Anti-Cancer Drugs 1997, **8**, pp. 686–695

A quinolinone derivative, vesnarinone (OPC-8212), significantly inhibits the *in vitro* and *in vivo* growth of human pancreatic cancer cell lines

Yoshinori Nio, Hiroshi Ohmori, Yoshimitsu Minari, Noriyuki Hirahara, Susumu Sasaki, Michio Takamura and Katsuhiro Tamura

First Department of Surgery, Shimane Medical University, 89-1, Enya-cho, Izumo, Shimane 693, Japan.
Tel: (+81) 853-23-2111 (extn 2615); Fax (+81) 853-25-3240.

A quinolinone derivative, vesnarinone, has been used as a cardiotonic agent. Previous studies have demonstrated that vesnarinone has potent antitumor activity. The present study was designed to assess the antitumor effects of vesnarinone on human pancreatic cancer cell lines *in vitro* and *in vivo*. The *in vitro* effects of vesnarinone on the human pancreatic cancer cell lines (PANC-1, MIA PaCa-2 and BxPC-3) were assessed by the MTT assay, the Trypan blue dye exclusion test and the Matrigel invasion chamber assay. The inhibition of *in vivo* tumor growth was evaluated on two human pancreatic cancer xenografts (BxPC-3 and SPa-1) transplanted s.c. into nude mice. The dose of vesnarinone for 50% inhibition of cell growth in a 7 day culture ranged between 10 and 20 μg/ml as verified by the Trypan blue dye exclusion test. The dose for 50% cytotoxicity after a 3 day culture in the MTT assay was 32 μg/ml for PANC-1 and 30 μg/ml for BxPC-3, but 50% cytotoxicity for MIA PaCa-2 was not achieved by the maximal dose of vesnarinone (50 μg/ml). Nomalsky optic microscopy and acridine orange staining demonstrated the vacuolization and crater-like changes in the cell nucleus after vesnarinone treatment. Moreover, staining of an apoptosis marker (Le^y protein) and nick end-labeling increased. Vesnarinone also inhibited cancer invasion in the Matrigel invasion chamber assay. *In vivo*, BxPC-3 and SPa-1 were s.c. transplanted into the nude mice, and vesnarinone (5 or 50 mg/kg) was daily administered orally for 21 days. In both lines, vesnarinone at 50 mg/kg achieved significant inhibition. The present study suggests that vesnarinone may be a new therapeutic agent for pancreatic cancer.

Key words: Pancreatic cancer, vesnarinone.

Introduction

Pancreatic cancer is highly progressive and resistant to various cancer therapies. The prognosis of patients with pancreatic cancer is extremely poor: 87% of patients with non-resectable tumors and 50% of patients who undergo pancreatectomy die within 1 year after diagnosis.[1] Although some authors have reported better results after chemotherapy with fluorouracil-related agents, the 1 year survival rate was still less than 40%.[2] Accordingly, the development of an effective therapy for pancreatic cancer is urgently needed.

A quinolinone derivative, vesnarinone (OPC-8212), with a chemical formula of 4-dihydro-6-[4-(3,4-dimethoxybenzoyl)-1-piperazinyl]-2(1*H*)-quinolinone and molecular weight of 395.46, has been widely used for the treatment of heart failure as a cardiotonic agent with no chronotropic effects.[3,4] Vesnarinone is structurally similar to flavonoid compounds, which are known as tumor cell differentiation inducers. Therefore, it is also expected to cause cancer cell differentiation and this was demonstrated on erythroleukemia cells.[5] A previous study indicated that vesnarinone may become a potential anticancer agent,[6] and the antiproliferative effects of vesnarinone on solid tumors including cervical cancer and melanoma have also been demonstrated.[7,8] In this report, we assessed the *in vitro* cytotoxic and cytostatic effects of vesnarinone on human pancreatic cancer cell lines and the *in vivo* antitumor activity of vesnarinone alone or in combination with chemotherapeutic agents on pancreatic cancer lines, which are transplanted into nude mice.

Materials and methods

Pancreatic cancer cell lines

Four human pancreatic tumor cell lines, PANC-1, MIA PaCa-2, BxPC-3 and SPa-1, were used in this study (Table 1). PANC-1, MIA PaCa-2 and BxPc-3 were purchased from Riken Gene Bank (Wako, Japan). PANC-1 and MIA PaCa-2 lines, which were established in 1975 and 1977, respectively, represent undifferentiated carcinomas.[9,10] BxPC-3, established in 1981,

Correspondence to Y Nio

Table 1. Human pancreatic cancer lines used in the present study

Line	Histology	Mean doubling time (days)	
		In vitro	*In vivo*
PANC-1	undifferentiated carcinoma	2.1	NT
MIA PaCa-2	undifferentiated carcinoma	3.1	NT
BxPC-3	poorly to moderately differentiated adenocarcinoma	4.5	7.1
SPa-1	moderately differentiated adenocarcinoma	–[a]	8.4

[a]SPa-1 cannot be cultured *in vitro*.
NT, not tested.

represents a poorly to moderately differentiated adenocarcinoma.[11] These cell lines have been passaged *in vitro* in plastic flasks. All lines were maintained in culture medium containing 10% fetal calf serum (Gibco, Grand Island, NY), 1% L-glutamate, 1% non-essential amino acids (Gibco), 1% pyruvic acid (Gibco) and 100 μg/ml gentacin (Gibco) in RPMI-1640 (Gibco). The medium was filtered through a 0.22 μm filter (Corning, Corning, NY). These three lines are plastic-adherent, and before the experiments they were treated with 0.25% trypsin–EDTA solution (Gibco) at 37°C for 5 min. The SPa-1 xenograft line was established by us from a surgical specimen of human pancreatic cancer (35-year-old male patient). The tumor was serially passaged by s.c. transplantation into the backs of male BALB/c nude mice, since it was impossible to maintain the tumor in a cell culture. Histologically, SPa-1 tumor is a moderately differentiated adenocarcinoma of the pancreas.

Nude mice

Male BALB/c athymic nude mice (4–8 weeks old) were purchased from CLEA Japan (Tokyo, Japan). They were housed in specific pathogen-free conditions at the Shimane Institute of Health Science.

Agents

Vesnarinone (OPC-8212, Arkin-Z) was kindly supplied by Otsuka Pharmaceutical (Tokyo, Japan). Vesnarinone is not water-soluble and was first dissolved in 1 N HCl at 1 mg/ml. The solution was diluted with the culture medium, adjusted to pH 7.3 with 1 N NaOH and then filtered through a 0.22 μm filter. Vesnarinone was not soluble in a pH 7.3 medium at concentrations higher than 50 μg/ml, which is its maximal concentration *in vitro*. For *in vivo* experiments, the vesnarinone powder was emulsified in 0.5% celogen F-SB (CMC, Daiichi Kogyo Seiyaku, Tokyo, Japan) for oral administration to the nude mice. A 0.5% CMC solution was prepared by dissolving 0.5 g of CMC in 100 ml of distilled water and then heating at 50°C.

Trypan blue dye exclusion test

For determination of the cell proliferation activity, 5×10^5 cells were cultured for 7 days in 10 ml of culture medium containing the drugs at various concentrations in 25 cm^2 plastic flasks (Corning) at 37°C under 5% CO_2 and the number of growing cells was measured. After culture, the cells were treated with 0.25% trypsin–EDTA solution (Gibco) for 5–10 min, and the cells were removed from the plastic by tapping and shaking the flasks. The recovered cells were washed twice and resuspended in 1 ml of culture medium, and the live and dead cells were counted using a hemocytometer after staining with Trypan blue. All experiments were set up in triplicate (three flasks) and mean values were used to calculate the inhibition rate. The results were expressed as percent inhibition of the control value.

MTT assay

Cell damage was assessed in a 3 day culture using the MTT [3-(4,5-dimethyl-2-thiazolyl)2,5-diphenyl-2*H*-tetrazolium bromide; Sigma, St Louis, MO] method.[12] MTT was dissolved in Hanks' balanced salt solution (HBSS; Gibco) at 5 mg/ml. The cells were incubated in 96-well flat-bottom microtiter plates (Corning) at 5×10^4/0.2 ml/well in the presence of the drug at various concentrations at 37°C under 5% CO_2. After culture with vesnarinone for 72 h, MTT solution (25 μl/well) was added and the mixture was incubated for 4 h. The supernatant was carefully taken up with a pipette and the cells were stained by treatment with 0.1 ml/well of propanol for 1 h. The number of cells was measured with a microplate reader (Model 450; Nippon BioRad, Osaka, Japan) at 570 nm. All experiments were set up in quadriplicate and mean values were used to calculate the inhibition rate. The results

were expressed as percent inhibition of the control value.

Morphological observation

The tumor cells were cultured overnight in a 100 mm plastic dish (Corning) with culture medium and adhered to a 20 × 20 mm cover glass. The cover glass was moved to another dish and treated with vesnarinone for the indicated period. After treatment with vesnarinone, the cover glass was washed in phosphate-buffered saline (PBS; Gibco) at 37°C and the resulting morphological changes to the cells were observed under a Nomalsky optic differential interference microscope (IMT2-NIC; Olympus Optic, Tokyo, Japan) and photographed. In addition, acridine orange (Sigma) was added to the cell suspension to a final concentration of 5 μg/ml and morphological changes to the nuclei caused by vesnarinone were determined.[13]

Immunohistochemical staining with an apoptosis-related BM-1 antibody

After treatment with vesnarinone, the cells were stained with an apoptosis-related antibody, BM-1 (anti-Le^y, BM-1/JIMRO; Japan Antibody Laboratory, Takasaki, Gunma, Japan).[14] A histofine SAB-PO kit (Nichirei, Tokyo, Japan) was used for the following procedures, all performed according to the manufacturer's instructions. The cells were cultured in a Lab-Tek Chamber Slide (Nunc, Naperville, IL) overnight and incubated with vesnarinone. The slides were then fixed in 100% methanol. Specimens were treated with 3% peroxidase in methanol for 10–15 min at room temperature to block endogenous peroxidase. After washing twice in PBS, the specimens were treated with blocking serum (10% normal rabbit serum) for 10–15 min at room temperature to block non-specific reaction and then with the primary antibodies as indicated by the instructions. The specimens were treated with BM-1 antibody for 1 h at 37°C, washed twice in PBS and then reacted with biotin-labeled anti-mouse IgM (μ chain specific; Nichirei) as the secondary antibody. After being washed twice in PBS, specimens were exposed to peroxidase-labeled streptoavidin (Nichrei) for 5 min at room temperature. After washing in PBS, the specimens were then treated with 0.2% peroxidase in Tris–HCl buffer containing 3,3-diamino-benzidine (DAB) for 5–20 min at room temperature. After washing in distilled water, the specimens were counterstained with hematoxylin and then covered in Entellan-new (Merck, Rathway, NJ) with cover glasses. For the evaluation of the positive cell ratios, a total of 300 cells in one optical field were counted three times and the mean values were taken as the percent positive. All experiments were set up in triplicate (three slides) and the mean values were used for calculation.

Nick end-labeling[15]

The cells were incubated with vesnarinone in eight-well Lab-Tek Chambers (Nunc) at 4×10^3/0.2 ml/well. After incubation, the cells were fixed with neutral formalin solution, washed with PBS, fixed again with a mixture of ethanol:acetic acid (2:1) for 5 min and then washed again with PBS. The cells were treated with 20 μg/ml of Proteinase K (Boehringer, Mannheim, Germany) for 20 min and then with 0.3% H_2O_2 for 7.5 min at room temperature. Next, cells were rinsed with TdT buffer and reacted with terminal deoxy transferase (0.3 U/μl; Gibco) and then with biotin-16-2′-deoxyuridine-5′-triphosphate (Boehringer). The resulting cell morphology was observed with an optical microscope after staining using the ABC method. For evaluation of the positive cell ratios, a total of at least 300 cells in one optical field were counted three times and the mean values were taken as the percent positive. All experiments were set up in triplicate (three slides) and the mean values were used for calculation.

Matrigel invasion chamber assay

The invasive activity of tumor cell lines was determined by Matrigel invasion chamber (Becton Dickinson, Bedford, MA) assay according to the instructions.[16,17] Five hundred microliters of chemoattractant (the supernatant of a 3T3 fibroblast cell culture incubated for 12 h from the semiconfluent state) was added to the external fluid in a well of a Falcon 24-well plate (Becton Dickinson) and 10^4/0.2 ml of the tumor cells was added to the interior of the chamber (Falcon cell culture insert with an 8 μm pore size PET membrane coated with Matrigel). Matrigel basement membrane matrix is a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma. It contains laminin, collagen type IV, heparin sulfate proteoglycan, entactin and growth factors, including transforming growth factor (TGF)-β, basic fibroblast growth factor and others which occur naturally in the EHS tumor. After a 24 h incubation, the cells that passed through the Matrigel-coated artificial mem-

brane were counted to determine their invasive activity. Vesnarinone was added to the chamber and incubated as described above. The chamber was taken off, the culture medium was removed by suction and the non-invasive cells attached to the upper surface of the membrane were removed with a cotton swab. The chamber was dipped in formalin solution, and the cells were fixed and stained with hematoxylin. After the artificial basal membrane was cut off with a scalpel and mounted on a glass slide, the cells that had invaded the underside of the membrane were counted.

Evaluation of the toxicity and antitumor effect on human pancreatic cancer xenograft in nude mice

BxPC-3 and SPa-1 tumors were maintained *in vivo* by transplanting them into nude mice. The tumors were aseptically removed from the nude mice and cut into 2–3 mm fragments in HBSS. One fragment each was s.c. transplanted into the backs of the nude mice. After transplantation as described above, the size of the tumors was measured serially with calipers. The tumor volume was calculated according to the following formula:[18]

$$\text{volume } (V) = \text{length} \times \text{width}^2 \times 1/2.$$

When the tumor had grown to 100–300 mm^3 (usually 2–3 weeks after transplantation), the experiments were started. The mice were randomly assigned to the indicated groups of eight to 10 animals. The following groups were created: group 1, control (CMC only); group 2, vesnarinone (5 mg/kg); group 3, vesnarinone (50 mg/kg). The agents were administered directly into the stomach of the mice using a stainless steel canule (KN-348; Natsume, Matsue, Japan), daily for 3 weeks.

The tumor size was measured serially and on day 21 the tumor growth rate (TGR) in each group was calculated in terms of an inhibition rate (IR). The TGR and IR were calculated according to the following formulae:

$$\text{TGF} = V_n/V_0$$

$$\text{IR} = (1 - \text{TGR}_t/\text{TGR}_c) \times 100(\%)$$

V_n represents the tumor volume estimated n days after the initial administration and V_0 is the tumor volume at the start of the experiment. TGR_t and TGR_c indicate the TGRs of the test and control groups, respectively. A regimen was considered effective when there was a significant difference in TGR between the control group and the relevant test group.

The toxicities of the regimens were assessed in terms of the percentage mortality, body weight loss and peripheral blood cell counts.

Statistics

The results of the Trypan blue dye exclusion test and the Matrigel invasion chamber assay were evaluated for significance using unpaired Student's t-test and p values less than 0.05 were considered significant. The relative inhibition of *in vivo* tumor growth was statistically analyzed according to the Scheffe method.

Results

Cytostatic and cytotoxic effects of vesnarinone on the *in vitro* growth of human pancreatic cancer cell lines

The *in vitro* cytostatic and cytotoxic effects of vesnarinone on three human pancreatic cancer cell lines were assessed by the Trypan blue dye exclusion assay and MTT assay.

After 7 days of culturing, untreated PANC-1, MIA PaCa-2 and BxPC-3 showed 10-, 4.9- and 2.7-fold increases in growth, respectively, which were calculated using the results of the Trypan blue dye exclusion assay. When vesnarinone was added to the culture medium, the growth of the cells was dose-dependently inhibited. The growth of BxPC-3 was significantly inhibited by vesnarinone at more than 1.0 μg/ml, and the growth of PANC-1 and MIA PaCa-2 was significantly inhibited at more than 5.0 μg/ml (Figure 1). The IC_{50} value of vesnarinone was 21.0 μg/ml for PANC-1, 18.0 μg/ml for MIA PaCa-2 and 10.0 μg/ml for BxPC-3.

The cytotoxicity curves for the three cell lines based on the MTT assay (3 days culture) are shown in Figure 2. The IC_{50} of vesnarinone for the three lines based on the MTT assay was 32 μg/ml for PANC-1 and 30 μg/ml for BxPC-3, but 50% cytotoxicity for MIA PaCa-2 was not achieved even with the maximal dose of vesnarinone (50 μg/ml).

Morphological changes to the human pancreatic cancer cells after incubation with vesnarinone

Acridine orange staining demonstrated the vacuolization and crater-like changes to the nuclei in the cells after vesnarinone treatment. Figure 3(a–f) shows

pictures of PANC-1 cells obtained by Nomalsky optical differential interference microscopy after incubation with vesnarinone for 48 h. The cells incubated with vesnarinone (Figure 3d–f) were vacuolated and produced small and large crater-like cave-ins (Figure 3e). When nuclei were stained with acridine orange, the marginal zone was stained, but the crater-like cave-ins were not. This suggests that the crater-like cave-ins might be formed from the nuclear chromatins aggregating along the marginal zone of the cell nucleus (Figure 3f). Similar phenomena were also observed for vesnarinone-treated MIA PaCa-2 and BxPC-3 cells.

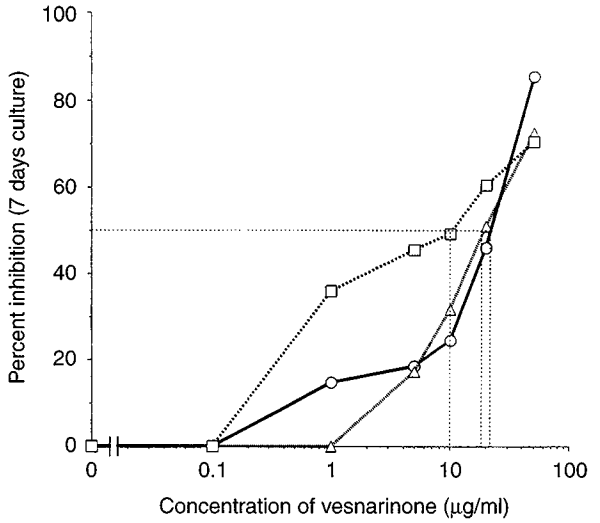

Figure 1. Inhibitory effects of vesnarinone on the growth of three pancreatic cancer cell lines (○, PANC-1; △, MIA-PaCa2; □, BxPC-3). After 10^4 cells were cultured for 7 days in 25 cm^2 plastic flasks, the number of cells was counted using the Trypan blue dye exclusion test.

Immunostaining of BM-1 and nick end-labeling after incubation with vesnarinone

In order to investigate the relation between cell damage and apoptosis caused by vesnarinone, kinds of immunocytochemical staining of the Le^y antigen and nick end-labeling were applied. It has been shown that an increase in nick end-labeling indicates DNA fragmentation caused by apoptosis,[15] while the Le^y antigen, visualized by BM-1 antibody, is reported to be specifically stained in the cytoplasm of cells undergoing apoptosis.[14]

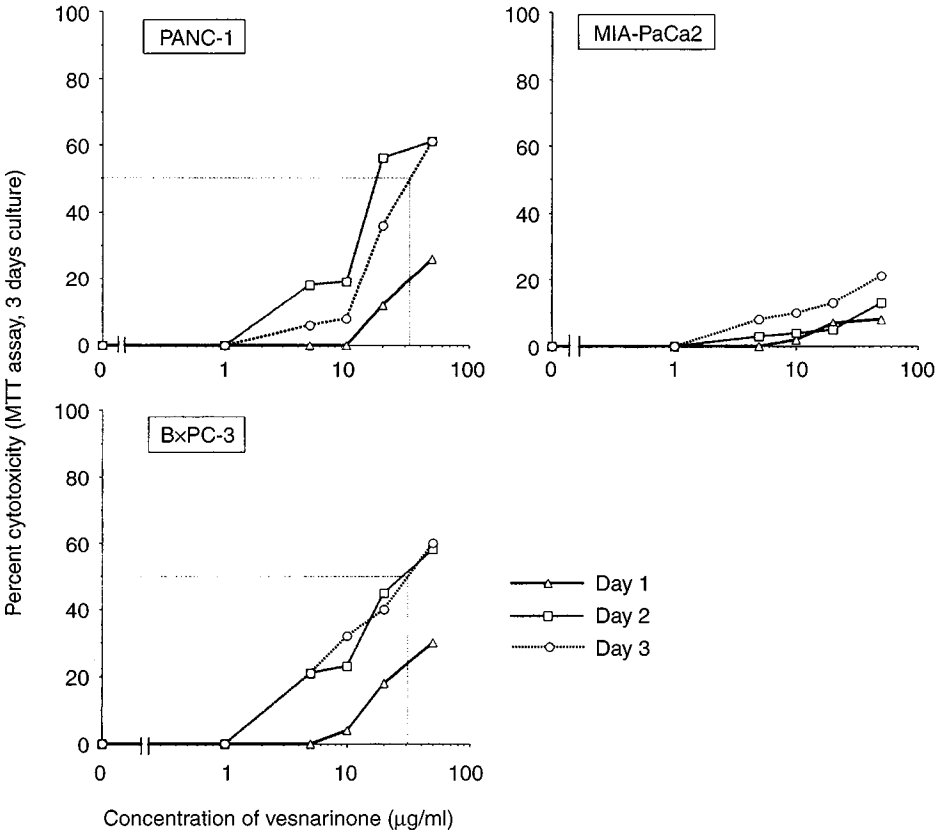

Figure 2. Cytotoxic effect of vesnarinone against three pancreatic cancer cell lines, assessed using the MTT assay. Tumor cells were cultured with vesnarinone at the indicated concentrations for 3 days in a 96-well flat-bottom microtiter plate and the cell viability was assessed using the MTT assay.

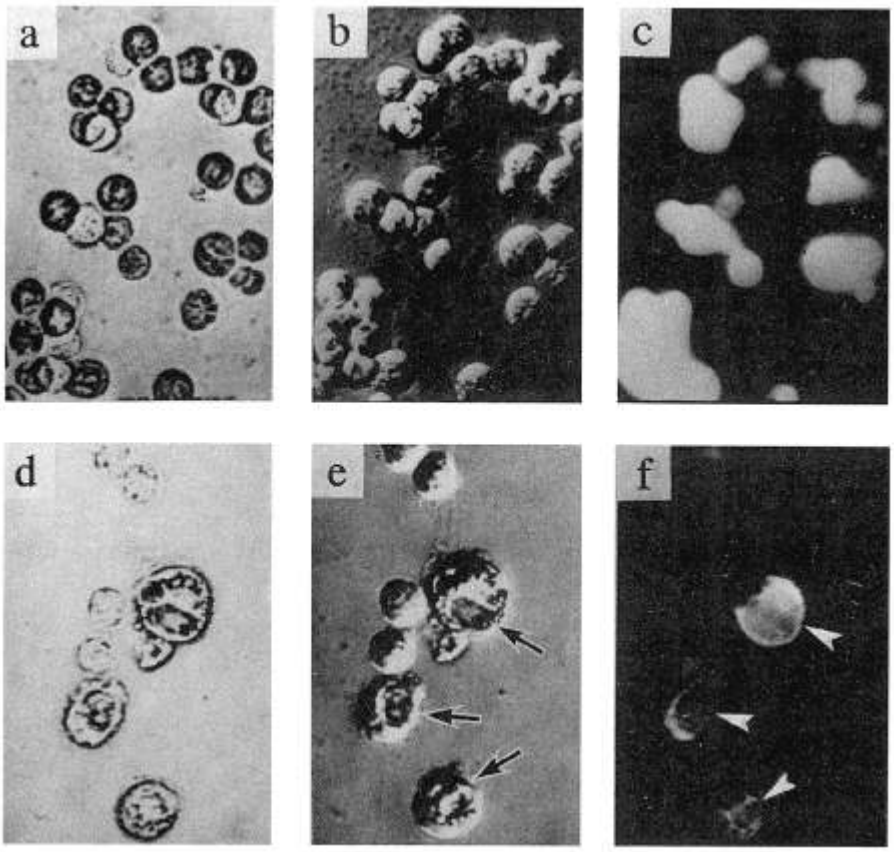

Figure 3. Morphological changes to PANC-1 caused by vesnarinone treatment. (a–c) Control cells. (d–f) Vesnarinone-treated cells (50 μg/ml for 48 h). (a and d) Pictures taken using an ordinary microscope. (b and e) Images obtained using a Nomalsky differential interference microscope. (c and f) Nuclear staining by acridine orange. Arrows refer to the crater-like cave-ins. Arrowheads indicate areas not stained by acridine in the nucleus.

Table 2 summarizes the results of immunostaining. After culturing cells with vesnarinone for 72 h, the number of cells with positive nick end-labeling and BM-1 staining increased. Figure 4(a–d) shows the immunostaining of nick end-labeling and BM-1 in PANC-1 cells.

Effect of vesnarinone on the invasivity of human pancreatic cancer cells

Since, of the three pancreatic tumor cell lines, only PANC-1 attaches poorly to the basal membrane, it could not be used for the Matrigel invasive chamber

Table 2. Immunostaining of human pancreatic cancer cell lines with BM-1 antibody and nick end-labeling

	Vesnarinone (μg/ml)	Positive cells (%)			
		0 (control)	10	20	50
BM-1	PANC-1	21.7±3.9	58.5±6.1**	43.0±14.3*	82.7±15.9**
	MIA-PaCa2	16.5±7.6	85.1±0.9**	97.8±3.1**	98.5±2.1**
Nick end-label	PANC-1	4.5±2.4	42.8±19.0*	47.8±3.2**	61.0±7.3**
	MIA-PaCa2	20.2±5.1	50.8±22.6*	70.9±1.3**	54.1±9.0**

*$p<0.05$; **$p<0.01$ versus vesnarinone (–).
BxPC-3 cells were not stained, because they produced mucin which caused strong non-specific staining.

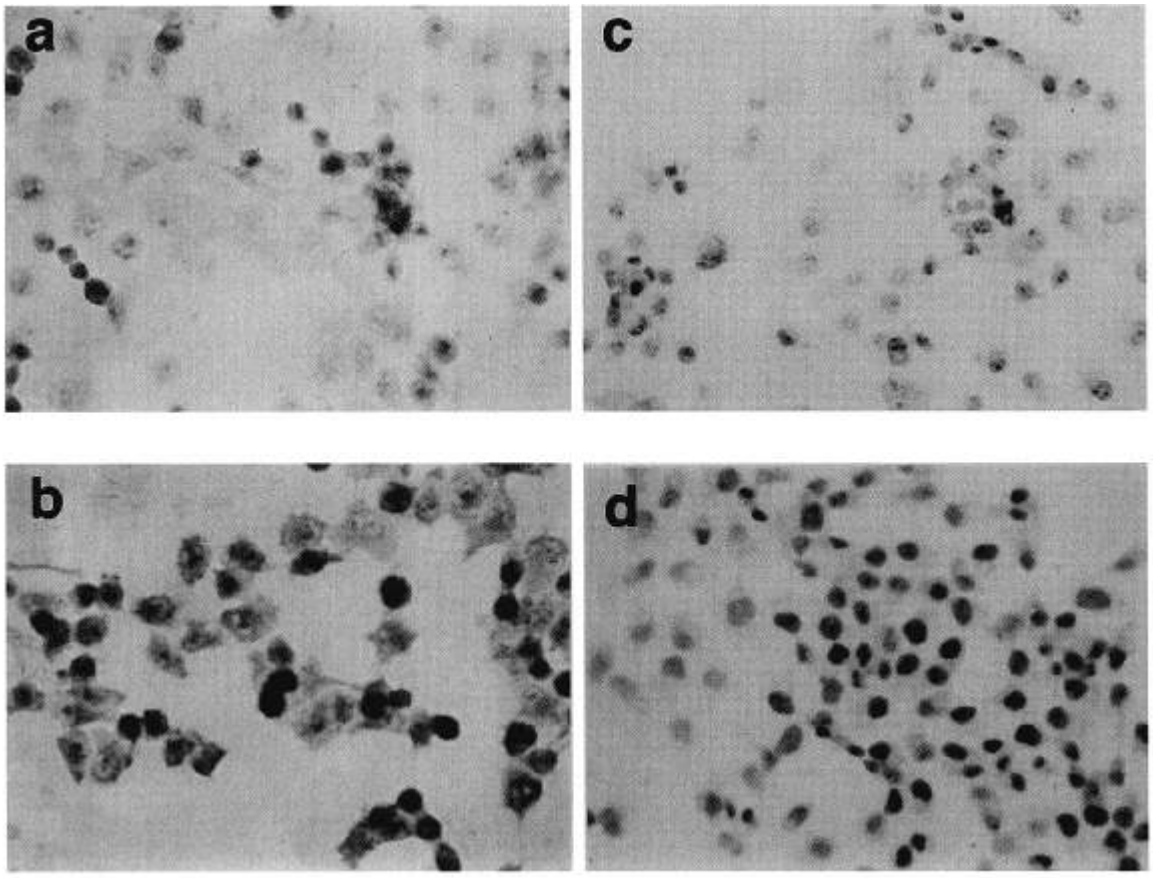

Figure 4. Immunostaining by the apoptosis-related antibody, BM-1 (anti-Le^y antibody) and detection of DNA fragmentation using the nick end-labeling method in PANC-1 cells after treatment with vesnarinone (× 180) (a) Control cells (BM-1). (b) Cells treated with vesnarinone (50 μg/ml) for 72 h (BM-1). (c) Control cells (nick end-labeling). (d) Cells treated with vesnarinone (50 μg.ml) for 72 h (nick end-labeling).

Table 3. The inhibitory effect of vesnarinone on invasivity of human pancreatic cancer cell lines assessed by the matrigel invasion chamber assay

Vesnarinone (μg/ml)	No. of invading cells (/field, × 200)	
	MIA PaCa-2	BxPC-3
0 (control)	90.3±20.0	73.3±4.2
1.0	56.0±24.8	71.0±8.5
5.0	53.0±10.4*	62.7±24.0
10.0	37.0±3.6**	47.3±15.6
20.0	22.3±7.2**	69.3±24.8
50.0	28.3±6.4**	6.0±1.0***

*p<0.05; **p<0.01; ***p<0.001 versus control.

assay. Accordingly, MIA PaCa-2 and BxPC-3 were used for the inhibition assay of the cell invasivity. The invasivity of both tumor cell lines was assessed after 2 h of incubation, at which time the invasivity reached its maximum. Vesnarinone significantly inhibited the invasivity of MIA PaCa-2 line at concentrations larger than 5 μg/ml to 24.4% of the activity shown by the untreated cells and reached its peak activity at 20 μg/ml (Table 3). On the other hand, the minimal concentration of vesnarinone required to significantly inhibit the invasivity of BxPC-3 was higher than 20 μg/ml; at this concentration the invasiveness was inhibited to 10% of the activity of the control cells.

In vivo antitumor activity of vesnarinone on human pancreatic cancer xenografts in nude mice

The BxPC-3 cell line was used for the *in vivo* study, because it responded best to vesnarinone *in vitro*. Furthermore, the human pancreatic cancer xenograft, SPa-1, was also used. Vesnarinone was orally administered and the antitumor effects of the agents were assessed after daily administration for 21 days (Table 4). The inhibition of the growth of BxPC-3 by vesnarinone at 5 mg/kg was not significant (mean

Table 4. The antitumor effect of vesnarinone on human pancreatic cancer lines transplanted s.c. into the back of nude mice

	Tumor growth rate (mean percent inhibition versus control) on day 21	
	BxPC-3	SPa-1
Control (CMC)	8.44±2.90 (n=10)	6.27±2.45 (n=9)
Vesnarinone (5 mg/kg)	5.41±2.25 (35.9%, n=9)	5.77±1.63 (8.0%, n=8)
Vesnarinone (50 mg/kg)	4.88±2.11 (42.2%, n=9)**	4.44±1.12 (29.2%, n=8)*

*p<0.05; **p<0.01 versus control.

Table 5. Side effects of vesnarinone in nude mice transplanted BxPC-3

	Body weight (ratio[a])[b]		WBC ($\times 10^2$/mm^3)
	Day 1	Day 21	Day 21
Control (CMC)	22.4±2.3	24.0±3.1 (1.07, n=10)	3.5±1.2 (n=8)[c]
Vesnarinone (5 mg/kg)	25.3±2.1	26.6±4.2 (1.05, n=9)	4.7±2.5 (n=9)
Vesnarinone (50 mg/kg)	24.8±2.8	25.9±4.3 (1.04, n=9)	3.4±1.3 (n=9)

[a]Mean ratio versus body weight before therapy.
[b]Body weight was measured including tumor.
[c]Blood cells were not counted in all mice, because the blood coagulated.

IR=36%). In contrast, vesnarinone at 50 mg/kg showed significant inhibition (mean IR=42%, $p<0.01$). The inhibitory effect of vesnarinone on SPa-1 tumors was similar to that observed on BxPC-3 tumors. Administration of vesnarinone at 5 mg/kg did not produce a significant inhibition of tumor growth, whereas significant inhibition was observed with vesnarinone at 50 mg/kg (mean IR=29%, $p<0.05$). Side effects of the agents are summarized in Table 5. No significant body weight loss or leucopenia was seen.

Discussion

Vesnarinone is used clinically in therapy for congestive heart failure.[3,4] Its chemical structure is related to that of flavonoids, which have been shown to induce apoptosis in tumor cells and to inhibit tumor proliferation.[19] The clinical application of vesnarinone as an anticancer agent has been expected. In fact, vesnarinone was reported to inhibit the proliferation of various tumor cells.[6,7] In the present study, we demonstrated that vesnarinone had cytotoxic and cytostatic activity against human pancreatic cancer lines *in vitro* and *in vivo*. The mechanisms responsible for the antitumor activity of vesnarinone are unclear. However, as described above, vesnarinone has been shown to induce apoptosis. In the present study morphological observations with an ordinary phase contrast microscope and a Nomalsky optical differential interference microscope demonstrated vacuoles in the nucleus and crater-like cave-ins in the vesnarinone-treated cells. Staining of the nucleus with acridine orange suggested that the condensation of chromatins at the margins caused vacuoles in the nucleus. Furthermore, increases in nick end-labeling and in staining of apoptosis-related antibody BM-1 after vesnarinone treatment also indicated that vesnarinone caused apoptosis in these pancreatic tumor cell lines. These results also suggest that vesnarinone induced nuclear damage, presumably caused by apoptosis, to the three human pancreatic tumor cell lines and consequently caused cell damage.

Vesnarinone has also been studied as a potential anti-AIDS drug and a report described that vesnarinone inhibited HIV-1 replication in virus-infected lymphocytes.[20] The mechanism of action is considered to be the inhibition of tumor necrosis factor (TNF) and interleukin-6 production in peripheral lymphocytes.[20] It has been reported that TNF triggers apoptosis induction through its receptor and also that the receptor has a chemical structure similar to that of the Fas antigen,[21] which is known to induce apoptosis.[22] Judging from these observations, the growth inhibition by vesnarinone may be derived from its action on the TNF receptor or on the TNF-like substances produced by tumor cells. At present, studies on alteration of TNF production and on TNF receptor expression in tumor cell membranes are in progress. In connection with these studies, there is a report that the level of TNF increased in the blood of

patients with congestive heart failure.[23] Thus, vesnarinone may have the same mechanism of action as cardiovascular and antitumor drugs. However, swelling of the cell, which was recognized in vesnarinone-treated cells, was not observed for ordinary apoptotic cells. Therefore, cell death caused by vesnarinone may be induced by a mechanism different from that of the apoptosis induced by ordinary chemotherapeutic agents. Consequently, additional experimental studies including electron microscopic observations may be needed to elucidate whether or not vesnarinone really induces apoptosis.

In this study, vesnarinone showed an inhibitory effect on the invasion as well as proliferation of tumor cells. Many reports have described the anti-invasive activity of various anticancer agents against tumor cells. Adriamycin was reported to inhibit tumor cell invasion *in vitro* at a concentration as low as 1.0 ng/ml. At this dose, it did not inhibit tumor growth.[24] Several studies demonstrated that the production of TGF-β is modulated by vesnarinone, resulting in the suppression of proteins regulating the cell cycle,[25] and that tyrosine phosphorylation of proteins is stimulated by vesnarinone, resulting in the activation of cytoskeleton molecules.[26] These results suggest that vesnarinone not only activates tyrosine phosphorylation pathways to inhibit tumor growth, but also affects morphological structures of tumor cells resulting in alterations of cell adhesion characteristics.

The *in vitro* inhibition activity of vesnarinone against three pancreatic tumor cell lines, expressed as IC_{50}, was 10–21 μg/ml in the growth inhibition assay (7 day culture). In clinical application for heart failure patients, the blood concentration of vesnarinone generally reaches about 9.5 μg/ml with a 60 mg dose over a 4 week schedule. Therefore, this usual dose and slightly higher doses are considered sufficient to inhibit tumor growth and tumor invasion. The *in vivo* experiments using human pancreatic cancer xenografts (BxPC-3 and SPa-1) transplanted into nude mice also demonstrated that a significant inhibition of tumor growth was obtained by the administration of vesnarinone at 50 mg/kg without serious side effects. The *in vivo* antitumor activity of vesnarinone appears to be comparable to that of conventional anticancer drugs such as adriamycin, mitomycin or cisplatin, because the percent inhibition of the growth of human pancreatic cancer lines, which were transplanted into nude mice, by these agents alone at a dose of 1/3 LD_{50}, was less than 50%.[27] Moreover, there were no evident side effects in the group receiving 50 mg/kg of vesnarinone. Vesnarinone is known to cause body weight loss in 1.7% of patients treated for heart failure. Chemotherapeutic agents produce much higher weight loss. The biological half life of vesnarinone is much longer in humans than in mice. When vesnarinone is administered at 1 mg/kg/day for 4 weeks in humans, a blood concentration of 9.5 ng/ml results;[6] in order to obtain the same concentration of vesnarinone in mice, the necessary dose would be about 50 times higher than in humans.[6] Accordingly, for clinical application in cancer therapy, vesnarinone may be administered at a dose 2- or 3-fold higher than that used for heart failure (usually 1 mg/kg/day). It is necessary to assess the side effects of vesnarinone at these higher doses.

Conclusion

Vesnarinone may be a beneficial agent for pancreatic cancer therapy. Furthermore, studies on the combination effect of vesnarinone with other anticancer agents and/or with radiation therapy must be pursued to determine whether vesnarinone is truly worthwhile of clinical application.

Acknowledgments

We gratefully appreciate Ms Tomoko Toga, Miyuki Ishihara, Yasuko Sonoyama and Rumiko Hayashi for their helpful assistance with the experiments.

References

1. Janes RH Jr, Niederhuber JE, Chmiel SJ, *et al.* National patterns of care for pancreatic cancer; results of survey on the Commission on Cancer. *Ann Surg* 1996; **223:** 261–72.
2. Palmer KR, Kerr M, Knowles G, Cull A, Carter DC, Leonard RC. Chemotherapy prolongs survival inoperable pancreatic carcinoma. *Br J Surg* 1994; **81:** 882–5.
3. OPC-8212 Mulicenter Research Group. A placebo-controlled, randomized, double-blind study of OPC-8212 in patients with mild chronic heart failure. *Cardiovasc Drugs Ther* 1990; **4:** 419–26.
4. Feldman AM, Bristow MR, Parmley WW, *et al.* Effects of vesnarinone on morbidity and mortality in patients. *N Engl J Med* 1993; **329:** 149–55.
5. Sato S, Tomoyasu S, Okabe-Kado J, *et al.* Induction of differentiation and enhancement of vincristine sensitivity of human erythroleukemia HEL cells by vesnarinone, a positive inotropic agent. *Exp Hematol* 1996; **24:** 37–42.
6. Nakai S, Kawai K, Aihara K, Adachi M. Antitumor effect of vesnarinone on leukemias and solid tumors by induction of cellular differentiation and apoptosis. *Biotherapy* 1995; **9:** 889–97.
7. Sato M, Harada K, Bando T, *et al.* Characteristics of antitumor activity of 4-dihydro-6-[4-(3,4-dimethoxy-benzoyl)-1-piperazinyl]-2(1*H*)-quinorinone (vesnarinone) against a human adenoid squamous carcinoma-forming

cell line grown in athymic nude mice. *Cancer Lett* 1995; **91:** 1–9.
8. Koide T, Komatsu T, Yamazaki N, Yamamoto A, Ishihara K. Anti-tumor effect of DTIC with or without vesnarinone on the growth of human malignant melanoma (SK-14) in nude mice. *J BCG BRM Ther* 1995; **19:** 39–50.
9. Lieber M, Mazzetta J, Nelson-Ress W, Kaplan M, Todaro G. Establishment of a continuous tumor-cell line (PANC-1) from a human carcinoma of the exocrine pancreas. *Int J Cancer* 1975; **15:** 741–7.
10. Yunis AA, Arimura GK, Russin DJ. Human pancreatic carcinoma (MIA PaCa-2) in continuous culture: sensitivity to asparaginase. *Int J Cancer* 1977; **19:** 218–35.
11. Tan MH, Nowak NJ, Loor RM, *et al.* Characterization of a new primary human pancreatic tumor line. *Cancer Invest* 1986; **4:** 15–23.
12. Cole SP. Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. *Cancer Chemother Pharmacol* 1986; **17:** 259–63.
13. Abrams JM, White K, Fesseler LI, Steller H. Programmed cell death during drosophila embryogenesis. *Development* 1993; **117:** 29–43.
14. Hiraishi K, Suzuki K, Hakomori S, Adachi M. Le^y antigen expression is correlated with apoptosis (programmed cell death). *Glycobiology* 1993; **3:** 381–90.
15. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992; **119:** 493–501.
16. Terranova VP, Hujanen ES, Loeb DM, Martin GR, Thornburg L, Glushko V. Use of reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumor cells. *Proc Natl Acad Sci USA* 1986; **83:** 465–9.
17. Albini A, Iwamoto Y, Kleinman HK, *et al.* A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987; **47:** 3239–45.
18. Ovejera AA, Houchens DP, Baker AD. Chemotherapy of human tumor xenografts in genetically athymic mice. *Ann Clin Lab Sci* 1978; **8:** 50–6.
19. Traganos F, Ardelt B, Halko N, Bruno S, Darzynkiewicz Z. Effect of genistein on the growth and cell cycle progression of normal human lymphocytes and human leukemic MOLT-4 and HL-60 cells. *Cancer Res* 1992; **52:** 6200–8.
20. Maruyama I, Maruyama Y, Nakajima T, *et al.* Vesnarinone inhibits production of HIV-1 in cultured cells. *Biochem Biophys Res Commun* 1993; **195:** 1264–71.
21. Suda T, Takahashi T, Goldstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 1993; **75:** 1169–78.
22. Nagata S, Goldstein P. The fas death factor. *Science* 1995; **267:** 1449–56.
23. Levine B, Kalman J, Mayer L, Fiilit HM, Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Eng J Med* 1990; **323:** 236–41.
24. Repesh LA, Drake SR, Warner MC, *et al.* Adriamycin-induced inhibition of melanoma cell invasion is correlated with decrease in tumor cell motility and increases in focal contact formation. *Clin Exp Metastasis* 1993; **11:** 91–102.
25. Harada K, Sato M. Induction of TGF-β on human salivary adenosquamous carcinoma cell line with vesnarinone. *Biotherapy* 1996; **41:** 416–8.
26. Yoshinaka Y, Katoh I, Kyushiki H, Sakamoto Y. Alteration of the cytoskeletal organization in tumor cell line by a cardiac drug, vesnarinone, through protein tyrosine phosphorylation. *Exp Cell Res* 1995; **219:** 21–8.
27. Imai S, Nio Y, Shiraishi T, Manabe T, Tobe T. *In vivo* inhibitory effect of anticancer agents on human pancreatic cancer xenografts transplanted in nude mice. *Anticancer Res* 1991; **11:** 657–64.

(Received 3 June 1997; accepted 11 June 1997)