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ORIGINAL ARTICLE

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Antitumor activity of Virulizin, a novel biological response modifier (BRM) in a panel of human pancreatic cancer and melanoma xenografts

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Abstract *Purpose*: To define the anticancer efficacy of Virulizin in vivo as a single agent or in combination with conventional drugs in human pancreatic tumor and melanoma xenografts. *Methods*: The therapeutic effect of Virulizin was evaluated in a series of human tumor xenografts in athymic nude mice. *Results*: Virulizin had a high level of antitumor activity against all the pancreatic tumors (BxPC-3, SU 86.86. and Mia-PaCa-2) and melanomas (C8161 and A2058), as indicated by suppression of tumor growth with an optimal T/C value of $\leq 40\%$ when administered as a single agent. No significant changes in Virulizin antitumor activity were observed when different schedules (3 days/week vs 7 days/week) or routes of administration (i.p. vs i.m.) were used. In combination therapy, Virulizin significantly enhanced the antitumor activity of gemcitabine and 5-fluorouracil against pancreatic tumors and of dacarbazine against metastatic melanomas, as reflected by a further decrease in tumor growth as compared to tumors in animals treated with the conventional drugs alone. *Conclusion*: These studies suggest that Virulizin effectively inhibits the growth of solid human pancreatic tumors and melanomas in the xenograft model.

Keywords Virulizin · Antitumor · Pancreatic cancer · Melanoma

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Introduction

Pancreatic cancer is commonly diagnosed at an advanced stage, and patients with this type of cancer have a poor prognosis and suffer debilitating disease-related symptoms [1]. Despite continued efforts to develop a variety of conventional treatment regimens, pancreatic cancer is usually either resistant or has a low rate of response to most conventional chemotherapies [2, 3, 4, 5, 6]. Currently 5-fluorouracil (5-FU) and gemcitabine are the two primary drugs for the treatment of pancreatic cancer. However, the impact of 5-FU on patient survival has been only minimal with response rates of less than 20% (4.2–5.5 months median survival and a 2% 1-year survival rate) [4, 7, 8]. Treatment with recently developed gemcitabine, a first-line therapy approved by the FDA (Food and Drug Administration, USA), also only provides a marginal improvement with approximately 6 months median survival with 1-year survival rates ranging from 18% to 23% [8, 9, 10, 11].

Patients with primary cutaneous melanoma have an expected survival of 95% at 10 years with proper surgical excision, but the prognosis for patients with malignant melanoma is poor as well (e.g. 13% 1-year survival and 3.1 months median survival for stage IV disease) [12]. Dacarbazine (DTIC) is the only drug approved by the FDA for treating melanoma despite relatively low response rates. Randomized clinical trials have shown a response rate to DTIC treatment of between 15% and 25% with a median duration of 5–6 months [13]. In another similar study of the survival of patients with high-risk melanoma, DTIC treatment gave no statistically significant survival improvement [14]. These studies indicate a substantial lack of effective therapeutic modalities for both pancreatic cancer and metastatic melanoma. Therefore, it is urgent and important to continue the development and evaluation of new therapeutic agents for these diseases.

Immunotherapy for cancers is based on stimulation of immune responses of the patient's immune system

against tumor cells. In the natural course of melanoma the host's immunological responses appear to play a role, as indicated by the observation that melanoma metastases can occasionally spontaneously regress for many months or years [15]. It has been documented that T cells and particularly macrophages are involved in the response to melanoma [16]. Therefore, immunotherapy approaches are the active areas of research in this disease, including recombinant cytokines (IL-2, IL-12 and IFN-α) and other biological response modifiers (BRM), such as vaccines and monoclonal antibodies [17].

Virulizin is a novel BRM obtained from bovine bile by a standardized extraction process (Lorus Therapeutics, Toronto, Canada). In vitro, Virulizin enhances a cell-mediated immune response against tumor cells by direct activation of macrophages/monocytes. Addition of Virulizin to in vitro cultures stimulates blood monocytes, peritoneal macrophages and alveolar macrophages to mediate a high level of cytotoxicity against tumor cells [18]. In preclinical studies, administration of Virulizin has been shown to result in delay of BxPC-3 human pancreatic tumor growth in mice with no signs of toxicity [19], and an increase in survival time of a murine melanoma model [18]. In clinical studies with over 450 patients, Virulizin has demonstrated an excellent safety profile. Preliminary data from phase I/II clinical trials in advanced pancreatic cancer and melanoma show that patients who are given Virulizin survive longer than historical controls [18]. Due to the immunotherapeutic properties of Virulizin, we predicted that administration of Virulizin would not only suppress human BxPC-3 pancreatic tumor growth but also the growth of other human tumors in the xenograft mouse model. In this study, we evaluated the antitumor activity of Virulizin in a panel of human pancreatic tumors and metastatic melanoma xenografts.

Materials and methods

Drugs

Virulizin (registered trademark of Lorus Therapeutics) is an aqueous solution obtained from bovine bile by a standardized process involving solvent extraction and heat hydrolysis. The drug contains a 5% (w/v) solid material mixture comprising inorganic salts (95–99% of the dry weight) and organic compounds of molecular weights of <3000 Da (1–5% of the dry weight), and is provided as a sterile, injectable formulation. Identification of all the organic compounds and inorganic components in the mixture is in process. Gemcitabine was purchased from Eli Lilly Canada (Toronto, Canada). DTIC was from Fauling (Montreal, Canada), and 5-FU was from Pharmacia and Upjohn (Toronto, Canada).

Human tumor cells, animals and tumor model

Human tumor cell lines (BxPC-3, SU 86.86., Mia-PaCa-2 and A2058) were purchased from the American Type Culture Collection (ATCC, Manassas, Va.). They were grown in culture medium and under conditions as recommended in the ATCC technical datasheet. C8161, a gift from Dr. D.R. Welch (Pennsylvania State University, Hershey, Pa.) [20], was grown in RPMI 1640 medium (Wisent, St. Bruno, QC, Canada) supplemented with 10% fetal bovine serum (Wisent) at 37°C under an atmosphere comprising 95% air and 5% CO_2. CD-1 athymic nude mice (6–8 weeks old, female) were purchased from Charles River (Montreal, Canada). These mice are T-cell deficient (no thymus at birth) and have a low antibody response [21], and would be able to accept the xenografted human tumor tissues, which are usually rejected by immunologically competent strains, without the need for immunosuppressive treatment. In addition, it may be more important that the tumor-bearing state of patients is known to induce immune dysfunction similar to these immune-deficient mice. Therefore, xenografts in CD-1 athymic mice provide an ideal model for evaluating the efficacy of immunotherapy. All the animals were maintained in the animal facility of Sunnybrook and Women's College Health Sciences Center (Toronto, Canada). The animal experimentation was performed following the NIH (National Institutes of Health, USA) and our Lorus Therapeutics Animal Care and Use guidelines.

Before reaching approximately 80% confluence in culture medium, the tumor cells were harvested and suspended in sterile phosphate-buffered saline. A volume of 100 µl containing tumor cells (3×10^6 BxPC-3 cells, 1×10^7 SU 86.86. and Mia-PaCa-2 cells, 5×10^6 C8161 and A2058 cells) was implanted subcutaneously into the right flank of the mice (20–28 g body weight). The animals were monitored on a daily basis. The treatment started when the tumor had reached a volume of 50–100 mm^3, and the animals were randomly separated into groups of at least 8–15 animals, so that the mean tumor size in each group was the same. Animals were treated with Virulizin and other substances such as saline or conventional drugs listed above until the endpoint of each experiment. The doses and schedules are described in the text.

Evaluations of antitumor activity

Tumors were measured using calipers and the volume estimated as length×width×height/2 [22]. The efficacy of a drug was evaluated in terms of tumor growth inhibition (T/C), tumor growth delay (T−C), tumor regression, and tumor weight (TW). T/C was calculated from the mean tumor volumes of the drug-treated (T) and control groups (C): T/C(%) = (mean tumor volume of drug-treated group/mean tumor volume of control group)×100. The optimal T/C value was the minimal T/C ratio which reflects the maximal tumor growth inhibition achieved [23]. T−C was defined as the difference in time for drug-treated (T) and control (C) tumors to reach a given volume. Tumor regression was defined as partial regression (PR) if the tumor volume decreased to ≥50%, or as minor regression (MR) if the tumor volume decreased from 0 to 50% of that at the start of treatment. The final TW was the mass of tumor tissue isolated by surgery from the animal on the last day of an experiment. Percentage inhibition was determined as (mean TW of controls−mean TW of drug-treated group)/(mean TW of controls)×100. The single-blind measurement for evaluating the efficacy of drug was applied to the initial experiment for each tumor. In addition, the same experiment was repeated by different people and at different times. The statistical analysis of the difference in TW between the groups was carried out by the Biostatistical Consulting Unit of the Department of Community Health Sciences at the University of Manitoba (Winnipeg, Canada). P values ≤ 0.05 were considered statistically significant.

Results

Human pancreatic carcinomas

BxPC-3

The therapeutic efficacy of Virulizin against human pancreatic tumors was first tested on gemcitabine-resistant BxPC-3 cells either as a single agent or in

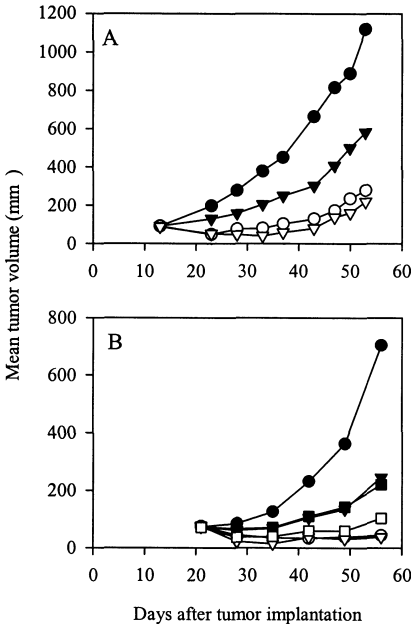

Fig. 1 Antitumor effect of Virulizin as a single agent or in combination with conventional drugs on human pancreatic BxPC-3 xenografted tumors. Tumor cells (3×10^6) were implanted into each athymic nude mouse on day 0. The following treatments were administered: 0.2 ml saline/day (i.p.), 0.2 ml Virulizin/day (i.p.), 2.5 mg gemcitabine in 0.1 ml saline (100 mg/kg) every 3 days (i.v.), and 0.75 mg 5-FU in 0.1 ml saline (30 mg/kg) every other weekday (i.v.). The treatments started (**A**) on day 13 ($n=9$) and (**B**) on day 21 ($n=10$) (● saline, ○ Virulizin, ▼ gemcitabine, ▽ gemcitabine plus Virulizin, ■ 5-FU, □ 5-FU plus Virulizin)

combination with the conventional drugs gemcitabine and 5-FU. The results are shown in Fig. 1 and summarized in Table 1. In these two separate experiments, administration of Virulizin alone resulted in a significant delay in tumor growth as compared to saline controls, as indicated by a significant optimal T/C value of 19% and T−C of 22 days, and a T/C value of 6% with tumor PR. The mean TW in Virulizin-treated animals was decreased by 74% ($P=0.0323$) and 95.5% ($P=0.0168$) at the endpoints of the experiments. In combination with gemcitabine, Virulizin further suppressed the growth of BxPC-3 tumors treated with gemcitabine, as demonstrated by a decrease in the optimal T/C value (46% to 11%, and 35% to 5%) as compared to the animals treated with gemcitabine alone. A decrease in TW following combination therapy was also recorded in these two experiments (671 mg to 198 mg, $P = 0.0204$; and 381 mg to 44 mg, $P = 0.0262$).

The antitumor activity of Virulizin in combination with 5-FU was also greater than that of 5-FU alone, as indicated by a decrease in the optimal T/C value from 31% (5-FU monotherapy) to 15% (combination therapy). Inhibition in TW was increased from 67% with 5-FU monotherapy to 85% with Virulizin in combination with 5-FU. In addition, the times for BxPC-3 to grow to a volume of 50–100 mm^3 were different in these two experiments (13 days in experiment A; 21 days in experiment B), which might have been due to handling by different people and/or at different times. Taken together, our studies reveal that Virulizin had a high level of antitumor activity against pancreatic BxPC-3 tumor in both monotherapy and combination therapy with conventional drugs, which was similar to that demonstrated previously [19].

The antitumor activity of Virulizin was next examined with different schedules and routes of administration. The results are summarized in Table 2. Comparing Virulizin antitumor activity in the different schedules, its activity remained high in the 3 days/week schedule as indicated by a 40% optimal T/C value and 61% inhibition of TW ($P=0.0016$ vs saline controls), but was not significantly different in the 7 days/week schedule ($P=0.2794$). When Virulizin was administered by

Table 1 Activity of Virulizin against BxPC-3 pancreatic tumor

Experiment	Treatment	Optimal T/C		T−C (days)[a]	Partial regression (days)	Tumor weight (mg)	Inhibition (%)	*P* value	
		%	Day					Vs saline	Vs Gemcitabine/5-FU
A ($n=9$)	Saline					1226			
	Virulizin	19	43	22		321	74	0.0323	
	Gemcitabine	46	43	8		671	45	0.1938	
	Virulizin + gemcitabine	11	33	27		198	84	0.0125	0.0204
B ($n=10$)	Saline					1022			
	Virulizin	6	56		35–56	46	95.5	0.0168	
	Gemcitabine	35	56	13		381	63	0.1232	
	Virulizin + gemcitabine	5	56		28–56	44	95.7	0.0231	0.0262
	5-FU	31	56	14		333	67	0.097	
	Virulizin + 5-FU	15	56		35–56	156	85	0.0136	0.1147

[a]Tumor growth delay (T−C) was calculated at a mean tumor size of 200 mm^3
No body weight loss was recorded in any animal treated with saline or Virulizin alone

Table 2 Activity of Virulizin against BxPC-3 pancreatic tumor with different routes and schedules of administration. BxPC-3 pancreatic tumor cells (3×10^6) were implanted into each animal on day 0. The treatment was started on day 21 and completed on day 53. Each injection was 0.2 ml of saline or Virulizin

Treatment	Schedule (days/week)	Route	Optimal T/C		Tumor weight (mg)	Inhibition (%)	*P* value
			%	Day			
Saline	3[a]	i.p.			1559		
Virulizin*	3[a]	i.p.	40	54	603	61	0.0016
Saline	7	i.p.			1590		
Virulizin	7	i.p.	33	42	513	68	< 0.0001
Saline	7	i.m.			1563		
Virulizin**	7	i.m.	26	58	305	80	< 0.0001

*P = 0.2794, **P = 0.4586, vs 7 days i.p.
[a]Monday, Wednesday, Friday
No body weight loss was noted in any animal in these experiments ($n = 20$)

intramuscular instead of intraperitoneal injection, its antitumor activity was high as well, as indicated by a 26% optimal T/C value and 80% inhibition of TW ($P < 0.0001$ vs saline controls). No significant difference in antitumor efficacy was noted between groups of mice injected intraperitoneally and intramuscularly ($P = 0.4586$). The results suggest that a less-stressful schedule (3 days instead of 7 days per week) does not result in a reduced antitumor efficacy of Virulizin in this animal model, and that the drug can be administered effectively both intraperitoneally and intramuscularly.

SU 86.86.

The antitumor activity of Virulizin in pancreatic tumor models was further evaluated using human carcinoma SU 86.86. cells in monotherapy and combination therapy with conventional drugs (gemcitabine and 5-FU). As shown in Fig. 2 and summarized in Table 3, Virulizin monotherapy resulted in a significant delay of tumor growth as compared to saline controls, as indicated by a T/C value of 11% with tumor PR, and a T/C value of 19% and a T−C of 19 days. The mean TW was decreased by 73% ($P = 0.0327$) and 59% ($P = 0.0451$) in the Virulizin-treated animals as compared to saline controls. Concurrent administration of Virulizin further increased the antitumor efficacy of gemcitabine and 5-FU against SU 86.86. In the first experiment, Virulizin increased the antitumor activity of gemcitabine, as indicated by a decrease in the optimal T/C value from 31% (gemcitabine only) to 10% (combination of gemcitabine and Virulizin), and by an increase in the inhibition of TW from 59% (gemcitabine only) to 88% (combination of gemcitabine and Virulizin; $P = 0.0387$). In the second experiment, even though the antitumor efficacy of gemcitabine alone was already very high, it was still slightly enhanced by combining with Virulizin, as reflected by a slight decrease in the optimal T/C value from 5% to 3%, and by a further increase in inhibition of TW from 94% to 97% ($P = 0.1834$). The antitumor efficacy of 5-FU alone was dramatically increased by combining with Virulizin, as indicated by a large decrease in the optimal T/C value from 68% (5-FU alone) to 5% (combination of 5-FU and Virulizin), and by a significant increase in inhibition of TW from 14% (5-FU alone) to 96% (combination of 5-FU and Virulizin; $P = 0.0022$). These results demonstrate that administration of Virulizin as a single agent or as part of combination therapy effectively suppresses the growth of SU 86.86. pancreatic tumors in a xenograft mouse model.

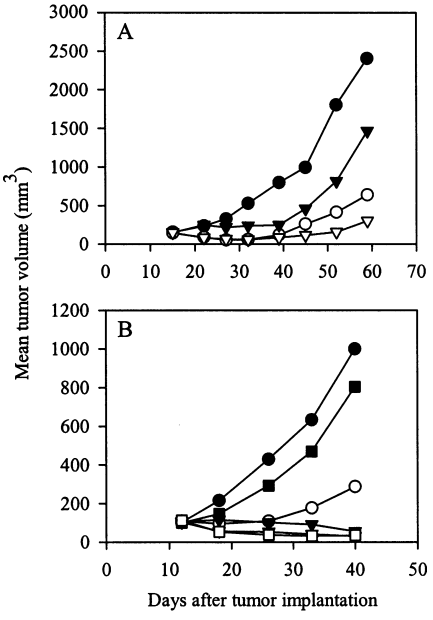

Fig. 2 Antitumor effect of Virulizin as a single agent or in combination with conventional drugs on human pancreatic SU 86.86. xenografted tumors. Ten million tumor cells were transplanted into each athymic nude mouse on day 0. The dose and route of administration for saline, Virulizin, Gemcitabine and 5-FU were the same as in Fig. 1. The treatments started (A) on day 15 (n = 10) and (B) on day 12 (n = 10). (●) Saline; (○) Virulizin; (▼) Gemcitabine; (▽) combination of Gemcitabine and Virulizin; (■) 5-FU; and (□) combination of 5-FU and Virulizin

Mia-PaCa-2

To confirm the antipancreatic tumor activity of Virulizin, the effect of Virulizin treatment as either

Table 3 Activity of Virulizin against SU 86.86. pancreatic tumor

Experiment	Treatment	Optimal T/C		T−C (days)[a]	Partial regression (days)	Tumor weight (mg)	Inhibition (%)	*P* value	
		%	Day					Vs saline	Vs Gemcitabine/ 5-FU
A ($n=10$)	Saline					1226			
	Virulizin	11	32		27–32	333	73	0.0327	
	Gemcitabine	31	39	13		504	59	0.0453	
	Virulizin + gemcitabine	10	39		27–42	143	88	0.0176	0.0387
B ($n=10$)	Saline					981			
	Virulizin	19	33	19		399	59	0.1045	
	Gemcitabine	5	40		20–40	61	94	0.0087	
	Virulizin + gemcitabine	3	40		20–40	30	97	0.0075	0.1834
	5-FU	68	26	5		841	14	0.7257	
	Virulizin + 5-FU	4	40		20–40	36	96	0.0258	0.0022

[a]Tumor growth delay (T−C) was calculated at a meantumor size of 300 mm^3
No body weight loss was noted in any animal treated with saline or Virulizin alone

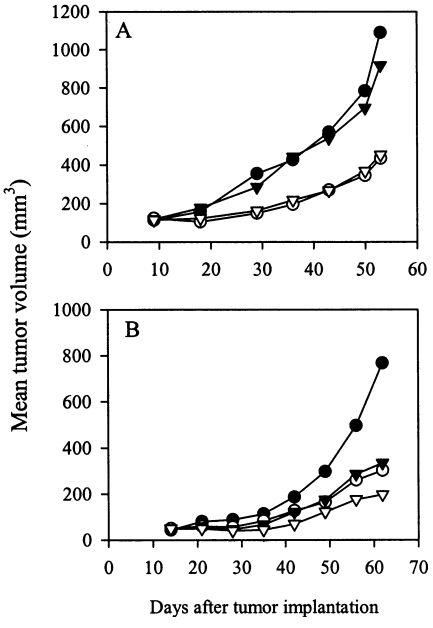

Fig. 3 Antitumor effect of Virulizin as a single agent or in combination with conventional drugs on human pancreatic Mia-PaCa-2 xenografted tumors. Ten million tumor cells were transplanted into each athymic nude mouse on day 0. The dose and route of administration for saline, Virulizin and Gemcitabine were the same as in Fig. 1. The treatments started on day 14 (n = 23). (●) Saline; (○) Virulizin; (▼) Gemcitabine; (▽) combination of Gemcitabine and Virulizin

monotherapy or in combination with gemcitabine was tested in human Mia-PaCa-2 pancreatic tumor, which is not sensitive to gemcitabine toxicity [24]. The combined data from two separate experiments are illustrated in Fig. 3 and summarized in Table 4. Administration of Virulizin as a single agent retarded tumor growth with an optimal T/C value of 39% and a T−C of 14 days as compared to saline-treated controls, suggesting high activity of Virulizin against Mia-PaCa-2 pancreatic tumor. Treatment with Virulizin alone resulted in 60% inhibition of mean TW ($P=0.0232$). The combination of Virulizin and gemcitabine showed a significantly greater antitumor effect against this tumor, as indicated by an optimal T/C value of 35% and a T−C of 18 days. TW was inhibited by 74% ($P=0.0043$). As compared to treatment with gemcitabine alone, the combination therapy demonstrated enhancement of antitumor efficacy with an optimal T/C value of 35%, which was lower than that (67%) obtained with gemcitabine alone. TW was decreased from 39% (gemcitabine alone) to 74% (combination therapy; $P=0.0169$). These results show that Virulizin suppressed the growth of Mia-PaCa-2 tumor, and its effect was much greater in combination with gemcitabine.

Metastatic melanomas

C8161

The antitumor efficacy of Virulizin against melanoma C8161 is illustrated in Fig. 4 and summarized in Table 5. Administration of Virulizin either as a single agent or in combination with DTIC resulted in a significant delay of tumor growth. Virulizin alone showed significant antitumor activity against C8161 melanoma with optimal T/C values of 35% (T−C 16 days) and 34% (T−C 14 days) in two separate experiments as compared to saline-treated controls. TW was significantly decreased by 72% ($P=0.0031$; experiment A) and 61% ($P=0.0085$; experiment B) by treatment with Virulizin. Virulizin enhanced the antitumor efficacy of DTIC with a decrease in the optimal T/C value from 9% (DTIC alone) to 2% (combination of DTIC and Virulizin), and a further decrease in TW from 93% (DTIC alone) to 99% (combination of DTIC and Virulizin; $P = 0.0339$). Our studies indicated that Virulizin is able to inhibit melanoma C8161 growth as a single agent, and the combination of DTIC and Virulizin exhibits a significantly greater antitumor effect than either drug alone.

Table 4 Activity of Virulizin against Mia-PaCa-2 pancreatic tumor

Experiment	Treatment	Optimal T/C		T−C (days)[a]	Tumor weight (mg)	Inhibition (%)	*P* value	
		%	Day				Vs saline	Vs gemcitabine
A ($n=8$)	Saline				725			
	Virulizin	40	53	17	359	50	0.1199	
	Gemcitabine	80	29	0	701	3	0.9162	
	Virulizin + gemcitabine	41	53	13	335	54	0.0495	0.1291
B ($n=15$)	Saline				1195			
	Virulizin	39	62	9	443	63	0.0602	
	Gemcitabine	43	62	8	597	50	0.1726	
	Virulizin + gemcitabine	26	62	20	231	81	0.0157	0.0456

[a]Tumor growth delay (T−C) was calculated at mean tumor size of 200 mm^3
No body weight loss was observed in any animal treated with saline or Virulizin alone

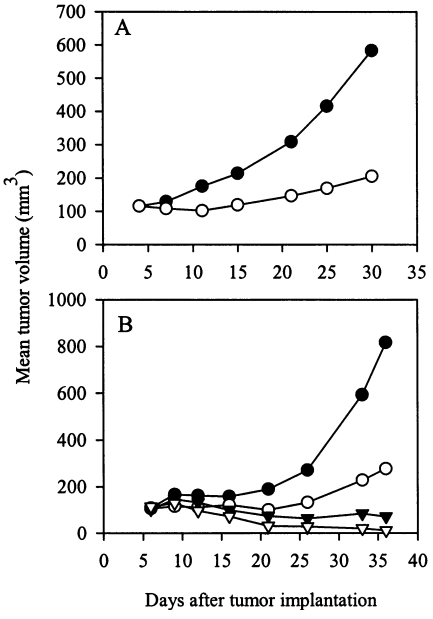

Fig. 4 Antitumor effect of Virulizin as a single agent or in combination with conventional drugs on human melanoma C8161 xenografted tumors. Five million tumor cells were transplanted into each athymic nude mouse on day 0. An i.p. injection given to each animal was 0.2 ml saline/day, 0.2 ml Virulizin/day, 2.0 mg DTIC in 0.2 ml saline (80 mg/kg)/day at the beginning of 5 days. The treatments started (A) on day 4 (n = 10) and (B) on day 6 (n = 10). (●) Saline; (○) Virulizin; (▼) DTIC; (▽) combination of DTIC and Virulizin

A2058

To further confirm the antitumor activity of Virulizin against melanoma, we evaluated its therapeutic effect in monotherapy and combination therapy with DTIC against human A2058 cells. As shown in Fig. 5 and summarized in Table 6, again Virulizin treatment resulted in a significant delay of A2058 melanoma growth as compared to saline controls in this model. Virulizin alone effectively inhibited tumor growth in both experiments, as reflected by an optimal T/C value of 34% (T−C 10 days) and 40% (T−C 4 days). TW was again significantly decreased by 66% ($P=0.0125$) and 45% ($P=0.0052$) in the Virulizin-treated animals. The antitumor effects elicited by the combination of Virulizin and DTIC were significantly more potent than those obtained using DTIC alone. The optimal T/C value was decreased from 28% (DTIC alone) to 20% (combination therapy), while TW inhibition was significantly increased from 47% (DTIC alone) to 71% (combination therapy; $P=0.0139$). These findings suggest that Virulizin effectively suppresses melanoma A2058 growth in both monotherapy and in combination with DTIC.

Discussion

Early preliminary studies indicated that Virulizin treatment delays the growth of human pancreatic BxPC-3 in a xenograft model [19], and increases the median survival time of mice bearing B16-F10 murine melanoma cells [18]. The present study further examined the antitumor efficacy of Virulizin in other human pancreatic tumor and human melanoma mouse experiments. Virulizin showed profound antitumor activity in a panel of human pancreatic tumor and metastatic melanoma xenografts. As a single agent, Virulizin exhibited high antitumor activity against all the pancreatic tumors and melanomas tested. In combination therapy, Virulizin significantly increased the antitumor activity of gemcitabine and 5-FU against pancreatic tumors and DTIC against melanomas, as shown by further decreases in tumor growth as compared to the effects of the conventional drugs alone. These results also demonstrate a lack of cross-resistance between Virulizin and these conventional drugs.

Our previous studies have demonstrated that Virulizin is a monocyte/macrophage activator. The potency of Virulizin is determined by its stimulation of TNF-α production in a human monocyte cell line (U-937). Early studies showed that Virulizin is unable to stimulate the proliferation or cytotoxicity of human peripheral lymphocytes against tumor cells, but is an excellent

Table 5 Activity of Virulizin against C8161 melanoma

Experiment	Treatment	Optimal T/C		T−C (days)[a]	Regression (days)[b]	Tumorweight (mg)	Inhibition (%)	*P* value	
		%	Day					Vs saline	Vs DTIC
A ($n=10$)	Saline					759			
	Virulizin	35	30	16		216	72	0.0031	
B ($n=10$)	Saline					900			
	Virulizin	34	36	14		347	61	0.0085	
	DTIC	9	36		16–36(MR)	67	93	0.0001	
	Virulizin + DTIC	2	36		20–36(PR)	12	99	< 0.0001	0.0339

[a]Tumor growth delay (T−C) was calculated at a mean tumor size of 200 mm^3
[b]*PR* partial regression, *MR* minor regression
No body weight loss was noted in any animal treated with saline or Virulizin alone

Table 6 Activity of Virulizin against A2058 melanoma

Experiment	Treatment	Optimal T/C		T−C (days)[a]	Tumor weight (mg)	Inhibition (%)	*P* value	
		%	Day				Vs saline	Vs DTIC
A ($n=10$)	Saline				1084			
	Virulizin	34	22	10	367	66	0.0125	
B ($n=10$)	Saline				1542			
	Virulizin	40	17	4	851	45	0.0052	
	DTIC	28	17	5	817	47	0.0034	
	Virulizin + DTIC	20	17	9	442	71	< 0.0001	0.0139

[a]Tumor growth delay (T–C) was calculated at mean tumor size of 300 mm^3
No body weight loss was noted in any animal treated with saline or Virulizin alone

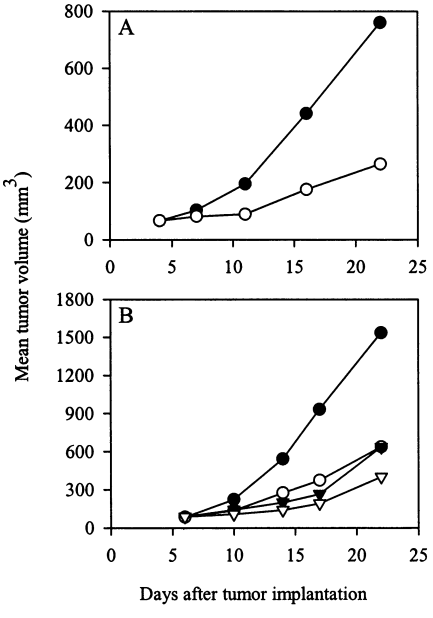

Fig. 5 Antitumor effect of Virulizin as a single agent or in combination with conventional drugs on human melanoma A2058 xenografted tumors. Five million tumor cells were transplanted into each animal on day 0. The dose and route of administration for saline, Virulizin and DTIC were the same as in Fig. 4. The treatments started (A) on day 4 (n = 10) and (B) on day 6 (n = 10). (●) Saline; (○) Virulizin; (▼) DTIC; (▽) combination of DTIC and Virulizin

stimulator of monocyte/macrophage-mediated tumor cell killing activity like granulocyte-macrophage colony stimulating factor (GM-CSF) [18]. Other studies have indicated that Virulizin does not act as a direct cytotoxic agent [19]: Virulizin added (at up to 50 µl in a total of 200 µl) to cultures of Calu-6 (human non-small-cell lung cancer cell line) cells or PANC-1 (human pancreatic carcinoma) cells did not show any cytotoxicity against the tumor cells. It was suggested that the antitumor activity of Virulizin in vivo could be due to its immunomodulating activity. The current and our previous studies [19] have indicated that administration of Virulizin effectively suppresses the growth of human tumors transplanted in athymic nude mice, which are deficient in T lymphocytes [25, 26]. It was proposed that the immunotherapeutic efficacy of Virulizin found in this model was likely due to macrophage activation. As enhancement of mitogenesis of T/B lymphocytes has been observed in Virulizin-treated mice [19], Virulizin-activated macrophages and their production of cytokines could act as intermediate by stimulating T/B lymphocytes. Additionally, recent studies have demonstrated that BxPC-3 and Mia-PaCa-2 are highly susceptible to apoptosis mediated by TRAIL (TNF-related apoptosis-inducing ligand), a member of the TNF receptor ligand family [27, 28], indicating that Virulizin-stimulated TNF-α production from monocytes/macrophages could directly induce apoptosis of these xenografted tumors in vivo.

Activation of the immune response is an attractive approach for the treatment of cancer because of its potentially greater specificity and less toxicity. The early research indicated that the residual T cells, NK cells and macrophages contribute active immune reactions against human tumor xenografts [29]. In the antitumor immunity against murine B16 melanoma, macrophage have been shown to be required for either processing of antigen or cytokine production [30]. In clinical studies of antibody therapy against tumor cells, macrophages have been found to play a role in the antibody-mediated tumor-killing mechanism [31, 32]. GM-CSF is a potent activator of natural cytotoxic activity of macrophages [33]. Dranoff and colleagues were the first to test the antitumor efficacy of more than ten known immune-activating cytokines in a non-immunogenic murine tumor, B16F10 melanoma. Only one cytokine, GM-CSF, stood out in mouse experiments as a potent therapy for this very aggressive tumor [34]. In a small-scale clinical trial, five out of seven patients with cutaneous melanoma metastases receiving perilesional intracutaneous injections of GM-CSF had a decrease in the total number of metastases, where an increase in infiltrated monocytes was noted [35]. In phase I/II clinical studies of advanced pancreatic adenocarcinoma, combination therapy of GM-CSF and dexverapamil was shown to be effective in pancreatic cancer [36, 37]. These studies support the feasibility of activating macrophages as a cell-mediated immunotherapy against both melanoma and pancreatic cancer.

Virulizin has been tested in many phase I/II and phase II clinical trials evaluating its safety and efficacy against advanced pancreatic cancer and melanoma. Three studies have involved the treatment of patients with pancreatic adenocarcinoma. The majority of patients participating in these studies had failed conventional treatment including surgery and chemotherapy. Thirlwell and colleagues reported that Virulizin treatment provided a 1-year survival rate of 31% and a 5.2-month median survival in 13 pancreatic cancer patients. No severe adverse events related to the treatment were observed [38]. In another phase I/II study, 19 out of 26 patients treated with different dose levels of Virulizin appeared to benefit from the treatment. The maximum dose level was well tolerated. Seven patients (37%) achieved stable disease and one patient in the last cohort achieved a complete response. The patients had a median survival of 6.7 months with a 1-year survival rate of 21%, and they also showed a significant improvement in quality of life [39]. In 1993, a phase II clinical trial was initiated to determine the efficacy and toxicity of Virulizin in treatment of patients with previously untreated unresectable or metastatic pancreatic adenocarcinoma. No severe toxicity of Virulizin was encountered. Among 17 patients, 6 (35%) had disease stabilization for more than 3 months, and 2 demonstrated tumor stabilization for 15 and 17 months. The 1-year survival rate was 18% with a median survival of 5 months [40].

Three phase II clinical trials have been conducted to evaluate the safety and efficacy of Virulizin in patients with malignant melanoma. In a total of 48 patients evaluated, the 1-year survival and median survival time in those with advanced disease (stage III or IV) were 24% (12 patients) and 6 months and, among those with distant metastases, the 1-year survival was 12.5% (4 patients). At least 69% of those treated with Virulizin showed no additional deterioration or had improved at 4 weeks with the response being only slightly reduced after 8 weeks [18]. As compared to the impact of 5-FU on patients with pancreatic cancer ($<20\%$ response, 4.2–5.5 months median survival and 2% of 1-year survival rate) [4, 7, 8], and the efficacy of DTIC treatment in the patients with malignant melanoma (either 15–25% response, 5–6 months median survival; or no statistical survival significance in favor of the treatment) [13, 14], the results of these clinical trials suggest that Virulizin treatment may be associated with an increase in survival advantage in patients with advanced pancreatic cancer or melanoma. A phase III clinical trial with patients with advanced pancreatic cancer is currently underway to further evaluate the potential benefit of Virulizin therapy. Our preclinical data suggest a positive outcome of the clinical trial.

In conclusion, human tumor xenografts are widely used as tumor models in an attempt to develop new and improved treatment strategies. Administration of Virulizin showed antitumor efficacy in the treatment of human pancreatic tumor and melanoma xenograft models. In addition, Virulizin could potentiate the efficacy of conventional drugs in the treatment of pancreatic cancer and melanoma in these models. Virulizin represents a novel immunotherapeutic agent that may have efficacy in the treatment of pancreatic cancer and melanoma in humans. Our preclinical studies and phase I/II clinical trials on both cancers suggest that Virulizin is a promising new immunotherapeutic agent for treatment of both cancers.

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