PERGAMON

European Journal of Cancer 36 (2000) 128–136

European Journal of Cancer

www.elsevier.com/locate/ejconline

# Antagonists of growth hormone-releasing hormone (GH-RH) inhibit *in vivo* proliferation of experimental pancreatic cancers and decrease IGF-II levels in tumours

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Received 16 July 1999; revised and accepted 18 August 1999

**Abstract**

Insulin-like growth factors (IGF-I and IGF-II) are implicated in the pathogenesis of pancreatic carcinoma. Antagonists of growth hormone-releasing hormone (GH-RH) suppress the GH-RH–GH–IGF-I axis and also act directly on tumours to reduce production of IGF-I or II. The aim of this study was to investigate the effects of two potent GH-RH antagonists in two experimental models of pancreatic cancer. Syrian golden hamsters with nitrosamine-induced pancreatic tumours were treated with 10 μg/day of GH-RH antagonist MZ-4-71 for 60 days. The therapy reduced the number of tumorous animals, decreased the weight of tumorous pancreata by 55%, and lowered AgNOR numbers in tumour cells. In two other experiments, GH-RH antagonists MZ-4-71 and MZ-5-156 significantly inhibited growth of SW-1990 human pancreatic cancers xenografted into nude mice, as shown by a reduction in tumour volume and tumour weights, and a decrease in AgNORs in cancer cells. IGF-I levels in serum and in pancreatic cancer tissue remained unchanged after therapy, suggesting that an effect on IGF-I is not involved in tumour inhibition. In contrast, IGF-II concentrations in tumours were significantly reduced by 50–60% after treatment with the GH-RH antagonists as compared with controls. *In vitro* studies showed that the concentration of IGF-II in the culture medium was increased after seeding of SW-1990 cells, indicating that this pancreatic cancer cell line produced and released IGF-II. This finding was also supported by the expression of IGF-II mRNA in the SW-1990 cells. Addition of $3\times10^{-6}$ M of GH-RH antagonist MZ-5-156 to the reduced-serum medium decreased cell proliferation, IGF-II mRNA expression in the cells and IGF-II concentration in the medium. Our findings indicate that inhibitory effects of GH-RH antagonists on the growth of experimental pancreatic cancers, may result from a decrease in the production and concentration of IGF-II in the tumours. 

*Keywords:* GH-RH antagonists; Pancreatic cancer; IGF-I; IGF-II; IGF-I receptor; AgNOR

## 1. Introduction

Pancreatic carcinoma is the fifth leading cause of cancer-related deaths in the developed world [1]. The survival rate of patients with pancreatic cancer is one of the shortest among various malignancies [1]. Most pancreatic tumours are diagnosed at an advanced stage when surgical intervention may be merely palliative [2], and there is no effective treatment currently available for advanced pancreatic cancer [3].

In the past decade, some hormonal manipulations have been tried in order to extend the survival and improve the quality of life of patients with pancreatic cancer. These treatment modalities were based on theoretical considerations, and were supported by extensive experimental studies [4,5]. Various hormones may affect pancreatic carcinoma. Analogues of luteinising hormone-releasing hormone (LH-RH) may inhibit the growth of cancers by suppressing sex steroids and by a direct action on tumour cells. Somatostatin analogues counteract the gastrointestinal hormones that may promote growth of pancreatic cancer cells, and also inhibit the GH–IGF-I axis. Antagonists of bombesin (BN)/gastrin-releasing peptide (GRP) prevent the stimulatory

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0959-8049/00/$ - see front matter 
PII: S0959-8049(99)00230-0

activity of BN/GRP on pancreatic tumours. In addition, these peptide hormone analogues reduce the effect of epidermal growth factor (EGF) on tumours by decreasing the number of EGF receptors (EGFR) on cancer cells [6]. On the basis of experimental findings, some of these hormone analogues were tried clinically in patients with inoperable pancreatic cancers. Treatment with LH-RH or somatostatin analogues led to clinical improvement in some patients, but the increase in survival rate was small [5]. Because of the unsatisfactory results of present treatment procedures for pancreatic cancer, it is imperative to seek new therapeutic approaches.

Various experimental and clinical studies show that EGF, IGF-I and IGF-II may play substantial roles in the progression of pancreatic cancers [5–8]. IGF-I was found to contribute to pancreatic regeneration after partial pancreatectomy [7]. Addition of IGF-I to the medium, enhances growth of ASPC-I and COLO-357 human pancreatic cancer cells. These cell lines express mRNA transcripts for IGF-I receptor (IGF-IR) which mediate the growth-promoting effect of IGF-I [8]. Many human pancreatic cancers contain higher levels of mRNA for IGF-I than the normal pancreas, which also suggests a possible role of IGF-I in the progression of pancreatic tumours [8]. Other studies indicate the significance of IGF-II in pancreatic carcinogenesis. Cultured islet tumour cells release IGF-II into the medium, and in pancreata of transgenic mice, proliferation activity of tumorous islet cells shows a high correlation with IGF-II mRNA expression [9]. Addition of IGF-II to the medium enhances the growth of several pancreatic cancer cell lines and this effect can be prevented by a monoclonal antibody (MAb) that blocks ligand binding to the IGF-I receptor [10], which mediates the proliferative effects of IGF-II.

IGF-I promotes the growth effects of growth hormone (GH). The production of hepatic IGF-I and its level in serum are controlled by GH [11,12]. Thus, the suppression of the GH-RH–GH–IGF-I axis might lead to growth inhibition of tumours that are stimulated by IGF-I. In view of the importance of IGFs in pancreatic carcinogenesis, we decided to study how GH-RH antagonists might affect pancreatic tumours. Among various potent GH-RH antagonists synthesised by us, analogues MZ-4-71 ([Ibu$^0$,D-Arg$^2$,Phe(4-Cl)$^6$,Abu$^{15}$, Nle$^{27}$]hGH-RH(1-28)Agm) and MZ-5-156 ([PhAc$^0$,D-Arg$^2$, Phe(4-Cl)$^6$,Abu$^{15}$,Nle$^{27}$]hGH-RH(1-28)Agm) were the most effective for inhibition of GH release in rats [13]. In previous studies, MZ-4-71 inhibited growth of human osteosarcomas growing in nude mice [14], lung cancers [15] and prostate cancers [16], the growth of which is known to be affected by IGFs.

In the present study, we investigated the effects of GH-RH antagonists MZ-4-71 and MZ-5-156 on experimental pancreatic cancers. Three *in vivo* studies were performed. The first investigation was in nitrosamine-induced pancreatic cancers in hamsters, which have some similarities to human cancer models [6,17–19], and express IGF-I receptors [17]. Two studies were carried out on SW-1990 human pancreatic cancers xenografted into nude mice [20,21]. In addition, we investigated the *in vitro* effects of MZ-5-156 on growth and IGF production of SW-1990 cells.

## 2. Materials and methods

### 2.1. Peptides

GH-RH antagonists MZ-4-71 and MZ-5-156 were synthesised in our laboratory by solid phase methods [13]. The peptides were dissolved in 20 μl dimethylsulphoxide (DMSO) and diluted with 10% propylene glycol in water. Amounts for one week treatment were prepared in advance, and stored in Sigmacoated (Sigma, St Louis, MO, USA) vials at 4°C.

### 2.2. Animals and tumours

Female Syrian golden hamsters (CH:RGH) weighing on average 102 g were obtained from the NCI Frederick Cancer Research Facility (Frederick, MD, USA). The animals were maintained in our Animal Research Facility in a temperature-controlled room with 12 h light/12 h dark cycle and allowed pelleted diet and water '*ad libitum*'. Pancreatic tumours were induced with N-nitrosobis(2oxopropyl)amine (BOP) (American Tokyo Kasei, Portland, OR, USA) as previously described [18,19]. Male athymic nude mice (Ncr nu/nu), approximately 6 weeks old on arrival, were obtained from the National Cancer Institute, Frederick, MD, USA and were maintained under pathogen-limited conditions. Three male nude mice were injected subcutaneously (s.c.) with 0.2 ml of SW-1990 human pancreatic cancer cell suspension [20,21] ($10^6$ cells/mouse) to obtain tumour donor animals (methods of cell culture are described below). After 4 weeks, the well-developed tumours were dissected, mechanically minced, and 2 $mm^3$ pieces of tumour tissue were transplanted s.c. by trocar needle into the right flank area of 80 male nude mice.

Three experiments were performed, and the animals received the following treatments.

#### 2.2.1. Experiment 1 (nitrosamine-induced pancreatic cancers in hamsters)

Group 1. Control, injection vehicle only; group 2. MZ-4-71, 10 μg. Each group contained 14 hamsters, and the injections were given once daily, intraperitoneally (i.p.). The treatment started 18 weeks after the last BOP administration and lasted for 60 days.

*2.2.2. Experiment 2 (SW-1990 cancers in nude mice)*

Group 1. Control, vehicle only; group 2. MZ-4-71, 40 μg/day; group 3. MZ-5-156, 10 μg/day, all injected once daily, i.p. Treatment started 13 days after transplantation of tumours, when tumour growth was apparent and lasted for 7 weeks.

*2.2.3. Experiment 3. (SW-1990 cancers in nude mice)*

Group 1. Control, vehicle only; group 2. MZ-4-71, 2×10 μg/day; group 3. MZ-5-156, 2×10 μg/day. All injections were given s.c. The treatment was started 10 days after tumour transplantation and was continued for 7 weeks.

In Experiments 2 and 3, all groups contained 10 mice. SW-1990 tumours were measured with microcalipers, and tumour volume was calculated as length× width×height×0.5236. At the end of the treatment period, the animals were anaesthetised with methoxyflurane (Metofane, Malinkrodt Vet. Mundelein, IL, USA) and sacrificed by decapitation. Trunk blood was collected, centrifuged and serum was frozen for hormone studies. Tumours were cleaned, weighed, and samples were taken for histological investigation, receptor analysis and IGF assays. All experiments were performed according to institutional ethical guidelines.

*2.3. Histological procedures*

Samples were fixed in 10% buffered formalin. Specimens were embedded in Paraplast (Oxford Labware, St Louis, MO, USA). Sections were stained with haematoxylin-eosin. In Experiment 1, the mitotic and apoptotic rates were expressed as the percentage of tumorous glands exhibiting mitotic or apoptotic cells [19]. In Experiments 2 and 3, mitotic and apoptotic cells were counted in 10 standard high-power microscopic fields containing, on average, 250 cells, and their number per 1000 cells were accepted as the mitotic and apoptotic indices. For demonstration of the nucleolar organizer region (NOR) in tumour cell nuclei, the AgNOR method was used, as described [22]. The number of AgNOR granules is an indicator of cell proliferation. The silver-stained black dots in 50 cells of each tumour were counted, and the AgNOR number per cell was calculated.

*2.4. Receptor assay*

Measurement of receptors for IGF-I in the membranes of SW-1990 tumours was performed as described [14]. The LIGAND PC computerised curve-fitting programme of Munson and Rodbard [23] was used to determine the types of receptor binding, the maximal binding capacity ($B_{max}$) of the receptors and the dissociation constant ($K_d$) values.

*2.5. Radioimmunoassays for GH, IGF-I and IGF-II*

Serum GH was determined using materials provided by Dr. A.F. Parlow (NIDDK's National Hormone & Pituitary Program, Torrance, CA, USA): rat GH-RP-2 AFP-3190B, rat GH-I-6 AFP-5676B and anti-rat GH-RIA-5/AFP-411S. The methods used for determination of IGF-I and IGF-II levels in serum, pancreatic tumour samples and cell culture media after acid ethanol cryoprecipitation have previously been described [24].

*2.6.* In vitro *studies*

The SW-1990 cancer cell line was purchased from ATCC (Rockville, MD, USA). Cancer cells were routinely maintained as a monolayer culture in L-15 medium containing 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin-B and incubated in sealed culture flasks at 37°C in a humidified atmosphere. Cells growing exponentially were harvested by incubation with 0.25% trypsin–EDTA solution. All culture media components were purchased from Gibco (Grand Island, NY, USA).

*In vitro* cell growth was estimated using the 3,(4,5-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [25]. Briefly, SW-1990 cells were seeded into 96-well microplates and cultured for 18 h. MZ-5-156 ($3\times10^{-7}$–$3\times10^{-6}$ M), IGF-I (10 and 25 ng/ml), IGF-II (500 and 800 ng/ml) and GH (5 and 25 ng/ml) were added to the medium. Control cultures received medium alone. After 67 or 90 h, the medium was removed and 200 μl fresh L-15 + 5% fetal bovine serum (FBS) medium containing 80 μg MTT (Sigma) was added. The microplates were incubated for 4 h at 37°C in darkness. The medium was removed, cells were washed twice with L-15 medium and 200 μl dimethyl sulphoxide (DMSO, Sigma) followed by 25 μl of Sorensen's glycine buffer (0.1 M glycine plus 0.1 M NaCl, pH 10.5). After a brief mixing, the plates were read at 540 nm on the plate reader (Beckman, Palo Alto, CA, USA). Results were calculated as % T/C, where T = optical density ($OD_{540nm}$) of treated cultures and C = $OD_{540nm}$ of control cultures ×100.

For measurement of IGF-I and IGF-II levels in media, SW-1990 cells were cultured as described above. Samples were taken before seeding the cells (base medium) and 24 h after seeding, right before treatments ($t0$). Then, the following substances were given to the medium (8 wells each): MZ-5-156 at $3\times10^{-7}$, $10^{-6}$ and $3\times10^{-6}$ M; GH at 5 and 25 ng/ml concentrations. The controls had medium only. Samples were taken from 4 wells 67 h after addition of the peptides ($t1$), and from the other 4 wells 90 h after treatment ($t2$). The experiment was done in duplicate. Since SW-1990 cells did not grow in serum-free medium, a reduced serum medium Dulbecco's modified Eagle medium (DMEM) F12

(HAM) + 2% FBS was used to test the effects of the peptides on the cells. After an initial growth period, the concentration of serum in the medium was further reduced before starting the assay.

### 2.7. IGF-II mRNA expression in SW-1990 cells

Cells were harvested as described above 4 h after addition of MZ-5-156 to the medium, and total RNA was extracted from control and treated cells using the MicroRNA Isolation Kit (Stratagene, La Jolla, CA, USA), and quantified spectrophotometrically at 260 nm.

#### 2.7.1. Reverse transcription (RT)

Three μg of total RNA was reverse-transcribed using the RT-PCR Kit (Stratagene). The RNA was diluted with RNAse-free distilled water up to a final volume of 38 μl in a tube containing 6 μM random hexamer primers. The mixture was heated for 5 min at 65°C, left at room temperature for 10 min, and then 25 mM Tris, pH 8.3, 37.5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM of each deoxynucleotide triphosphate (dNTP), 40 U RNAse Block Inhibitor, and 40 U Molony Murine leukaemia virus reverse transcriptase were added for a total reaction volume of 50 μl. This mixture was incubated at 37°C for 1 h and then the reaction was terminated by heating at 94°C for 5 min.

#### 2.7.2. PCR amplification

PCRs for IGF-II and β-actin (internal control) were carried out in a final volume of 100 μl containing 10 mM Tris (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, sense and antisense primers at a concentration of 0.12 μM each, and 2.5 U Taq 2000 polymerase (Stratagene). The primers for human IGF-II were [26]: 5′-AGTCGATGCTGGTGCTTCTCACCT-TCTTGGC-3′ (sense) and 5′-TGCGGCAGTTTTGC-TCACTTCCGATTGCTGG-3′ (antisense). The primers for human β-actin were [27]: 5′-TCATGAAGTGTGA-CGTGGAC-3′ (sense) and 5′-ACCGACTGCTGTCA-CCTTCA-3′ (antisense). After 5 min of denaturation at 94°C, PCR was performed in a GeneAmp PCR System 2400 (Perkin-Elmer, Norwalk, CT, USA). A step programme of 35 cycles of 95°C for 15 s and 60°C for 30 s was used for IGF-II and for β-actin, a step programme of 32 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s was used. Following both of these programmes, a final step of 72°C for 10 min was added. Negative controls containing RNA were run in parallel to confirm that the samples were not contaminated with genomic DNA. 10 μl of the PCR products were separated on 1.8% agarose gel and visualised by ethidium bromide on a UV transilluminator. The banded areas were scanned and quantified using an imaging densitometer.

### 2.8. Statistical methods

Statistical analyses of the data were performed using Duncan's new multiple range test and Student's two-tailed *t*-test. All *P* values are based on two-sided hypothesis testing. SigmaPlot computer software (Jandel, San Rafael, CA, USA) was used for preparation of the figures.

## 3. Results

### 3.1. Effect of MZ-4-71 and MZ-5-156 on pancreatic cancers in vivo

In Experiment 1, treatment with MZ-4-71 reduced the average weight of pancreata by 55%. Because of the multinodularity of cancers and the confluence of nodules, the weight of tumorous pancreata (including both tumour weight and the weight of the rest of the pancreas) was recorded on the basis of histological examination [19]. The average weight of the tumorous pancreata was decreased by 55% in the group receiving MZ-4-71 compared with controls (Table 1). Five of the control pancreatic cancers (42%) were macroscopically invasive tumours, infiltrating neighbouring organs and areas (stomach, duodenum, intestines, liver and retroperitoneum), but none of the treated cancers showed such invasive characteristics (Table 1).

Table 1
Effect of daily i.p. treatment for 60 days with 10 μg/day of GH-RH antagonist MZ-4-71 on nitrosamine-induced pancreatic cancers in hamsters (Experiment 1)

| Groups | No. of tumorous hamsters *n* (total) | Weights of all pancreata (mg) mean ± SEM | Weights of tumorous pancreata (mg) mean ± SEM | Invasive tumour macroscopically *n* (%) | No. of hamsters with ascites *n* (%) | No. of animals with metastasis in lungs *n* (%) | Number of AgNORs/cell mean ± SEM |
|---|---|---|---|---|---|---|---|
| 1. Control | 12 of 14 | 1424 ± 342 | 1574 ± 383 | 5 (42) | 4 (33) | 3 (25) | 7.06 ± 0.13 |
| 2. MZ-4-71 | 9 of 14 | 647 ± 84[a] | 708 ± 127[a] | 0 (0) | 0 (0) | 2 (22) | 6.24 ± 0.15[b] |

i.p., intraperitoneal; SEM, standard error of the mean.
[a] $P < 0.05$.
[b] $P < 0.01$.

Table 2
Effect of treatment with GH-RH antagonists MZ-4-71 and MZ-5-156 on growth of SW-1990 human pancreatic cancer xenografts in nude mice, binding characteristics of IGF-I receptors, as well as concentrations of IGF-I and IGF-II in the tumours (experiments 2 and 3)

| Groups | Final tumour volume ($mm^3$) | Tumour weights (mg) | No. of AgNORs/cell | IGF-I receptors | | IGF-I | IGF-II |
|---|---|---|---|---|---|---|---|
| | | | | $K_d$ (nM) | $B_{max}$ (fmol/mg protein) | Concentration in tumours (pg/100 μg protein) | |
| Experiment 2 | | | | | | | |
| 1. Control | 1291 ± 301 | 1467 ± 374 | 7.23 ± 0.25 | 0.71 ± 0.16 | 91.4 ± 2.4 | nd | nd |
| 2. MZ-4-71 40 μg/day i.p. | 902 ± 156 | 1297 ± 258 | 6.14 ± 0.27[a] | 0.82 ± 0.13 | 113.3 ± 9.8 | nd | nd |
| 3. MZ-5-156 10 μg/day i.p. | 612 ± 220[a] | 931 ± 330 | 6.26 ± 0.26[a] | 0.68 ± 0.24 | 129.1 ± 8.6 | nd | nd |
| Experiment 3 | | | | | | | |
| 1. Control | 1894 ± 785 | 2501 ± 1085 | 8.53 ± 0.82 | 0.60 ± 0.04 | 96.3 ± 15.9 | 330 ± 73 | 524 ± 88 |
| 2. MZ-4-71 2×10 μg/day s.c. | 369 ± 208[a] | 634 ± 333[a] | 6.95 ± 0.45[a] | 0.58 ± 0.14 | 87.2 ± 9.5 | 364 ± 25 | 210 ± 40[a] |
| 3. MZ-5-156 2×10 μg/day s.c. | 671 ± 343[a] | 790 ± 418[a] | 6.30 ± 0.15[a] | 0.91 ± 0.03 | 111.7 ± 12.3 | 320 ± 15 | 266 ± 30[a] |

Binding characteristics were obtained from 12-point displacement experiments in two independent analyses, each done in duplicate.
Values are mean ± SEM.
nd, not done; i.p., intraperitoneal; s.c., subcutaneous; SEM, standard error of the mean.
[a] $P < 0.05$.

In Experiment 2, SW-1990 cancers were reduced by therapy with MZ-4-71 (tumour volume reduced by 30%) and were significantly smaller in the group receiving MZ-5-156, as shown by tumour volume data (47% of control) (Fig. 1 and Table 2). In Experiment 3, treatment of nude mice bearing SW-1990 cancers with MZ-4-71 or MZ-5-156 significantly inhibited tumour growth, final tumour volumes being reduced by 81% and 65% and weights by 75% and 68%, respectively (Fig. 2, and Table 2). The animals tolerated well the treatment with both MZ analogues. In all three *in vivo* experiments, body, heart, liver, spleen, kidney and sex organ weights of treated animals were not different from control values.

The histological structure of pancreatic cancers was similar in treated and control groups. The frequency of mitosis and apoptosis was not changed significantly by the treatments (data not shown), but AgNOR counts were lower in the treated groups (Tables 1 and 2).

### 3.2. Serum IGF-I, IGF-II and GH levels

Serum IGF-I and IGF-II levels were not changed by the treatment with MZ-4-71 or MZ-5-156. GH levels were decreased by 54, 42 and 53% in the groups treated with MZ-4-71 in Experiment 1, 2 and 3, respectively, and by 48 and 30% in the groups receiving MZ-5-156 in Experiment 2 and 3, respectively (data not shown). In

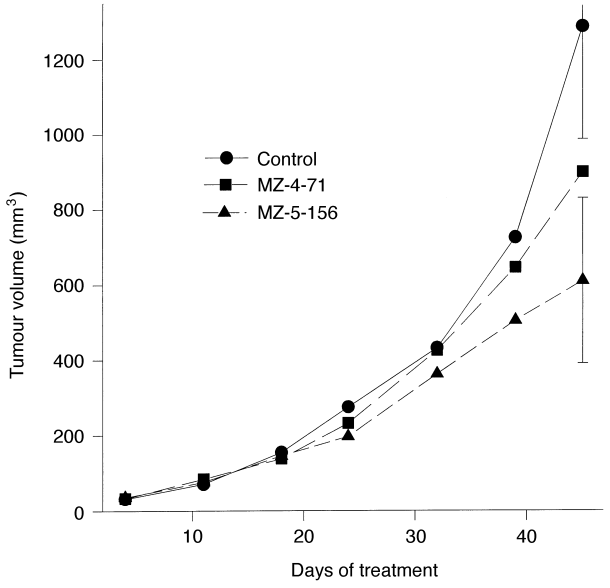


Fig. 1. Tumour volume changes of SW-1990 human pancreatic cancers xenografted into athymic nude mice in Experiment 2. The vertical bars show standard error of the mean (SEM).

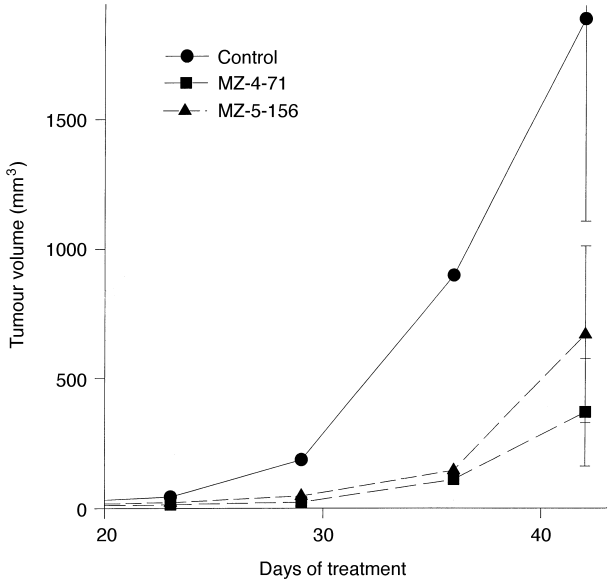


Fig. 2. Tumour volume changes of SW-1990 human pancreatic cancers in athymic nude mice in Experiment 3. The vertical bars represent standard error of the mean (SEM).

other treated groups, GH levels showed statistically non-significant reductions.

### 3.3. Analysis of IGF-I and IGF-II concentrations in tumour tissue

IGF-I and IGF-II levels were measured in SW-1990 cancers in Experiment 3. IGF-I concentration was similar in control and treated tumours. IGF-II levels, however, were significantly decreased in SW-1990 cancers (by 60% and 49%) after treatment with MZ-4-71 or MZ-5-156, respectively (Table 2).

### 3.4. Receptor studies

In the control tumours of Experiments 2 and 3, radiolabelled IGF-I was bound to a single class of high affinity and low capacity binding sites. Treatment with GH-RH antagonists MZ-4-71 or MZ-5-156 did not have significant effect on the affinity or capacity of receptors for IGF-I in membranes of SW-1990 cancers (Table 2).

### 3.5. In vitro studies

The IGF-I concentration in the serum-containing base medium was $0.77 \pm 0.023$ ng/ml. The IGF-I level was decreased significantly 24 h after seeding of SW-1990 cells to $0.50 \pm 0.029$ ng/ml, and remained low at 91 and 114 h after seeding ($0.38 \pm 0.028$ and $0.48 \pm 0.018$, respectively). Addition of the GH-RH antagonist MZ-5-156 or GH did not result in significant changes of IGF-I levels in the media (Fig. 3).

The original serum-containing base medium had $6.46 \pm 0.03$ ng/ml of IGF-II. 24 h after seeding of SW-1990 cells, IGF-II concentration in the medium rose to $6.89 \pm 0.08$, ($P = 0.048$). Subsequently, after a slight decrease at 91 h after seeding ($6.49 \pm 0.09$ ng/ml), IGF-II levels became significantly higher after 114 h ($7.95 \pm 0.11$ ng/ml, $P = 0.009$ compared with the base medium). Addition of MZ-5-156 or GH to the serum-containing medium 24 h after seeding of the SW-1990 cells caused no changes in IGF-II concentration (Fig. 4). The reduced-serum medium contained a very low level of IGF-II ($0.67 \pm 0.02$ ng/ml) which was significantly increased to $1.67 \pm 0.15$ ng/ml ($P < 0.001$) 24 h after seeding of the cells showing that the SW-1990 cells released IGF-II. Addition of $3 \times 10^{-6}$ M analogue MZ-5-156 lowered the IGF-II concentration in the medium to $1.55 \pm 0.13$ ng/ml after 68 h; this was significantly lower than the levels in the medium incubated with $3 \times 10^{-7}$ M or $10^{-6}$ M of MZ-5-156 ($1.88 \pm 0.15$ and $1.78 \pm 0.32$ ng/ml), respectively (Fig. 5a).

Addition of 10 or 25 ng/ml of IGF-I, 500 or 800 ng/ml of IGF-II and 5 or 25 ng/ml of GH to the media of SW-1990 cells produced no changes in cell growth (T/C%). The proliferation of SW-1990 cells in serum-containing medium was inhibited only by $10^{-5}$ M concentrations of MZ-5-156 (data not shown). However, in a reduced-serum medium, $3 \times 10^{-6}$ M levels of MZ-5-156 decreased cell growth significantly (Fig. 5b).

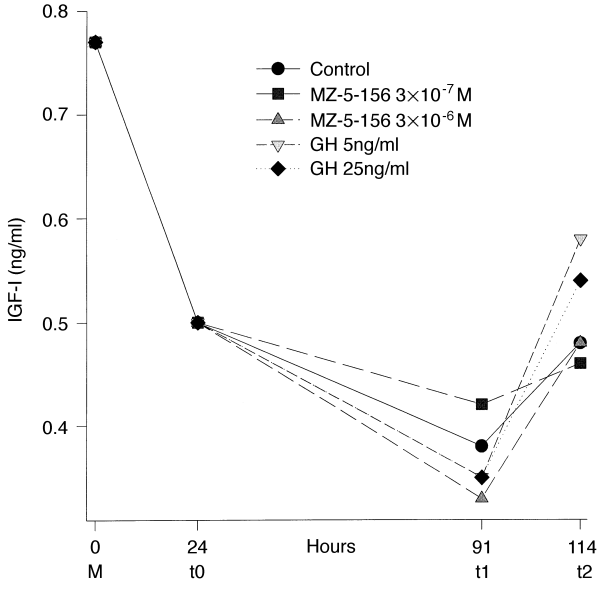


Fig. 3. IGF-I concentration in serum-containing medium of SW-1990 cells cultured *in vitro*. M, medium without cells; *t*0, medium 24 h after SW-1990 cells were seeded, immediately before treatment; *t*1 and *t*2, 67 and 90 h, respectively, after addition of peptides to the medium. The controls had medium only.

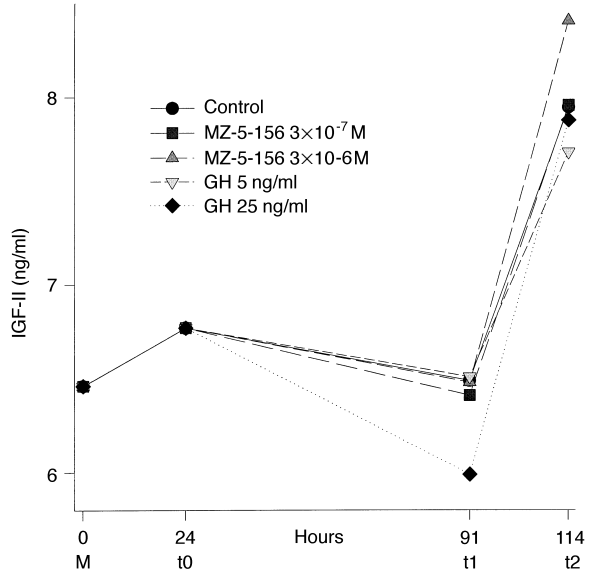


Fig. 4. IGF-II concentration in serum-containing medium of SW-1990 cells cultured *in vitro*. M, medium without cells; *t*0, medium 24 h after seeding of SW-1990 cells immediately before treatment; *t*1 and *t*2, 67 and 90 h, respectively, after addition of peptides to the medium. The controls had medium only.

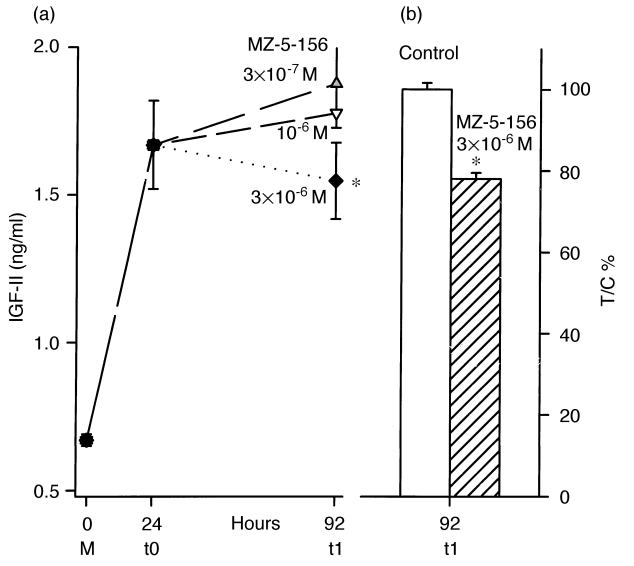


Fig. 5. Effect of addition of MZ-5-156 on: (a) IGF-II concentration in reduced-serum medium; and (b) on proliferation of SW-1990 cells in reduced-serum medium. (a) M, medium without cells; *t*0, medium 24 h after seeding of SW-1990 cells (control); *t*1, 68 h after addition of MZ-5-156 to the medium. $3\times10^{-6}$ M of MZ-5-156 significantly decreased IGF-II concentration in the medium (*) compared with the media containing $3\times10^{-7}$ M or $10^{-6}$ M MZ-5-156 $P<0.05$. (b) MZ-5-156 at $3\times10^{-6}$ M concentration significantly decreased proliferation of SW-1990 cells in reduced-serum medium 68 h after addition of the peptide (*t1*) ($P<0.05$).

### 3.6. IGF-II mRNA expression

IGF-II mRNA expression was analysed in SW-1990 cells cultured *in vitro* in reduced-serum medium using RT-PCR and the products were quantified using a densitometer. The level of IGF-II mRNA was $84.7\pm2.64\%$ of control values in the cells treated with $3\times10^{-6}$ M MZ-5-156 (data not shown). The difference between treated and control data was statistically significant ($P=0.021$, Student's *t*-test).

## 4. Discussion

IGFs have been implicated in the development and progression of pancreatic cancers [8,10] although the role of individual components of the GH-RH–GH–IGF axis and the exact mechanisms of tumour promoting action have not been clarified. IGFs, GH and even GH-RH, acting by endocrine, paracrine or autocrine pathways, could be involved in pancreatic tumour growth. According to the classical hypotheses, IGF-I may function as a circulating hormone secreted by the liver [12], mediating the normal growth promoting effect of GH. IGF-I is also produced in various other tissues, as for example in osteoblasts, where its levels are likewise regulated by GH [28]. IGFs may also be involved in the progression of pancreatic cancers [8–10]. IGF-I was found to be an autocrine growth factor for the human pancreatic cancer cell line Mia-PaCa2. These cells produce IGF-I, their growth can be stimulated by exogenous IGF-I and inhibited by MAb to IGF-I receptor [29]. One study revealed correlations between IGF-I concentration in sera and cancer promotion [30]. Daily treatment of athymic nude mice with GH raised serum IGF-I levels, and the addition of the serum from these mice to human pancreatic cancer cell cultures increased cell growth [30]. In contrast, serum IGF-I or IGF-II levels were found to be normal in patients with pancreatic carcinoma [31].

In addition to IGF-I, IGF-II may also be involved in stimulation of various tumours, mainly by autocrine–paracrine pathways. IGF-II has a well established role in the growth of myogenic sarcomas, Wilms' tumour and neuroblastoma, and is somewhat involved in other tumours such as osteosarcoma, breast cancer, hepatocellular carcinoma, brain tumours and colorectal cancer [32]. Some studies also report a possible significance of IGF-II in pancreatic carcinogenesis [9,10]. However, the regulation of IGF-II is less clear than that of IGF-I. The expression of IGF-II in various tissues appears not to be regulated by GH [12], but some other studies show that GH may act on the production of both IGF-I and IGF-II [33].

It is well established that the growth-promoting effects of both IGF-I and IGF-II are mediated through the IGF-I receptor [34]. The presence of genetically engineered dominant negative IGF-I receptor inhibits growth of cells *in vitro*, induces apoptosis *in vivo* and inhibits tumorigenesis in rats [35]. Inversely, overexpression of IGF-I receptors is sufficient for increased cellular proliferation and cell transformation [36]. The IGF-I receptor activated by its ligand has three functions: it is necessary for optimal growth, for the establishment and maintenance of the transformed phenotype, and it protects cells from apoptosis [11,34]. The IGF-I receptor can bind IGFs from the circulation or those produced locally by cancer cells and neighbouring cells. Thus, IGFs can act by endocrine, paracrine or autocrine pathways, depending on the type of cancer. Thus lung cancers express IGF-I, and the locally produced IGF-I in an autocrine fashion provides a growth stimulus for lung cancer cells in addition to the circulating IGF-I. In contrast, breast tumour cells express small amounts of IGF-II, and breast stromal cells produce IGF-I, which act on growth in an autocrine and paracrine manner, respectively [37].

The effect of IGF-I is mainly regulated by IGF-binding proteins (IGFBP), first of all IGFBP3. IGF-I binds to this protein with higher affinity than to the IGF-I receptor, thus IGFBPs may modulate the bioavailability of IGF-I for tissues [32,38,39]. The presence of IGFBPs also affects the measurement of serum IGF levels. Therefore, our IGF assays were performed after acid-

ethanol cryoprecipitation extraction to eliminate the effects of IGFBPs [24]. This procedure has provided satisfactory results in comparative studies [40,41].

In addition to its growth-promoting effects mediated through IGFs, GH also may have a direct action on tissues, since GH receptors were detected at various sites of the body, including the gastro-intestinal tract and pancreas [42]. Furthermore, a local direct effect of GH-RH itself on growth cannot be excluded. Although GH-RH receptors have not been found in extra-pituitary sites, GH-RH may bind to other neuropeptide receptors in tissues.

The exact mechanism of action of our GH-RH antagonists on pancreatic cancer is not clear. In previous studies, GH-RH antagonist MZ-4-71 decreased GH response to exogeneous GH-RH, lowered the GH content and GH-RH receptor concentrations of the pituitaries in rats [43]. Basal serum GH and IGF-I levels also declined after a bolus injection of the antagonist. GH-RH antagonists lower GH and, in most cases, IGF-I levels in sera of tumorous animals simultaneously with tumour inhibition [14–16]. A recent investigation, however, related the inhibitory effect of GH-RH antagonist MZ-5-156 on prostate cancers to a decrease in production of IGF-II in tumours [24].

In the present study, daily s.c. injections of GH-RH antagonists decreased GH levels in sera of most mice, but IGF levels remained steady. Local IGF-I concentration in SW-1990 cancers was also unchanged by the treatments. Hence, tumour inhibition appears not to be caused by decreases in systemic supply or deficient local availability of IGF-I. The IGF-I receptor concentration in the pancreatic cancers was also not affected by MZ-4-71 or MZ-5-156. IGF-II levels in the tumours, however, were significantly decreased after therapy with GH-RH antagonists. The reduction of IGF-II in tumours may be explained by a local action of the antagonist itself, or an effect mediated through the reduced serum GH levels.

The results of our *in vitro* studies provide additional information to the mechanism of tumour inhibitory action of the GH-RH antagonists. The decrease in IGF-I levels in the medium after seeding of SW-1990 cells showed that IGF-I was used up by the cells or was bound to IGF-I receptors on the pancreatic cancer cells. The IGF-II level, however, was significantly increased in the medium 90 h after seeding of cells, demonstrating that IGF-II was secreted by SW-1990 cells in greater amounts than what could be bound by the receptors. This suggests that endogenous IGF-II provides enough stimulus for growth, and also explains why exogenous IGF-II did not enhance the proliferation of SW-1990 cells. Previous *in vitro* studies showed that GH-RH antagonists decreased IGF-II production of various tumour cell lines including SW-1990 pancreatic cancer [44]. In the present experiment, IGF-II mRNA expression in SW-1990 cells was decreased by MZ-5-156 demonstrating a possible direct effect on the cells. The GH-RH antagonists were active *in vitro* only at relatively high concentrations, and caused only modest changes in cell growth and IGF-II production when the cells grew in reduced-serum medium. It is well known that many characteristics of cancer cells under *in vitro* conditions are different from those *in vivo* and some *in vivo* effects are difficult to reproduce under *in vitro* conditions. The finding that SW-1990 cells produce and release IGF-II, indicates that IGF-II may be a growth factor for this tumour and it may act in an autocrine manner. MZ-5-156 and MZ-4-71 inhibit the growth of experimental pancreatic cancers, and this effect may be linked to a decrease in IGF-II concentration in the tumours. Although the complete mechanism of action of the GH-RH antagonists still remains to be elucidated, these hormone analogues may become candidates for treatment of patients with pancreatic cancer.

## Acknowledgements

We thank Esther Li, Elena Glotser and Dora Rigo for their technical assistance. The gifts of materials for RIA provided by Dr. A.F. Parlow, Dr. Underwood and Dr. J. van Wyk from NIDDK are greatly appreciated. This work was supported by a grant from ASTA MEDICA to Tulane University School of Medicine and by the Medical Research Service of the Veterans Affairs Departments (all to AVS).

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