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Dose-Dependent Growth Inhibition In Vivo of PC-3 Prostate Cancer With a Reduction in Tumoral Growth Factors After Therapy With GHRH Antagonist MZ-J-7-138

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BACKGROUND. Antagonists of growth hormone-releasing hormone (GHRH) inhibit the growth of various cancers and affect tumoral growth factors.
METHODS. We investigated the effect of a new GHRH antagonist MZ-J-7-138 at doses of 1.25, 2.5, 5 and 10 μg/day s.c. on the growth of PC-3 human androgen independent prostate cancers xenografted s.c. into nude mice. Binding assays were used to investigate GHRH receptors. The levels of IGF-II and VEGF in tumors were measured by radioimmunoassays.
RESULTS. Treatment with 2.5, 5, and 10 μg/day MZ-J-7-138 caused a significant dose-dependent growth reduction of PC-3 tumors. The greatest inhibition of 78% was obtained with 10 μg/day. The suppression of IGF-II protein levels in tumors was seen at all doses of MZ-J-7-138, but only 10 μg dose induced a significant inhibition. MZ-J-7-138 also reduced VEGF protein levels, the inhibition being significant at doses of 5 and 10 μg. Specific high affinity binding sites for GHRH were found on PC-3 tumors using ^{125}I-labeled GHRH antagonist JV-1-42. MZ-J-7-138 displaced radiolabeled JV-1-42 with an IC_{50} of 0.32 nM indicating its high affinity to GHRH receptors. Real-time PCR analyses detected splice variant 1 (SV1) of GHRH receptor (GHRH-R) as well as pituitary type of GHRH-R and GHRH ligand.
CONCLUSION. Our results demonstrate the efficacy of GHRH antagonist MZ-J-7-138 in suppressing growth of PC-3 prostate cancer at doses lower than previous antagonists. The reduction of levels of growth factors such as VEGF and IGF-II in tumors by GHRH antagonist was correlated with the suppression of tumor growth. *Prostate 68: 1763–1772, 2008.*

KEY WORDS: **androgen independent prostate cancer; GHRH; splice variants of GHRH receptors; antitumor peptide analogs**

Abbreviations: GHRH, growth hormone-releasing hormone; pGHRH-R, pituitary GHRH receptor; SV, splice variants of GHRH receptor; SV1, SV2, SV3, SV4—splice variants 1, 2, 3, and 4 of GHRH receptor; PCa, prostate carcinoma; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor.

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INTRODUCTION

Prostate cancer is the most common non-cutaneous malignant tumor in men [1,2]. Androgen-dependent prostate cancer constitutes ≈70% of all cases of prostate neoplasms [1]. The suppression of androgenic activity with surgical orchiectomy or agonists of luteinizing hormone-releasing hormone is considered the most adequate first line treatment. However hormonal therapy is successful in only 70–80% of cases and the median duration of response is usually only 12–24 months [2]. Currently the management of androgen-independent metastatic prostate cancer remains a complex and difficult problem and there is no curative treatment [1,2]. The chemotherapy with Docetaxel based combinations can lead to significant improvement in survival time. However, median survival does not exceed 20 months from the start of chemotherapy [3]. Therefore, there is a great need for new and better therapies.

On the basis of recent advances in the understanding of the role of neuropeptides and growth factors in the progression of prostate carcinoma and other cancers [1,4], we have developed antagonists of GHRH for the treatment of various cancers including prostate cancer [1,5–8]. GHRH antagonists inhibit the growth of various human tumors xenografted into nude mice, including androgen-independent prostate cancers [1,5–9] mainly by direct effects on cancer cells [9–13], although indirect effects mediated by the inhibition of the endocrine pituitary GH/hepatic IGF-I axis also occur [5,8,10]. The direct effects of GHRH antagonists on cancer cells are mediated by splice variants (SV) of the pituitary type of GHRH receptors (pGHRH-R), which are present on human prostate cancer specimens and on cell lines [5,6,11,12,14–18]. In addition to blocking GHRH receptors, antagonists of GHRH also inhibit the tumoral expression of IGF-I and -II. These effects contribute to their antiproliferative mechanism [5,6]. A decrease of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), IGF-I and -II protein levels and their mRNA expression, and a downregulation of EGF receptors was found after treatment of experimental androgen-independent prostate cancers with GHRH antagonists [11–13,19].

Although early GHRH antagonists such as MZ-4-71 or MZ-5-156 significantly inhibited the growth of PC-3 and DU-145 human androgen-independent prostate cancers in vivo [19,20], they were deemed unsuitable for clinical development, as they required high doses and frequent administration (2×20 μg/day in nude mice), due to the lack of sufficient potency, protracted activity, and chemical and enzymatic stability. Subsequently, our laboratory developed more potent and longer acting GHRH antagonists, in order to reduce the doses necessary for human therapy and to make the potential clinical use of GHRH antagonists technically and economically feasible [7–9]. One of the most potent GHRH antagonists prepared to date is MZ-J-7-138, which was developed by using a number of design criteria, including: (1) replacement of the enzymatically vulnerable Arg and Lys residues present in native GHRH peptide by enzymatically stable alternative residues while also preserving the subnanomolar binding affinities of the analogs to GHRH receptors; (2) replacement of the Asn and Met residues, prone to chemical hydrolysis, by other amino acids; (3) incorporation of a fatty acyl moiety into the molecule in order to enhance its binding to serum albumin, this strategy being employed to reduce the blood clearance due to enzymatic degradation and renal filtration of peptide drugs [7,8,21].

In the present study, using the PC-3 cells xenografted into nude mice as a preclinical model for testing the efficacy of anticancer drugs, we investigated the minimum effective dose and dose–response relationship of GHRH antagonist MZ-J-7-138 for the treatment of androgen-independent prostate cancer in vivo. Tumoral IGF-II and VEGF were also evaluated in view of evidence from earlier studies that tumor inhibition by GHRH antagonists affects multiple tumoral growth factors and their signaling. Our study should help towards a preliminary estimation of the doses required for human therapy and serves as a starting point for the design of future clinical trials.

MATERIALS AND METHODS

Peptides and Reagents

The GHRH antagonist MZ-J-7-138 was synthesized in our laboratory by solid phase methods. The chemical structure of MZ-J-7-138 is [CH_3-$(CH_2)_6$-CO-Tyr^1,D-Arg^2,$Phe(4\text{-}Cl)^6$,Ala^8,His^9, $Tyr(Et)^{10}$,His^{11}, Orn^{12},Abu^{15}, His^{20}, Orn^{21},Nle^{27},D-Arg^{28},Har^{29}]hGHRH(1–29)NH_2, where Phe(4-Cl) is 4-chlorophenylalanine, Tyr(Et) is O-ethyltyrosine, Abu is α-aminobutyric acid, Nle is norleucine, Har is homoarginine, Orn is ornithine. For daily injection, MZ-J-7-138 was dissolved in 0.1% dimethylsulfoxide (DMSO) in 10% aqueous propylene glycol solution (vehicle solution).

Cell Lines and Animals

Male athymic (Ncr nu/nu) nude mice, approximately 5–6 weeks old on arrival, were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD) and housed in laminar air flow cabinets under pathogen-free conditions with a 12-hr light/12-hr dark

schedule and fed autoclaved standard chow and water ad libitum.

The PC-3 androgen-independent prostate cancer cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture as described [22,23]. 1.5 million cells were injected s.c. at each flank to three donor animals. PC-3 tumor tissue was harvested aseptically from donor animals. Male nude mice were xenografted subcutaneously with 3 mm^3 pieces of PC-3 tumor tissue using a trocar needle. When tumors had grown to a mean volume of approximately 60 mm^3, the animals were randomly assigned to treatment groups and control of nine animals each.

In Vivo Studies

The antagonist MZ-J-7-138 was injected s.c. daily at doses of 1.25, 2.5, 5, 10 μg/day and controls received the injection vehicle. Tumor volumes (length × width × height × 0.5236) and body weights were recorded every week. Tumor volume doubling time (TDT) and tumor inhibition (TI) were calculated using the formula:

$$\mathrm{TDT} = \text{days of treatment} \times \log 2/(\log \text{volume}^{\text{final}} - \log \text{volume}^{\text{initial}})$$
$$\mathrm{TI} = 100\% \ (\Delta\text{volume}^{\text{control}} - \Delta\text{volume}^{\text{treatment}})/\Delta\text{volume}^{\text{control}}$$
$$\Delta\text{volume} = \text{final volume} - \text{initial volume}$$

At the end of the experiments, mice were anesthetized with Phenobarbital and sacrificed by cutting the abdominal aorta. Tumors were carefully excised, weighed, snap frozen and stored at −70°C for further investigations. Blood was collected and a complete necropsy was performed of all animals. Serum was collected and frozen. Liver, heart, lungs, kidneys, spleen were carefully removed and weighed. All experiments were in accordance with the institutional guidelines for the welfare of animals in experiments. The institutional Animal Care and Use Committee reviewed the animal experiments and gave full approval.

Total RNA Isolation and Real-Time PCR Analysis

Total RNA was extracted following TRI-Reagent® protocol (Sigma-Aldrich, St. Louis, MO) from six representative PC-3 tumors grown in nude mice (three controls and three tumors from animals treated with s.c. injection of MZ-J-7-138 10 μg/day). RNA was also extracted from human pituitary purchased from the National Hormone and Peptide Program (A.F.Parlow, Harbor-UCLA Medical Center). The yield and quality of total RNA was determined spectrophotometrically using 260 nm and 260/280 nm ratio, respectively. Two micrograms of RNA with a final volume of 40 μl were reverse transcribed into cDNA with the QuantiTect® Reverse Transcription Kit (Qiagen, Valencia, CA) using the GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA).

We evaluated the mRNA expression of human pituitary GHRH receptor (pGHRH-R), splice variant 1 of GHRH-R (SV1), GHRH and beta-actin. Probes and primers for pGHRH-R, GHRH and beta-actin and sense and antisense specific primers for SV1 were described previously [18].

All real-time PCR reactions were performed in the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). All thermal cycling conditions were described previously [18]. All samples were run in triplicate and each well of PCR reaction contained 25 μl as final volume including 2 μl of cDNA, 200 nM of gene specific primers and 400 nM of probes. iQ™ Supermix (Bio-Rad Laboratories) was used in the PCR reactions for pGHRH-R, GHRH and beta-actin and iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) for SV1. The efficiencies of all primers (Invitrogen Life Technologies, Carlsbad, CA) and probes (Integrated DNA Technologies, Coralville, IA) were tested prior to the experiments and they were all efficient in the range of 95–105%. Normal human pituitary was used as positive control and human beta-actin as housekeeping gene. Negative samples were run in each reaction consisting of no-RNA in reverse transcriptase reaction and no-cDNA in PCR reaction.

The relative expression ratio of pGHRH-R, SV1, and GHRH in samples of the xenografted tumors was calculated as described before [18]. To calculate the relative expression of pGHRH-R, SV1, and GHRH we used the probe efficiency and the threshold cycle (CT) of beta-actin, as housekeeping gene, and normal human pituitary (Pi) as positive control.

Radioimmunoassays (RIA) for Tumoral VEGF and Tumoral IGF-II

Human VEGF and IGF-II levels were determined by RIA in tumor tissue homogenates [13]. VEGF and IGF-II were extracted from tumor tissue, using a modified acid-ethanol cryoprecipitation method [24]. The total protein content was determined by using the Bio Rad protein assay kit (Bio-Rad Laboratories). The standard for VEGF was a recombinant human VEGF, 38.2 kDa,

consisting of 165 amino acids. Antihuman VEGF was an affinity-purified, polyclonal antibody. Both VEGF and antihuman VEGF were purchased from PeproTech, Inc. (Rocky Hill, NJ). The standard was used in the range of 0.006 and 12.8 ng per tube. The antibody was used at a final dilution of 1:200,000. VEGF was iodinated by the lactoperoxidase method and purified by high-performance liquid chromatography, using a reverse-phase Vydac C4 column. The assay buffer for VEGF consisted of 0.01 M sodium phosphate pH 7.6, 0.025 M EDTA, 0.14 M NaCl, and 1% BSA. The antibody and tracer were added simultaneously and incubated overnight at 4°C. Bound and free fractions were separated by the polyethylene–glycol double-antibody method. IGF-II concentration was measured by RIA using human recombinant IGF-II standard (Bachem, Torrance, CA) in the range of 2–2,000 pg per tube. IGF-II was iodinated by the lactoperoxidase method, and purified as described previously [19]. Anti-rat IGF-II (10 μg/ml) monoclonal antibody (Amano International Enzyme, Troy, VA) was used at a final dilution of 1:12,500. This antibody crossreacts 100% with human IGF-II and rat IGF-II, and 10% with human IGF-I. The RIA results from all samples were evaluated in a computer-controlled gamma Counter (Packard Cobra System, Perkin Elmer Life & Analytical Science, Shelton, CT). The intraassay and the interassay coefficients of variation were less than 10% and 15%, respectively.

Receptor Binding Assays

Preparation of the membrane fractions from PC-3 human prostate cancers and receptor binding studies were performed as described previously [17]. Binding characteristics of receptors for GHRH were determined by in vitro ligand competition assays based on the binding of radiolabeled GHRH antagonist JV-1-42 to tumor membrane fractions [17]. Receptor binding affinity (IC_{50}) of GHRH antagonist MZ-J-7-138 to membranes of subcutaneously grown PC-3 tumors from the control group was also measured in displacement experiments based on the competitive inhibition of [^{125}I]JV-1-42 binding by various concentrations (10^{-12}–10^{-6} M) of MZ-J-7-138. IC_{50} is defined as the dose of MZ-J-7-138 causing 50% inhibition of the specific binding of [^{125}I]JV-1-42.

Statistical Analysis

Data are expressed as means ± SE. The Sigma Stat Software was used for the statistical analysis of data. Differences between mean values were evaluated by ANOVA followed by Holm–Sidak test, $P < 0.05$ being considered significant. For analysis of results from RIA, a two tailed Student's *t*-test with Microsoft® Excel Software was performed between groups.

RESULTS

Effect of GHRH Antagonist MZ-J-7-138 on the Growth of PC-3 Human Androgen Independent Prostate Cancer in Nude Mice

As shown in Figure 1 and Table I, MZ-J-7-138 at the dose of 1.25 μg/day did not produce any inhibition of tumor growth compared to the control group. The lowest dose which caused a significant growth suppression of 52% by the end of the fourth week was 2.5 μg/day. MZ-J-7-138 at the dose of 5 μg/day led to a significant decrease in tumor growth of 65%. The highest dose of MZ-J-7-138, 10 μg/day significantly suppressed PC-3 tumor growth exerting a final tumor inhibition of 78%.

Already after the first week, the groups receiving 5 and 10 μg/day MZ-J-7-138, showed a better growth inhibition than the 1.25 μg/day group. After the fourth week significant differences were observed between the treatment groups of 10 μg/day versus 2.5 μg/day and 1.25 μg/day. The tumor doubling time increased from 6.2 days in control group to 12.4 days by the treatment with 10 μg/day. No significant differences were found in the body and organ weights of animals receiving various treatments versus controls, indicating the lack of toxicity of GHRH antagonist.

Effect of GHRH Antagonist MZ-J-7-138 on the Expression of IGF-II and VEGF

The treatment with GHRH antagonist MZ-J-7-138 decreased the expression of tumoral proteins of VEGF and IGF-II (Figs. 2 and 3). Administration of 10 μg/day

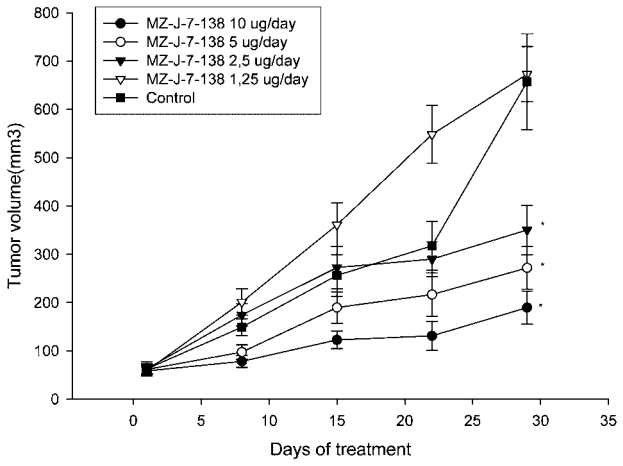

Fig. 1. Growth of PC-3 tumors, treated with different doses of the GHRH antagonist MZ-J-7-138. Significant differences from the control are marked by asterisks ($P < 0.001$).

TABLE I. Effect of GHRH Antagonist MZ-J-7-138 on the Growth of Human PC-3 Androgen Independent Prostate Cancers in Nude Mice

Treatment	Tumor volume (mm^3)		Tumor volume doubling time (days)	Tumor weight (mg)
	Initial	Final (% inhibition)		
Control	62.44 ± 14.63	657.02 ± 99.39	6.18	762.22 ± 271.71
MZ-J-7-138 (1.25 μg/day)	58.78 ± 7.74	672.84 ± 57.07 (0.35%)	6.05	737.50 ± 298.89
MZ-J-7-138 (2.5 μg/day)	62.85 ± 10.07	350.25 ± 26.60 (52%)*,†	8.47	710.00 ± 204.95
MZ-J-7-138 (5 μg/day)	61.46 ± 9.42	$271{,}67 \pm 44.07$ (65%)*,†	9.79	592.50 ± 70.59*
MZ-J-7-138 (10 μg/day)	58.47 ± 7.96	189.37 ± 34.33 (78%)*,†	12.39	385.71 ± 53.14*

*$P = <0.001$ versus controls.
†$P = <0.05$ versus lower dose step.

induced a significant reduction to 47.3% for IGF-II and to 55% for VEGF versus the control groups ($P < 0.05$ in both cases). Lower doses of the antagonist caused a smaller reduction in both proteins (Figs. 2 and 3). VEGF protein levels were reduced to 75% of the control group by 5 μg/day ($P < 0.05$), to 80% by 2.5 μg/day (NS) and to 82% by 1.25μg/day (NS). Treatment with 1.25, 2.5, and 5 μg/day of MZ-J-7-138 reduced tumoral IGF-II protein to 66.8%, 64%, and 57.2% respectively of the control group (NS). Significant differences between the different doses (10 μg/day vs. 2.5 μg/day and 1.25 μg/day) were seen in the VEGF expression.

Effect of GHRH Antagonist MZ-J-7-138 on the mRNA Expression for pGHRH-R, SVI and GHRH

The presence of mRNA for the SV1 of the GHRH receptor and GHRH ligand in PC-3 tumors was reported previously [25]. In this study, using real-time PCR we demonstrated the presence of mRNA for pituitary type of GHRH receptor pGHRH-R. The reaction efficiencies for the probes of pGHRH-R, GHRH, and beta-actin were 103.7%, 101.3% and 97.4% respectively. The relative expression of pGHRH-R in PC-3 tumors treated with MZ-J-7-138 was significantly elevated, being 2.75-fold higher than the control group. Moreover, real-time PCR also revealed a non-significant downregulation of SV1 receptor and significant downregulation of GHRH after treatment with GHRH antagonist, the ratios being 0.63 and 0.25, respectively. The results are shown in Table II.

Binding Assays for GHRH Receptors in PC-3 Tumors

The presence of GHRH receptors was assessed by radioligand binding assays in control tumors. Binding studies demonstrated the presence of a single class of specific, high affinity (dissociation constant, $K_d = 0.63 \pm 0.08$ nM) binding sites for GHRH antagonist JV-1-42 in the membrane preparation of PC-3 tumors with a mean maximal binding capacity (B_{max}) of 178.5 ± 24.7 fmol/mg membrane protein.

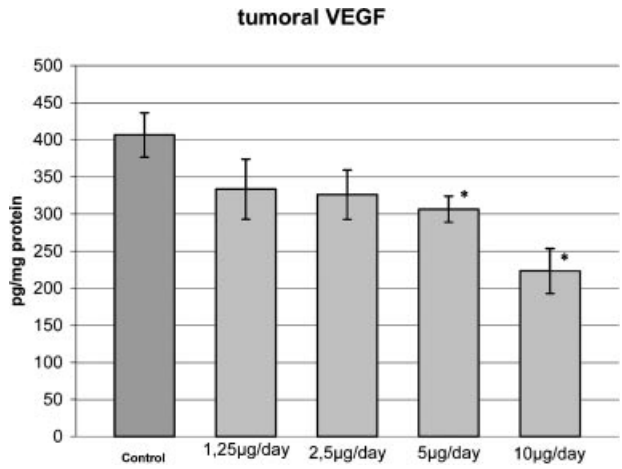

Fig. 2. Effect of GHRH antagonist MZ-J-7-138 at different doses on the protein levels of tumoral VEGF after 4 weeks of daily treatment. Significant differences from the control are marked by asterisks ($P < 0.05$).

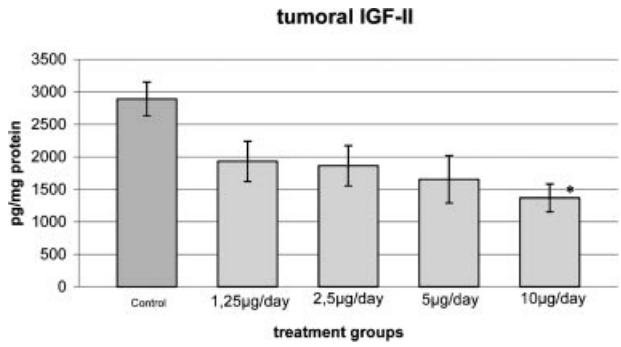

Fig. 3. Effect of GHRH antagonist MZ-J-7-138 at different doses on the protein levels of tumoral IGF-II after 4 weeks of daily treatment. Significant differences from the control are marked by asterisks ($P < 0.05$).

TABLE II. Real-Time PCR Values of the Relative Expression of mRNA for pGHRH-R, SVI, and GHRH in PC-3 Tumors After Treatment With GHRH Antagonist MZ-J-7-138

	Control	MZ-J-7-138	
	C_T values, mean ± SEM	C_T values, mean ± SEM	Ratio
pGHRH-R	38.18 ± 0.37	36.86 ± 0.25	2.75*
SV1	33.87 ± 0.46	34.66 ± 0.42	0.63
GHRH	36.94 ± 0.30	39.09 ± 0.71	0.25*
β-actin	19.74 ± 0.09	19.88 ± 0.03	—

The ratio represents the gene expression level in the treatment group as compared to the control group.
*$P < 0.05$ versus controls.

In competition displacement experiments on subcutaneously grown PC-3 tumors from the control group, GHRH antagonist MZ-J-7-138 was able to displace the [^{125}I]JV-1-42 radioligand with an $IC_{50} = 0.32$ nM. This IC_{50} value indicates a high affinity binding of MZ-J-7-138 to GHRH receptors expressed on PC-3 tumors.

DISCUSSION

Treatment of relapsed androgen-independent prostate cancer remains a major challenge [1–6]. New therapeutic modalities are being developed based on antagonists of LHRH, GHRH and targeted cytotoxic peptide analogs [5–7]. Polypeptide growth factors such as GHRH, gastrin-releasing peptide, IGF-I and -II, VEGF, bFGF, EGF, and their receptors are widely expressed in prostate cancer [6,16,17,25–30]. Some of these growth factors aberrantly stimulate the androgen receptor pathway [4,28,31] and contribute to the androgen-independent growth of prostate cancer cells. The involvement of GHRH in the growth of various human neoplasms prompted the development of GHRH antagonists for the endocrine therapy of these cancers [1,5–9]. GHRH antagonists can inhibit the growth of various human experimental prostate cancers indirectly by reducing pituitary GH and hepatic IGF-I secretion and directly by blocking tumoral GHRH receptors and decreasing tumoral IGF-I and -II [6–12].

The PC-3 tumor model represents an advanced stage androgen independent prostate cancer and has been extensively utilized in our previous investigations for the assessment of antitumor effects of less potent GHRH antagonists such as MZ-4-71, MZ-5-156, JV-1-38, MZ-J-7-118 [1,10,11,13,20,21,32]. In the present study we used the latest and most potent GHRH antagonist MZ-J-7-138 developed very recently. Our results indicate that the minimum effective dose of GHRH antagonist MZ-J-7-138 is 2.5 μg/day for the inhibition of PC-3 cancers in nude mice, and a clear relationship of dose-dependent tumor inhibition is observed in the dose range of 2.5–10 μg/day. This pattern of dose–response relationship where greater inhibition of tumor growth is observed with higher doses of GHRH antagonist represents an advantage compared to some of the other peptide hormones used in cancer treatment. By comparison, several studies found that the response of cells to exposure of somatostatin analog octreotide produces a biphasic dose–response curve, implying that overdosing or underdosing of somatostatin analogs may result in suboptimal antitumor activity [33]. The present study and previous work also indicates that MZ-J-7-138 and other GHRH antagonists are devoid of toxic side-effects and have an extremely broad therapeutic window [7]. Thus, here we obtained significant inhibition of PC-3 tumor growth with 2.5 μg/day of MZ-J-7-138, but in other studies in nude mice, this antagonist was used at a 32-fold higher dose level of 2 × 40 μg/day, without any visible side-effects or toxicity [34].

A comparison between the effects of MZ-J-7-138 and older antagonists on the growth of various human prostate cancers in nude mice is given in Table III. Collectively, the data presented in Table III indicate that new antagonists such as MZ-J-7-118 and MZ-J-7-138 are effective against androgen-independent prostate cancers PC-3 and DU-145 at doses 8- to 16-fold lower than early GHRH antagonists MZ-4-71 and MZ-5-156 that had to be used at doses of up to 2 × 20 μg/day to inhibit tumor growth. In addition, MZ-J-7-138 is the first GHRH antagonist that was effective alone as a therapeutic agent for the treatment of MDA-PCa-2b human androgen-sensitive prostate cancers in nude mice [35]. Thus, MZ-J-7-138 at a dose of 5 μg/day inhibited the growth of MDA-PCa-2b tumors to a similar degree as LHRH antagonist Cetrorelix at a dose of 100 μg/day [35]. Earlier antagonists such as JV-1-38 or MZ-J-7-118 were not effective when used alone against MDA-PCa-2b cancers, although in combination with androgen deprivation therapies such as LHRH antagonist Cetrorelix they enhanced the antitumor effects of the latter agents [12,35,36]. Recently, we also performed experiments to directly compare the effects of MZ-J-7-138 with other highly potent GHRH antagonists including MZ-J-7-118. In these studies, we found that MZ-J-7-138 more potently inhibited the growth of PC-3 cancers in vivo and showed an increased binding affinity to the GHRH receptors on PC-3 tumor membranes, as compared to other antagonists tested (Zarandi M, Schally AV, Varga JL et al., unpublished data).

TABLE III. Comparative Effects of Various GHRH Antagonists on the Growth of Human PC-3 and DU-145 Androgen Independent and MDA-PCa-2b Androgen-Sensitive Prostate Cancers in Nude Mice

PC-3 tumors xenografted s.c. into nude mice			
GHRH antagonist (reference no.)	Dose	Inhibition of tumor volume	Other human prostate cancers xenografted s.c. into nude mice
MZ-4-71 (Ref. 20)	2 × 20 μg/day	70%**	DU-145 (Ref. 20): MZ-4-71 (2 × 20 μg/day) significantly inhibited tumor volumes by 81% (**)
MZ-5-156[a] (Ref. 10)	20 μg/day[a] (Exp. 1)	53% n.s.[a]	DU-145 (Ref. 19): MZ-5-156 (2 × 20 μg/day) significantly inhibited tumor volumes by 57% (*)
JV-1-38 (Ref. 10)	20 μg/day[a] (Exp. 1)	65%*,[a]	DU-145 (Ref. 12): JV-1-38 (20 μg/day) significantly inhibited tumor volumes by 57% (*)
	20 μg/day (Exp. 2)	65%*	MDA-PCa-2b (Ref. 12): JV-1-38 (20 μg/day) did not inhibit tumor volumes
JV-1-38 (Ref. 11)	20 μg/day	49%**	MDA-PCa-2b (Ref. 36): in two separate experiments
JV-1-38[b] (Ref. 21)	10 μg/day[b]	0% n.s.[b]	JV-1-38 (20 μg/day) did not significantly affect tumor volumes
MZ-J-7-114[b] (Ref. 21)	5 μg/day[b]	43%*,[b]	
MZ-J-7-118 (Ref. 32)	5 μg/day	56%*	DU-145 (Ref. 13): MZ-J-7-118, at 2.5 μg/day and 5 μg/day, inhibited tumor volumes by 67% (*) and 82% (*), respectively
MZ-J-7-118 (Ref. 13)	5 μg/day	65%*	MDA-PCa-2b (Ref. 35): MZ-J-7-118 (5 μg/day) slightly inhibited tumor volumes by 24% (n.s.)
MZ-J-7-138 (present study)	2.5 μg/day	52%***	MDA-PCa-2b (Ref. 35): MZ-J-7-138 (5 μg/day) significantly inhibited tumor volumes by 50% (**)
	5 μg/day	65%***	
	10 μg/day	78%***	

n.s., not significant.
[a]GHRH antagonists MZ-5-156 and JV-1-38 directly compared in the same experiment.
[b]JV-1-38 and MZ-J-7-114 directly compared in the same experiment. Accurate comparisons of the potencies of GHRH antagonists are only possible when they are used in the same experiment. Nevertheless, inhibitory results obtained in different experiments are helpful for a rough estimation of the order of potencies and effective doses of various antagonists.
*$P < 0.05$ versus controls.
**$P < 0.01$ versus controls.
***$P < 0.001$ versus controls.

The present data allow an extrapolation from the doses effective in nude mice to the doses recommended for clinical trials, based on extensive preclinical (nude mice) and clinical data available with other classes of peptide drugs for cancer, such as somatostatin analogs, and agonists and antagonists of LHRH. In the nude mouse model, GHRH antagonist MZ-J-7-138 is effective at much lower doses (2.5–5 μg/day) against androgen-independent PC-3 and androgen-sensitive MDA-PCa-2b prostate cancers than other types of peptide drugs that are already in clinical use or showed efficacy in clinical trials. Thus, somatostatin analog RC-160 (Vapreotide) inhibited the growth of PC-3 prostate cancers in nude mice at 100 μg/day, but was ineffective at the lower dose of 50 μg/day [11,22]. Likewise, LHRH agonist triptorelin (Decapeptyl), and LHRH antagonist SB-75 (cetrotide, Cetrorelix) need to be used in a dose range of 50–100 μg/day for a significant inhibitory effect on the growth of MDA-PCa-2b androgen-sensitive tumors in nude mice [35,36]. Thus, GHRH antagonist MZ-J-7-138 is effective against prostate cancers in nude mice at dose levels that are 10–40 times lower than those of the mentioned somatostatin and LHRH analogs. For the clinical therapy of prostate cancer, Decapeptyl is used at a dose of 0.125 mg/day (3.75 mg/month), Cetrorelix showed clinical efficacy at a dose of 2 × 0.5 mg/day, and Vapreotide produced clinical responses in patients with advanced prostate cancer at a dose of 3 × 1 mg/day [5,37–40]. Thus, supposing that GHRH antagonists will be active in human cancer therapy at dose levels 10–40 times lower than these analogs of somatostatin and LHRH, the clinically effective doses of GHRH antagonists would fall into the range of 0.0125–0.3 mg/day. A clinical therapy based on sub-milligram daily doses of GHRH antagonist would be feasible both from a pharmaceutical technological and economical standpoint, as it would permit the use of depot formulations containing

a full monthly dose of peptide. The cost of the pharmaceutical preparation at such low doses would also be reasonable.

The present study demonstrates the dose-dependent inhibitory effect of GHRH antagonist MZ-J-7-138 on the tumoral growth factors VEGF and IGF-II in PC-3 prostate cancer. In the past few years, different GHRH antagonists have been tested at various doses in studies on growth inhibition of prostate cancer xenografts in vivo [1,5–13,19–21,32,35].

The effects of previous GHRH antagonists on tumor inhibition and expression of tumoral growth factors have been discussed [6–9]. GHRH antagonists used in the earlier studies by our group were administered at doses as high as 20–40 μg/day, because of their lower tumor inhibitory potency and at these doses they significantly affected the expression of tumoral growth factors. With more potent GHRH antagonists such as MZ-J-7-118, effective tumor inhibition was demonstrated at lower doses like 5 μg/day, with a decrease in VEGF, but not in the levels of IGF-II [13]. In this study with the most potent GHRH antagonist MZ-J-7-138 developed so far in our laboratory, we were able to clearly demonstrate dose-dependent effects on both tumor inhibition and growth factor suppression. We observed significant ($P < 0.05$) decrease in VEGF protein levels at the doses of 5 μg and 10 μg/day. IGF-II was also significantly decreased by the highest dose of 10 μg/day, but 5 μg/day caused a non-significant reduction ($P < 0.07$). The inhibition of tumoral VEGF and IGF-II is in agreement with the data obtained previously in our laboratory, where a reduction of growth factors was detected with high doses of GHRH antagonists. However, the present study shows that the tumor inhibition obtained with a low dose of GHRH antagonist is not associated with a significant decrease of tumoral growth factors, and it may be chiefly due to the direct inhibitory effect of this antagonist on the tumoral GHRH receptors.

In previous studies [25], we could not detect the expression of mRNA for the pituitary GHRH-R in PC-3 cell line using conventional RT-PCR. Moreover, GHRH binding sites could not be detected with [His^{1}, Nle^{27}]hGHRH(1–32)NH_2 ligand which binds with high affinity to the pituitary isoform of GHRH-R. Thus, it was concluded that PC-3 cell line does not express pituitary GHRH-R. However, in the present investigation using the more sensitive real-time PCR method, we detected the expression of mRNA for pituitary GHRH-R. Based on the low expression of mRNA for pituitary GHRH-R, it is likely that the corresponding receptor protein is expressed only at minimal levels in PC-3 cells, which would explain the negative results obtained in previous receptor binding studies. Specific high affinity binding sites for GHRH were found on PC-3 tumor membranes using ligand competition assays with ^{125}I-labeled GHRH antagonist JV-1-42 which detects both SV-1 and the pituitary type of GHRH receptors. In competition assays GHRH antagonist MZ-J-7-138 displaced the [^{125}I]JV-1-42 radioligand with an $IC_{50} = 0.32$ nM, which indicates a high affinity binding of MZ-J-7-138 to GHRH receptors on PC-3 tumors. However, future investigations are warranted to better characterize the expression of pituitary GHRH-R in PC-3 cell line, especially at protein level.

The effect of treatment with GHRH antagonist MZ-J-7-138 (10 μg/day) on the expression of mRNA for pituitary GHRH-R and SV1 was also investigated. The expression of mRNA for SV1, which is probably the main GHRH receptor expressed at high levels in PC-3 tumors, was slightly and not significantly downregulated after treatment. Conversely, the expression of mRNA for pituitary GHRH-R was significantly upregulated, but it may contribute very little to total binding. Finally, we also studied the expression of GHRH ligand and found that the mRNA level of GHRH peptide was strongly downregulated after treatment with the GHRH antagonist to about one quarter of the level found in control tumors.

Our results demonstrate the effectiveness of a new GHRH antagonist in reducing tumor growth of androgen independent prostate cancer in a dose-dependent manner. The reduction of growth factors like VEGF and IGF-II by this GHRH antagonist was evident at higher doses and after prolonged treatment. Further investigations with GHRH antagonists are required to determine their potential utility for the therapy of prostate cancer. It is likely that the synthesis of still more potent GHRH antagonists might be necessary to convert this class of compounds into a therapeutic tool.

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