San Diego, CA. Drugs were prepared so that the required dose would be delivered in 0.1 ml/10-g body weight and introduced at the specified time intervals after tumor cell inoculation which was considered Day 0.

Mice. Five- to 6-wk-old female C3H/He mice purchased from Sprague-Dawley (Indianapolis, IN) were housed in suspended metal cages with no more than 5 mice/cage. They were fed Wayne Rodent Blox (Continental Grain Co., Chicago, IL) and HCl-acidified (pH 2.7 to 3) tap water *ad libitum* and were placed in rooms with controlled temperature (22.2–24.4°C), humidity (40%), and 12-h light-dark cycles. Mice, used at least 1 wk after their arrival, were weighed on Day 1 after tumor cell inoculation and then every other day until the end of each experiment. No differences occurred in mouse weight between groups except as related to tumor size.

Tumors. MBT-2 (16), derived from a FANFT-induced bladder carcinoma kindly provided by Dr. Mark Soloway (University of Tennessee, Memphis, TN), was maintained *in vivo* by s.c. transfer to C3H mice every 2 wk. Nonnecrotic parts of the tumor were dissociated using a 0.05% trypsin-0.02% EDTA preparation for 2 to 5 min. Tumor cells were then washed twice in Hanks' balanced salt solution and suspended at the desired concentration of viable cells. Cells were inoculated in 0.1-ml volume s.c. in the right inguinal region. Usually a group consisted of 15 mice.

Tumor growth s.c. was assessed by measuring by a caliper the longest dimension and a dimension perpendicular to it. A formula to quantify tumor size, namely (Diameter 1) (Diameter 2) (0.523), was applied (17). Measurements were made every other day until Day 21, at which time mice with tumors were killed, and their tumors were measured, dissected, and weighed. Tumor-free mice on Day 21 were kept alive to Days 40 and then 100. Mice were then killed, and tumors were measured, dissected, and weighed. In all groups, mean tumor size was based on mice which developed tumors. Viable MBT-2 cells (10^5) were inoculated except when indicated. Since tumors are heterogeneous the same cell number may give rise to tumors of different mean size in

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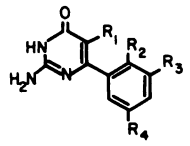
[2] To whom requests for reprints should be addressed, at the Department of Human Oncology, Wisconsin Clinical Cancer Center, K4/414 Clinical Science Center, 600 Highland Avenue, Madison, WI 53792.

[3] The abbreviations used are: IFN, interferon; poly(I)·poly(C), polyriboinosinic-polyribocytidylic acid; FANFT, *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; CY, cyclophosphamide; MBT-2, mouse bladder tumor; ABPP, 2-amino-5-bromo-6-phenyl-4(3*H*)pyrimidinone; ABMFPP, 2-amino-5-bromo-6-(3-fluorophenyl)-4(3*H*)pyrimidinone; ABDFPP, 2-amino-5-bromo-6-(2,5-difluorophenyl)-pyrimidin-4(3*H*)one; AIDFPP, 2-amino-5-iodo-6-(2,3-difluorophenyl)pyrimidin-4(3*H*)one; IFN-β, β-interferon.



	R_1	R_2	R_3	R_4
ABPP	Br	H	H	H
ABMFPP	Br	H	F	H
ABDFPP	Br	F	H	F
AIDFPP	I	F	F	H

Fig. 1. Structures of pyrimidinones, ABPP, ABMFPP, ABDFPP, and AIDFPP.

vehicle-treated control mice in the course of different experiments.

MBT-2 cells were also directly implanted in the bladder (18). Mice were anesthetized with avertin (tribromoethanol). A 24-gauge Teflon catheter (Travenol Laboratories, Inc., Deerfield, IL) was introduced to the bladder via the urinary opening. A stainless steel wire electrode was inserted through the catheter so that only 1 mm protruded outside the tip of the catheter thus coming in direct contact with the inner wall of the bladder. The electrode was activated by a Bovie unit for 4 s at the lowest coagulating setting. The electrode was then withdrawn while the catheter remained in place. MBT-2 cells (10^5) in 0.1-ml volume were instilled through the same catheter. For intravesical treatment with ABPP, mice were anesthetized with avertin, and 400 mg of ABPP per kg in a volume of 0.1 ml were instilled in the bladder. On the day of tumor evaluation, mice were killed with ether, 0.2 ml of WARF fixative (24% ethanol, 10% formaldehyde and 2% acetic acid in H_2O) were instilled in the bladders, and bladders were dissected. The next day bladders were examined macroscopically for growing tumors, cut in half, carefully dried on a blotting paper, and weighed. Some bladders were examined histologically. On the day of tumor analysis, some of the bladders still retained some ABPP in their lumen.

Statistical Methods. Student's two-tailed *t* test was used to assess the statistical significance of differences between pairs of means.

RESULTS

ABPP and ABMFPP. ABPP or ABMFPP injected i.p. at 100 or 250 mg/kg once every 4 days starting on Day 1 after MBT-2 cell inoculation resulted in significant inhibition of tumor growth (Fig. 2). Both ABPP and ABMFPP were equally effective. The administration of 250 mg/kg resulted in a statistically significant greater decrease in tumor size than 100 mg/kg on all days of measurement ($P < 0.05$). There was no effect on tumor incidence at the doses tested; all mice developed tumors.

The dose of 500 mg of ABPP per kg was examined and was well tolerated. Body weights were statistically indistinguishable in various groups; for example, after 3 wk of ABPP treatment i.p., mice weighed 20.6 ± 0.27 g compared to 20.0 ± 0.86 g for controls. The higher dose caused significantly more inhibition of MBT-2 growth than 100 mg/kg (Fig. 3). We compared effects of ABPP injected i.p. with the same dose (500 mg/kg) given p.o. via a 24-gauge curved animal feeding needle (Fig. 3). Treatment p.o. resulted in inhibition comparable to or greater than i.p. treatment on all days (Fig. 3). On Day 21 after tumor inoculation, tumor weight and tumor size were significantly smaller in mice treated p.o. ($P = 0.024$ and 0.046, respectively).

ABPP (100 mg/kg) inhibited tumor growth at a level comparable to that caused by murine IFN-β (5000 units) injected s.c. daily starting on the day after MBT-2 inoculation (compare Figs. 3 and 4). Poly(I)·poly(C) (10 mg/kg) injected i.p. every other day starting on Day 1 inhibited tumor growth more efficiently than even ABPP (500 mg/kg) (compare Figs. 3 and 4).

MBT-2 cells (10^5) were instilled in the bladder after a slight mucosal injury caused by a coagulating cautery. More than 95% of the bladders developed tumors; only 2 of 70 bladders showed no signs of tumor cell inoculation. No tumors were found in the peritoneal cavity, indicating proper bladder instillation. ABPP at 400 mg/kg was administered twice/wk starting on Day 4 after MBT-2 instillation for a total of 5 treatments. It was given p.o. or instilled directly in the bladder. Control mice received the vehicle p.o. Mice treated with the vehicle had mainly large tumors in their bladders (Table 1). The mean weight of bladders in the control group was significantly higher than that of mice treated with ABPP p.o. ($P = 0.03$) or

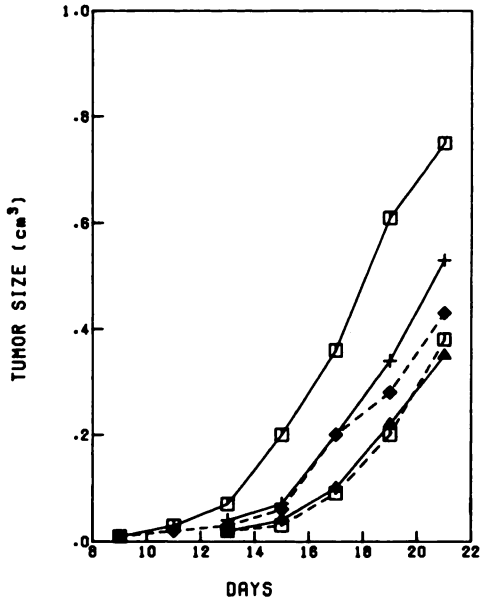


Fig. 2. Inhibition of MBT-2 growth by ABPP or ABMFPP at 100 or 250 mg/kg. Mice were inoculated s.c. with 10^5 MBT-2 cells in the inguinal region on Day 0. ABPP or ABMFPP was injected i.p. once every 4 days starting on Day 1. Tumor weights in g (mean ± SE) are also provided for comparison. □——□, vehicle, 0.90 ± 0.234 g; +, ABMFPP (100 mg/kg), 0.47 ± 0.177 g; □- - -□, ABMFPP (250 mg/kg), 0.27 ± 0.052 g; ◆, ABPP (100 mg/kg), 0.35 ± 0.087 g; ▲, ABPP (250 mg/kg), 0.27 ± 0.077 g.

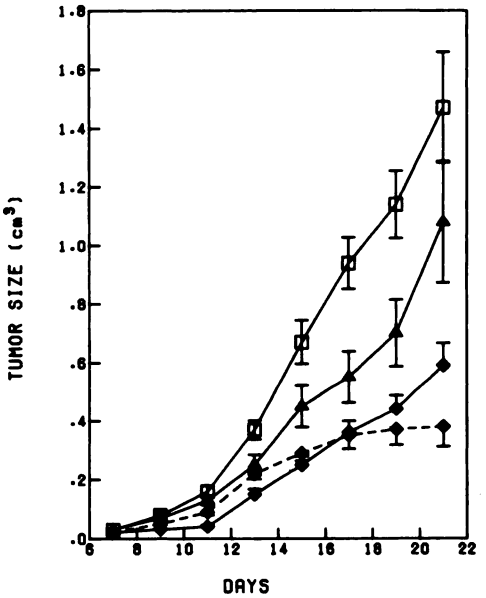


Fig. 3. Effect of route of ABPP administration (p.o. or i.p.) on level of inhibition of MBT-2 growth. Mice were inoculated with 10^5 MBT-2 cells on Day 0. ABPP was introduced i.p. or p.o. once every 4 days starting on Day 1; *bars*, SE. Tumor weights in g (mean ± SE) are also provided for comparison. □, vehicle, 2.04 ± 0.340 g; ▲, ABPP (100 mg/kg) i.p., 1.07 ± 0.356 g; ◆- - -◆, ABPP (500 mg/kg) i.p., 0.67 ± 0.118 g; ◆——◆, ABPP (500 mg/kg) p.o., 0.32 ± 0.088 g.

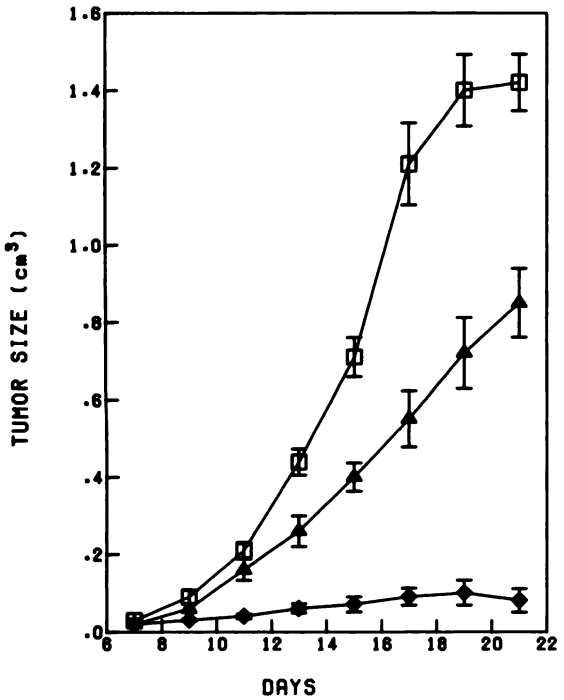


Fig. 4. Effect of murine β-interferon or poly(I)·poly(C) on MBT-2 growth. Mice were inoculated with 10^5 MBT-2 cells on Day 0, and treatment started on Day 1; *bars*, SE. Tumor weights in g (mean ± SE) are also provided for comparison. □, PBS i.p. every other day, 2.23 ± 0.192 g; ▲, 5000 units β-interferon s.c. daily, 0.95 ± 0.167 g; ◆, poly(I)·poly(C) (10 mg/kg) i.p. every other Day 1, 0.04 ± 0.018 g.

intravesically ($P = 0.0095$) (Table 1). Intravesical application of ABPP resulted in lighter bladders than in mice treated p.o. (43.5 ± 5.9 mg *versus* 54.5 ± 7.9 mg). The difference was, however, not statistically significant ($P = 0.27$).

ABDFPP and AIDFPP. We tested a high and a low dose of two aryl pyrimidinones, ABDFPP and AIDFPP, particularly effective in inducing high circulating IFN (2), for effects on MBT-2 cells inoculated s.c. Analogous to studies with ABPP or ABMFPP, no ill effects of treatment were observed as judged by appearance or changes in body weight. The drugs were tested in mice inoculated with different tumor loads: 10^4 or 10^5 MBT-2 cells.

In mice inoculated with 10^4 MBT-2 cells, it was observed that both compounds at 100 mg/kg or 250 mg/kg injected i.p. every other day starting on Day 1 inhibited tumor growth (Fig. 5, *A* and *B*). When ABPP (200 mg/kg i.p. every 4 days) effects were compared with the higher dose of ABDFPP or AIDFPP (250 mg/kg), the latter two compounds were more effective. Sizes of tumors in ABPP-treated mice, although larger, were not statistically different from those treated with the lower dose of either ABDFPP or AIDFPP (100 mg/kg). This suggests the latter compounds were more effective inhibitors of MBT-2 growth than ABPP.

No statistically significant difference in tumor size of mice treated with the same dose of either ABDFPP or AIDFPP existed, suggesting both compounds had similar effects on MBT-2 growth. Since, however, ABDFPP always resulted in smaller tumors it may be slightly more effective. There was also no statistically significant difference on most days between tumor size of mice treated with the high or low dose of each compound, although consistently the higher dose resulted in smaller tumors.

Most of the drug-treated mice had palpable tumors on Day 9. The majority of the tumors continued to grow although at a slower pace than those of control mice (Fig. 5, *A* and *B*).

Table 1 *Effect of intravesical instillation or p.o. application of ABPP (400 mg/kg) on growth of 10^5 MBT-2 cells instilled in the bladder*

Treatment[a]	*n*	Bladder wt (mg)	Normal bladder	Microscopic or small tumors	Medium or large tumors
Vehicle p.o.	18	108.2 ± 21.6[b]	2	4	12
ABPP p.o.	21	54.5 ± 7.9	0	15	6
ABPP intravesical	17	43.5 ± 5.9	0	12	5

[a] Treatment, twice/wk, started on Day 4 of MBT-2 instillation and continued for a total of 5 treatments. Mice were assayed on the next day of the last drug application, 19 days after tumor cell instillation.
[b] Mean ± SE.

However, as the treatment continued, tumors in some of the mice disappeared (Table 2). Tumor-free mice on Day 21 were kept alive. Some of them developed tumors which grew progressively; others remained tumor free until Day 40. The higher dose of each compound resulted in a higher number of tumor-free mice (Table 2). Mice with regressed tumors on Day 40 continued to be free of tumors until Day 100 when the experiment was terminated (Table 2).

All mice inoculated with 10^5 cells had palpable tumors on Day 5 compared to Day 7 for mice inoculated with the lower cell dose. Vehicle-treated mice, inoculated with 10^5 cells, had significantly larger tumors throughout the period of tumor growth than the ones inoculated with 10^4 cells, *e.g.*, 1456 mm^3 compared to 931 mm^3 on Day 21. The difference was significant ($P < 0.001$) by Day 7. All treatments were highly effective in inhibiting MBT-2 growth (Fig. 5, *C* and *D*). Mice treated with ABPP had significantly smaller tumors than those of control mice by Day 13; for 10^4 cells differences in size were not significant until Day 19. Mice treated with ABDFPP or AIDFPP (100 mg/kg) and inoculated with 10^5 cells had significantly smaller tumors than those treated with ABPP during most of the period of tumor growth (Fig. 5).

Similar to what was observed with mice inoculated with 10^4 cells, there was no statistically significant difference for tumor size of mice treated with the same dose of ABDFPP or AIDFPP, or, on most days, the high dose and low dose of each compound. The higher dose consistently resulted, however, in smaller tumors.

Treatment with compounds ABDFPP or AIDFPP in mice inoculated with 10^5 cells resulted in 100% incidence in all groups by Days 5 and 7 (Table 3). The higher doses of both ABDFPP and AIDFPP resulted in more cures than the lower dose. An equivalent number of cures occurred in mice inoculated with 10^5 cells to those inoculated with 10^4 cells (15 *versus* 13). While vehicle-treated mice had significantly larger tumors in mice inoculated with 10^5 cells than in mice inoculated with 10^4 cells, most tumor measurements of a particular pyrimidinone treatment in both groups of mice were not statistically different (compare Fig. 5, *A* and *B*, with Fig. 5, *C* and *D*). Thus, final tumor size after a given pyrimidinone treatment was equivalent in mice inoculated with either 10^4 or 10^5 MBT-2 cells.

All tumor-free mice along with 15 fresh C3H/He mice were inoculated on Day 70 with 5×10^4 MBT-2 cells in the left inguinal region (site opposite the first injection). This particular tumor inoculum was not highly tumorigenic; only 9 of 15 tumors resulted in the previously uninoculated mice on Day 21. None of the treated mice (0 of 30), however, developed any tumors by Day 21 when the experiment was terminated.

DISCUSSION

Pyrimidinones as a single modality were strongly inhibitory in the transplantable MBT-2 bladder model. Both ABPP and

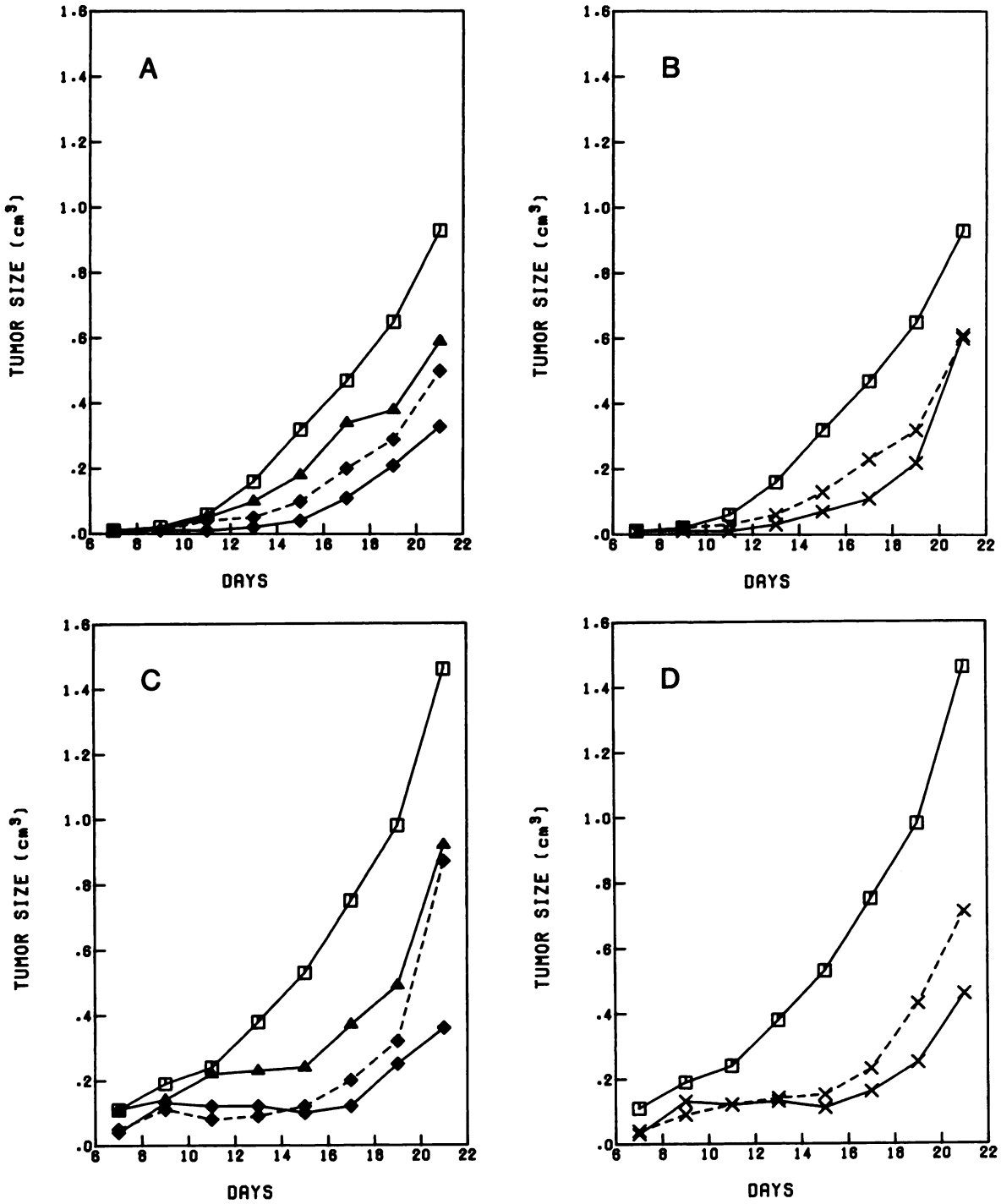


Fig. 5. Effect of i.p. injection of 100 or 250 mg of ABDFPP or AIDFPP per kg as well as 200 mg of ABPP per kg on the growth of tumors in mice inoculated on Day 0 with 10^4 (*A* and *B*) or 10^5 (*C* and *D*) MBT-2 cells and treatment started on Day 1. Tumor weights were not recorded in this experiment. □, vehicle every other day; ▲, ABPP (200 mg/kg) once every 4 days; ◆– – –◆, ABDFPP (100 mg/kg) every other day; ◆——◆, ABDFPP (250 mg/kg) every other day, ×– – –×, AIDFPP (100 mg/kg) every other day; ×——×, AIDFPP (250 mg/kg) every other day.

Table 2 *Tumor incidence; number of tumor-free mice in different treatment groups on different days in mice inoculated s.c. with 10^4 MBT-2 cells (15 mice/group)*

	Day									
Treatment	7	9	11	13	15	17	19	21	40	100
Vehicle	2	1	0	0	0	0	0	0		
ABDFPP (100 mg/kg)	4	2	4	2	4	4	4	4	1	1
ABDFPP (250 mg/kg)	11	8	5	4	2	5	5	5	3	3
AIDFPP (100 mg/kg)	1	1	0	1	3	5	4	5	3	3
AIDFPP (250 mg/kg)	9	11	5	5	5	5	6	8	6	6
ABPP (200 mg/kg)	4	1	1	1	2	2	2	2	2	2

Table 3 *Tumor incidence; number of tumor-free mice in different treatment groups on different days in mice inoculated s.c. with 10^5 MBT-2 cells (15 mice/group)*

	Day										
Treatment	5	7	9	11	13	15	17	19	21	40	100
Vehicle	0	0	0	0	0	0	1	1	1	1	1
ABDFPP (100 mg/kg)	0	1	1	1	2	3	3	5	4	3	3
ABDFPP (250 mg/kg)	0	0	1	2	2	3	5	7	6	6	6
AIDFPP (100 mg/kg)	0	0	1	2	2	2	2	3	2	2	2
AIDFPP (250 mg/kg)	2	0	3	1	2	2	4	5	5	4	4
ABPP (200 mg/kg)	0	0	0	0	0	0	0	0	1	1	1

ABMFPP, while active, did not effect cures. ABPP (100 mg/kg) resulted in inhibition similar to 5000 units of IFN-β injected s.c. daily. Administration of ABPP p.o. was as effective as i.p. treatment, indicating equal bioavailability of this highly insoluble compound for the two routes. Intravesical instillation of ABPP resulted in slightly better inhibition of MBT-2 tumors initiated in the bladder wall than the p.o. route. The pyrimidinones ABDFPP and AIDFPP resulted in similar reduction in MBT-2 tumor growth. They were, however, more potent than ABPP or ABMFPP. Moreover, their application resulted in cures of up to 40% of mice with previously palpable tumors. Cured mice remained tumor free for more than 100 days, at which time observation ceased. Cured mice (Day 70) were resistant to a second inoculum of MBT-2 cells, suggesting development of immunity to MBT-2 (19). Two hundred fifty mg of either compound per kg caused more tumor-free mice than 100 mg/kg. This was true for mice with a low inoculum of MBT-2 cells (10^4) or the high inoculum (10^5). This increased potency of ABDFPP and AIDFPP to inhibit MBT-2 growth and to cause some cures may be related to their increased ability to induce IFNs (5–10 times higher peak serum levels when compared to ABPP and ABMFPP).[4]

Poly(I)·poly(C) at 10 mg/kg was much more inhibitory of MBT-2 tumor size than any of the pyrimidinones tested. Yet treatment with poly(I)·poly(C) did not result in cures (19). The pyrimidinones ABDFPP or AIDFPP did not inhibit ultimate tumor size as efficiently as poly(I)·poly(C), but the treatment resulted in cures in some of the mice even at 100 mg/kg.

Antitumor effects in the mouse were much more pronounced when treatment with ABPP or other pyrimidinones was combined with a single injection of CY (9). CY was postulated to reduce the tumor load to allow pyrimidinone treatment to be effective. Our results, however, suggest treatment with pyrimidinones can be effective against larger tumor cell inocula. Thus, treatment with the same dose and schedule of mice inoculated with 10^4 MBT-2 cells or 10 times as much resulted in comparable tumor incidence in both groups (compare Tables 2 and 3). Tumor size in vehicle-treated mice inoculated with 10^4 and 10^5 cells was significantly different. While mice inoculated with the higher tumor cell number had larger tumors, treatment with ABDFPP or AIDFPP resulted in reduced tumor size to the same degree. Similar results were obtained in a previous study on the effects of poly(I)·poly(C) on MBT-2 growth (19).

The antitumor effect for transplantable, FANFT-derived MBT-2 of bladder origin represents the most pronounced antitumor effects of pyrimidinones as a single modality in experimental systems. The present study suggests tumors of bladder origin are particularly sensitive to the antitumor effects of these potent, low-molecular-weight compounds. We are now investigating the effects of pyrimidinones in combination with cytoreductive agents on MBT-2. Possible protection by pyrimidinones against carcinogenesis induced by a bladder-specific nitrofuran (FANFT) is also under study. Whether the sensitivity of MBT-2 reflects the tissue of origin or a unique susceptibility of MBT-2 cells to host effector mechanisms will require additional investigation.

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