Growth hormone-releasing hormone (GHRH) antagonists inhibit the proliferation of androgen-dependent and -independent prostate cancers

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The antiproliferative effects of an antagonist of growth hormone-releasing hormone (GHRH) JV-1-38 were evaluated in nude mice bearing s.c. xenografts of LNCaP and MDA-PCa-2b human androgen-sensitive and DU-145 androgen-independent prostate cancers. In the androgen-sensitive models, JV-1-38 greatly potentiated the antitumor effect of androgen deprivation induced by surgical castration, but was ineffective when given alone. Thus, in castrated animals bearing MDA-PCa-2b cancers, the administration of JV-1-38 for 35 days virtually arrested tumor growth (94% inhibition vs. intact control, $P < 0.01$; and 75% vs. castrated control, $P < 0.05$). The growth of LNCaP tumors was also powerfully suppressed by JV-1-38 combined with castration (83% inhibition vs. intact control, $P < 0.01$; and 68% vs. castrated control, $P < 0.05$). However, in androgen-independent DU-145 cancers, JV-1-38 alone could inhibit tumor growth by 57% ($P < 0.05$) after 45 days. In animals bearing MDA-PCa-2b and LNCaP tumors, the reduction in serum prostate-specific antigen levels, after therapy with JV-1-38, paralleled the decrease in tumor volume. Inhibition of MDA-PCa-2b and DU-145 cancers was associated with the reduction in the expression of mRNA and protein levels of vascular endothelial growth factor. The mRNA expression for GHRH receptor splice variants was found in all these models of prostate cancer. Our results demonstrate that GHRH antagonists inhibit androgen-independent prostate cancers and, after combination with androgen deprivation, also androgen-sensitive tumors. Thus, the therapy with GHRH antagonist could be considered for the management of both androgen-dependent or -independent prostate cancers.

Prostate cancer is the commonest noncutaneous malignancy in American males and ranks second as a cause of cancer-related deaths among men in the United States (1). Although 80% of the patients initially respond to androgen deprivation therapy, induced by orchiectomy, agonists of luteinizing hormone-releasing hormone, or antiandrogens, the remission generally lasts only for 18–36 mo and is followed by the progression to androgen-independent prostate cancer (2). Androgen-independent growth could be stimulated by locally produced growth factors such as insulin-like growth factors-I and -II (IGF-I and -II), epidermal growth factor, vascular endothelial growth factor (VEGF), and others (3, 4).

Extensive *in vivo* studies demonstrated that antagonistic analogs of growth hormone (GH)-releasing hormone (GHRH) inhibit growth of various human cancer cell lines such as osteosarcomas, glioblastomas, and lung carcinomas, as well as renal, breast, ovarian, pancreatic, and colorectal cancers transplanted into immunodeficient mice (2, 5). These studies support the view that, in addition to inhibition of pituitary GH-hepatic IGF-I axis, GHRH antagonists can directly suppress autocrine/paracrine production of IGF-I and -II in tumors (6–8), or block the effects of locally produced GHRH, which acts as a growth factor (9). This direct inhibition of tumor growth is likely mediated by splice variants (SV) of GHRH receptors, which were detected in human prostate cancer cell lines LNCaP,

MDA-PCa-2b, and PC-3 (10–12) and in surgical specimens from patients with locally advanced prostate cancer (13).

In the present preclinical study, we investigated the antiproliferative effects of a recent GHRH antagonist, JV-1-38 (14), alone or in combination with surgical castration in nude mice bearing xenografts of androgen-sensitive LNCaP and MDA-PCa-2b prostate cancers as well as androgen-insensitive prostate cancers DU-145 and C4-2b. We also evaluated the effect of treatment with JV-1-38 on tumoral IGF-I, IGF-II, and VEGF and the serum levels of prostate-specific antigen (PSA).

Materials and Methods

Peptides. GHRH antagonist JV-1-38 was synthesized in our laboratory by solid phase method and purified as described (14). For daily s.c. injection, the compound was dissolved in 0.1% dimethyl sulfoxide in 10% aqueous propylene glycol solution.

Animals. Male athymic (Ncr nu/nu) nude mice, ≈ 6 wk old, were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD) and housed and fed as described (6–9). All experiments were performed in accordance with institutional guidelines of animal care.

Cell Lines and Tumors. Human androgen-independent DU-145 and androgen-sensitive LNCaP human prostatic cancer cell lines were obtained from American Type Culture Collection (15, 16). MDA-PCa-2b human androgen-sensitive prostate cancer cell line was established at the University of Texas, M. D. Anderson Cancer Center (Houston) (17) and grown *in vitro* as described (11, 17, 18). C4-2b androgen-independent prostate cancer line, derived from LNCaP cell line, was obtained from Urocor (Oklahoma City, OK) and grown as described (19).

In Vivo Studies. Experiments I and II. MDA-PCa-2b cell line was initiated and implanted s.c. as described (11, 18). In experiments I and II, the treatment was started 21 days after tumor transplantation when tumors had reached an average tumor size of ≈ 200 and 125 mm^3 , respectively. Nude mice were divided into four groups, with eight mice each and received the following treatment: group 1, sham operation plus vehicle solution (control); group 2, castration plus vehicle solution; group 3, sham operation plus JV-1-38; group 4, castration plus JV-1-38. JV-1-38 was administered s.c. at a dose of $20 \mu\text{g/day}$. This dose was chosen on the basis of previous studies in various cancer models (2, 5, 7, 8, 15). Castration was performed via a scrotal incision under isoflurane (Abbott) anesthesia. The mice were killed when tumor size exceeded $1,500 \text{ mm}^3$, group 1 (control) and group 3

Abbreviations: GHRH, growth hormone-releasing hormone; SV, splice variant; PSA, prostate-specific antigen; IGF, insulin-like growth factor; VEGF, vascular endothelial growth factor; NS, nonsignificant.

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(JV-1-38) after 35 days, group 2 (castration) after 63 days, and group 4 (castration plus JV-1-38) after 79 days. In experiment II, the animals were killed on day 17 for collection of tumor tissue.

Experiments III and IV. Subcutaneous xenografts of LNCaP cancers were induced as described (18). When tumors had grown to a mean volume of $\approx 190 \text{ mm}^3$, the animals were randomly assigned into three groups with eight mice each as follows: group 1 (control); group 2 (castration), and group 3 combination (JV-1-38 and castration) and received the same treatment schedule as in experiment I. Experiment III was terminated on day 35 (group 1), day 42 (group 2), and day 49 (group 3) and experiment IV after 7 days of treatment, to collect tumor samples.

Experiment V. C4-2b cells (5×10^6 per injection site) were suspended in $100 \mu\text{l}$ of Matrigel matrix, and the tumors were initiated in nude mice as described (18, 19). Treatment was started when tumors had grown to 100 mm^3 . There were two groups of six mice each: control group received the vehicle and experimental group JV-1-38 at $20 \mu\text{g}/\text{day}$.

Experiment VI. Androgen-independent DU-145 prostate cancer cell line was transplanted according to previous techniques (16). When tumors had grown to $\approx 50 \text{ mm}^3$, the treatment was started with the animals being divided into two groups of nine mice each, as follows: group 1 (control); group 2 (JV-1-38). Dosage and treatment schedule corresponded to experiment V. Experiment VI was terminated on day 45.

In all experiments, body weights and tumor volumes were monitored weekly (6–9, 16). Tumor growth inhibition was calculated by using the formula: $100\% \times (\text{TVol}_{\text{control}} - \text{TVol}_{\text{treated}}) / \text{TVol}_{\text{control}}$, where TVol = final tumor volume – initial tumor volume. The mice were killed under isoflurane anesthesia, and the tumors were excised, cleaned, weighed, snap frozen, and stored at -70°C . In experiment I, blood was collected from the tail vein at weekly intervals. In all other experiments, trunk blood was collected from the abdominal aorta at the end of the observation period. Serum was separated for quantitative measurement of total PSA. Liver, heart, lungs, kidneys, spleen, and reproductive organs were carefully removed under a dissecting microscope.

mRNA Isolation and RT-PCR. The methods of isolation of mRNA and RT-PCR for the analysis of mRNA expression of VEGF-A, IGF-I, IGF-II, and GHRH receptor SVs were described (8, 10, 11).

Western Blots for IGF-I, IGF-II, and VEGF-A. Tumors were homogenized in ice-cold extraction buffer (50 mM Tris-HCl (pH 7.6), containing 1% Triton X-100, 200 mM NaCl, and 10 mM CaCl_2). Chilled samples were stirred for 30 min, and the supernatant was obtained by centrifugation ($12,000 \times g$, 20 min). The total fraction was frozen at -70°C until use. Immunodetection of IGF-I, IGF-II, and VEGF was performed as described (20) by using mouse monoclonal anti-human IGF-I (Diagnostic Systems Laboratories, Webster, TX), goat polyclonal anti-human IGF-II and anti-actin (Santa Cruz Biotechnology), and rabbit polyclonal anti-human VEGF (Santa Cruz Biotechnology) antibodies diluted 1:500–1:3,000. The bands were analyzed with a zoom digital camera (DC290) with EDAS 290 imaging system (Kodak), and the relative protein levels were normalized vs. the corresponding levels of actin.

PSA Measurement. Quantitative measurement of total PSA in serum was performed by using the reagents and protocol of DSL-9700 Active PSA Coated-Tube IRMA kit provided by Diagnostic Systems Laboratories as reported (15, 18).

Statistical Analysis. Data are expressed as mean $\pm$ SE. Differences between the values were evaluated with ANOVA followed by two-tailed Student's *t* test, $P < 0.05$ being considered significant.

Fig. 1. The effect of castration, GHRH antagonist JV-1-38 ($20 \mu\text{g}/\text{day}$ s.c.), or their combination on the growth of s.c. xenografted MDA-PCa-2b (A), LNCaP (B), and DU-145 (C) human prostate carcinomas in nude mice. The treatment lasted for 79 days (A), 49 days (B), or 45 days (C). Vertical bars represent standard error. *, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control; #, $P < 0.05$ vs. castration.

Results

GHRH Antagonist JV-1-38 Potentiates the Antitumor Effects of Castration in LNCaP and MDA-PCa-2b Human Androgen-Sensitive Prostate Cancers Xenografted into Nude Mice. Experiment I was designed to test whether GHRH antagonist JV-1-38 could enhance the inhibitory effect of castration on growth of MDA-PCa-2b tumors in nude mice (Fig. 1A). JV-1-38 alone did not affect tumor growth in intact mice, and the observations of this group and the controls had to be terminated on day 35 due to the tumor size. At that time, tumor growth in the group that received castration alone was inhibited by 65% ($P < 0.01$), and tumor volume doubling time was extended by 104% to 24.6 ± 1.3 days from 12.1 ± 1.3 days for the controls. Combined treatment with

JV-1-38 and castration virtually arrested tumor growth as measured on day 35 (94% inhibition, $P < 0.01$ vs. control) and extended tumor doubling time to 67.7 ± 11.7 days (460% of the control value; Fig. 1A). Combination therapy also powerfully enhanced the effects of castration (75% inhibition of tumor growth and 175% extension of tumor doubling time vs. castration group, $P < 0.05$) after 35 days of treatment (Fig. 1A). The inhibition of tumor growth in the group given the combined treatment was 53.5% on day 63 ($P < 0.05$). Tumor doubling time was extended by 44% after 63 days of treatment as compared with castration alone. The mice treated by castration alone had to be killed on day 63, when the average tumor size exceeded 1,500 mm³, whereas the group given combination therapy could be kept alive until day 79 (Fig. 1A).

In experiment III, we investigated the antiproliferative effects of JV-1-38 on androgen-sensitive LNCaP cancers xenografted into nude mice (Fig. 1B). On Day 35, castration alone inhibited growth of s.c. implanted LNCaP tumors by 47% [nonsignificant (NS)] and extended tumor doubling time by 20% (NS) as compared with controls (Fig. 1B). Because previous work showed that treatment with GHRH antagonists alone could not inhibit growth of orthotopically implanted LNCaP tumors (15), a group treated with JV-1-38 alone was not included. However, the combination of orchiectomy with JV-1-38 powerfully reduced tumor growth by 83% ($P < 0.01$ vs. control) and significantly enhanced the effect of castration (68% inhibition, $P < 0.05$ vs. castration) after 35 days of treatment (Fig. 1B). Combined treatment also prolonged tumor doubling time to 39.4 ± 10.7 days, as compared with 13.2 ± 1.3 days in the castration group and 11.0 ± 1.2 days in the control group (Fig. 1B). Nude mice in control group had to be killed on day 35 because of the tumor size, but those treated with castration or castration plus JV-1-38 could be kept alive until days 42 and 49, respectively. Treatment with JV-1-38 alone or combined with castration was well tolerated without signs of toxicity, and no differences in body and organ weights could be observed compared with the control group and to the castration group respectively.

Antagonist JV-1-38 Inhibits Growth of the Androgen-Insensitive DU-145 Prostate Cancers in Nude Mice. In experiment VI the volume of DU-145 tumors was significantly reduced by JV-1-38 after 21 days of treatment (Fig. 1C). At the end of the experiment on day 45, tumor volume was decreased by 57% ($P < 0.05$ vs. control), and tumor doubling time was extended by 35% ($P < 0.02$) to 10.6 ± 0.6 days from 7.8 ± 0.5 days in the control group (Fig. 1C). JV-1-38 was well tolerated and did not change organ or body weights compared with the control groups. Tumor growth in the LNCaP subline C4-2b (experiment V) was not affected by JV-1-38 after 28 days (data not shown).

Effects of JV-1-38 on the Expression of mRNA for VEGF, IGF-I and -II in MDA-PCa-2b, LNCaP, C4-2b, and DU-145 Prostate Cancer Lines. In experiments II, IV, V, and VI, we studied the effects of GHRH antagonist JV-1-38 on the mRNA expression of IGF-I and -II and VEGF in androgen-sensitive MDA-PCa-2b and LNCaP prostate cancers and in androgen-independent C4-2b and DU-145 prostate cancers grown in nude mice (Fig. 2). MDA-PCa-2b tumors (experiment II) showed no significant changes in tumoral mRNA expression for IGF-I after 17 days of treatment with JV-1-38 (Fig. 2A, Table 1), but castration alone and combined treatment increased it (Fig. 2B, Table 1). Therapy with JV-1-38 also decreased the expression of mRNA for IGF-II, but mRNA levels for VEGF did not change (Fig. 2A, Table 1). Castration suppressed the mRNA expression for IGF-II and for VEGF. The combined treatment augmented this inhibitory effect on mRNA expression for IGF-II and lowered the expression of mRNA for VEGF (Fig. 2B, Table 1).

In experiment IV, the mRNA expression for IGF-II and

Fig. 2. The effect of GHRH antagonist JV-1-38, castration, or combination (castration and JV-1-38) on the expression of mRNA for IGF-I (514 bp), IGF-II (538 bp), and VEGF (201 bp) in human MDA-PCa-2b (A and B), C4-2b (C), and DU-145 (D) prostate cancers grown in nude mice. Shown are RT-PCR products for human IGF-I, IGF-II, VEGF, and β -actin (internal control, 459 bp) from representative samples. Lane M is the molecular marker.

VEGF in LNCaP tumors was measured after 7 days of treatment with JV-1-38 or castration alone, or their combination. The mRNA expression for IGF-II was increased by JV-1-38, but castration or its combination with JV-1-38 did not alter it (data not shown). The mRNA expression for VEGF was enhanced by treatment with JV-1-38, castration, or combined therapy (data not shown).

In experiment V, in C4-2b tumors, JV-1-38 reduced the IGF-I mRNA levels and the mRNA expression for IGF-II and VEGF in (Fig. 2C, Table 1). In experiment VI, RT-PCR analysis of DU-145 tumors from animals treated with JV-1-38 revealed a decrease in VEGF levels, although IGF-II mRNA expression was not changed (Fig. 2D, Table 1). IGF-I mRNA expression could not be detected by RT-PCR in the DU-145 tumors.

Effects of GHRH Antagonist JV-1-38, Castration, or Their Combination on the Expression of IGF-I and -II and VEGF Proteins in MDA-PCa-2b and DU-145 Human Prostate Cancers. IGF-I and -II and VEGF were investigated by Western blotting in MDA-PCa-2b tumors treated with JV-1-38 alone, castration, or their combination (experiment II) and in DU-145 tumors treated with JV-1-38 (experiment VI). Thus, MDA-PCa-2b tumors from animals treated with castration showed a slight and nonsignificant decrease in IGF-II protein levels and an increase in IGF-I and VEGF protein levels (Fig. 3A, Table 1). JV-1-38 caused a slight decrease in IGF-II and VEGF protein levels, but IGF-I levels were not changed. Combined treatment with JV-1-38 and castration resulted in a strong inhibition of IGF-II levels and also reduced VEGF in MDA-

Table 1. Effect of GHRH antagonist JV-1-38 (20 μ g/day) on mRNA expression and protein levels of IGF-I, IGF-II, and VEGF in human prostate carcinoma lines grown in nude mice

Treatment groups	mRNA, % of control			Protein, % of control		
	IGF-I	IGF-II	VEGF-A	IGF-I	IGF-II	VEGF-A
Exp. II (MDA-PCa-2b tumors)						
Control	100.0 $\pm$ 3.7	100.0 $\pm$ 6.7	100.0 $\pm$ 4.5	100.0 $\pm$ 7.5	100.0 $\pm$ 14.4	100.0 $\pm$ 0.8
JV-1-38	104.6 $\pm$ 3.4	78.9 $\pm$ 4.8*	99.0 $\pm$ 9.1	103.7 $\pm$ 15.4	70.9 $\pm$ 6.1	90.8 $\pm$ 9.3
Castration	120.8 $\pm$ 2.6**	50.0 $\pm$ 3.2**	77.5 $\pm$ 8.1*	109.2 $\pm$ 3.2	72.0 $\pm$ 13.0	112.0 $\pm$ 12.8
Combination (castration and JV-1-38)	119.3 $\pm$ 4.4*	32.5 $\pm$ 6.0**,***	66.8 $\pm$ 8.9**	95.3 $\pm$ 2.8	36.8 $\pm$ 4.5**	62.5 $\pm$ 10.3
Exp. V (C4-2b tumors)						
Control	100.0 $\pm$ 7.3	100.0 $\pm$ 11.3	100.0 $\pm$ 7.30	NA	NA	NA
JV-1-38	49.0 $\pm$ 6.7**	30.8 $\pm$ 1.0**	46.1 $\pm$ 13.4**			
Exp. VI (DU-145 tumors)						
Control	ND	100.0 $\pm$ 22.6	100.0 $\pm$ 7.3	100.0 $\pm$ 13.4	100.0 $\pm$ 22.5	100.0 $\pm$ 23.0
JV-1-38	ND	104.0 $\pm$ 8.7	68.2 $\pm$ 7.1	68.06 $\pm$ 10.8	126.7 $\pm$ 7.3	44.1 $\pm$ 2.3*

mRNA levels were normalized to β -actin mRNA, whereas protein levels were normalized to actin protein and are expressed as percentages of control values. The data are the mean of at least three independent experiments. *, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control; ***, $P < 0.05$ vs. castration; NA, not available; ND, not detectable.

PCa-2b cancers (Fig. 3A, Table 1). In DU-145 tumors, IGF-I and VEGF protein levels were inhibited after treatment with JV-1-38 compared with the control group (Fig. 3B, Table 1), but IGF-II levels were not changed (Fig. 3B, Table 1).

The Expression of mRNA for GHRH Receptor SVs in C4-2b and DU-145 Tumors. RT-PCR analyses of cDNA isolated from C4-2b and DU-145 tumor tissue, using primers specific for the splice variants of tumoral human GHRH receptors, yielded two products of 720 and 566 bp in C4-2b cancers and one band of 720 bp in DU-145 tumors (Fig. 4). The 720-bp band corresponds to SV₁ and the 566-bp product to SV₂ isoforms of GHRH receptors, previously described in LNCaP, MDA-PCa-2b, and PC-3 prostate cancers (10, 11). Splice variants SV₃ and SV₄ previously found in normal prostate tissue (10) could not be detected in the C4-2b and DU-145 tumors.

Effects of GHRH Antagonist on PSA Secretion from LNCaP, MDA-PCa-2b, and C4-2b Tumors *in Vivo*. In experiment I in MDA-PCa-2b tumors, serum PSA levels of sham operated animals (controls) rose from the initial value of 4.6 ± 2.5 to 43.8 ± 5.5 ng/ml serum by day 35 (Fig. 5). Treatment with JV-1-38 did not change PSA levels compared with controls, but on day 35 castration reduced

them by 83% to 7.8 ± 1.2 ng/ml ($P < 0.01$). Combined treatment (castration and JV-1-38) decreased PSA by 89% to 5.0 ± 1.2 ng/ml ($P < 0.01$; Fig. 5). The PSA secretion index was the same in control animals and those treated with JV-1-38 (0.026 ng PSA/ml serum per mm³ tumor volume). Castration and combined treatment decreased the PSA secretion index to 0.013 and 0.017, respectively. Castration of nude mice bearing LNCaP tumors (experiment III) reduced serum PSA levels from 463.6 ± 90.7 ng/ml for control to 280.4 ± 66 ng/ml, a 33% decrease (NS). Combined treatment (castration and JV-1-38) caused a 76% reduction in PSA levels to 113.0 ± 29.6 ng/ml ($P < 0.01$). However, the PSA secretion index did not differ significantly between the groups, being 0.21 for the controls and 0.22 for both treated groups. The PSA secretion index in experiment V (C4-2b tumors) was 0.45 in the control group and 0.44 in mice treated with JV-1-38 (NS).

Discussion

Since the discovery of ectopic GHRH production and the demonstration of the presence of biologically or immunologically active GHRH and its mRNA in various malignant tumors, much evidence has accumulated supporting the involvement of GHRH and its receptors in the pathophysiology of human cancers (reviewed in refs. 2 and 5). Our initial rationale for the use of GHRH antagonists in the treatment of experimental human cancers was based on the assumption that the blockade of the pituitary GH/hepatic IGF-I axis might inhibit the growth of IGF-I-dependent cancers such as osteosarcomas xenografted into nude mice (2, 5, 21). However, various subsequent studies revealed that the inhibitory effects of GHRH antagonists on tumor growth may be also produced by suppression of tumoral IGF-I/IGF-II levels or interference with local GHRH (2, 5–9). Such effects were thought to be exerted directly through GHRH receptors on tumors (5, 10). The isolation and sequencing of

Fig. 3. Effect of JV-1-38, castration, or their combination on protein levels of IGF-I (16.1 kDa), IGF-II (12.3 kDa), and VEGF (29.6 kDa) in MDA-PCa-2b tumors (A) and effect of JV-1-38 on the IGF-I, IGF-II, and VEGF protein levels in DU-145 tumors grown in nude mice (B). Actin (62 kDa) was used as internal control. Three representative samples of MDA-PCa-2b tumors and five DU-145 tumors from each group are shown.

Fig. 4. The expression of mRNA for SV of GHRH receptors in C4-2b (lanes 1 and 2) and DU-145 (lane 3) human prostate cancers grown in nude mice, as revealed by RT-PCR. LNCaP (lane 4) was used as positive control. M is the molecular marker.

Fig. 5. The effect of castration, GHRH antagonist JV-1-38, or their combination on the serum PSA levels in nude mice bearing MDA-PCa-2b human androgen-sensitive prostate cancers. Vertical bars represent standard error. *, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control.

cDNAs encoding tumoral GHRH receptors showed that they were SVs of the pituitary GHRH receptors (10). Previously, we demonstrated the mRNA expression of SV of GHRH receptors in primary specimens of human prostate cancers (13), as well as in LNCaP, MDA-PCa-2b, and PC-3 prostate cancers grown in nude mice (11). This study shows the presence of the SV receptor in the LNCaP subline C4-2b as well as in DU-145 tumors.

Human androgen-sensitive LNCaP and MDA-PCa-2b prostate cancer cell lines have retained functional differentiation, show common features of clinical prostate cancer (androgen sensitivity and PSA expression; ref. 22), and express a mutated androgen receptor (AR; missense mutation T877A), which was also found in surgical specimens of prostate cancer patients (23, 24). The LNCaP subline C4-2b expresses both PSA and AR, but is unresponsive to androgens (25). The androgen-independent prostate cancer cell line DU-145 represents one of the most advanced forms of prostate cancer (22). The GHRH antagonist used in this study, JV-1-38, belongs to a recent class of GHRH antagonists (14) with a higher and more protracted activity than the antagonists MZ-4-71 or MZ-5-156 previously investigated in PC-3, DU-145, and other human cancer models in nude mice (2, 5–7). The present study demonstrates that GHRH antagonist JV-1-38 enhances powerfully the effects of surgical castration and delays the time of relapse after androgen ablation in LNCaP and MDA-PCa-2b tumors. Nevertheless, JV-1-38 alone cannot inhibit the growth of these androgen-sensitive tumors. Our findings are in accordance with previous studies on orthotopically implanted LNCaP tumors in nude mice, where JV-1-38 inhibited tumor growth only in combination with castration (15). However, JV-1-38 alone could induce a strong inhibition of human androgen-independent DU-145 tumors. Previously, we also demonstrated that the growth of androgen-independent PC-3 prostate cancers was inhibited by various GHRH antagonists (2, 5, 8). Thus, different cell signaling mechanisms seem to operate in androgen-independent and androgen-dependent prostate cancers (26).

Much evidence exists that mechanisms such as androgen receptor mutation or the activation of intracellular proliferation cascades evoked by growth factors such as IGF-I, IGF-II, or epidermal growth factor could be involved in the progression of prostate cancer toward androgen independence (2, 3, 27). The inhibition of IGF-dependent or GHRH-dependent mitogenic loops by GHRH antagonists could delay or prevent the activation of some of these pathways. In previous investigations,

GHRH antagonist MZ-4-71 significantly decreased tumor growth, the tumoral concentrations of IGF-I and -II, and serum levels of GH and IGF-I in nude mice bearing PC-3 and DU-145 tumors (16). Subsequent studies in androgen-independent prostate cancers PC-3 and DU-145 *in vivo* and *in vitro* revealed a direct inhibition of tumoral mRNA expression for IGF-I and -II by GHRH antagonists MZ-4-71 and MZ-5-156 (6–8, 28).

There is a lack of consensus regarding the influence of IGF-I on the etiology of prostate cancer (29–34). Chan *et al.* (32) reported an association between serum IGF-I levels and the risk of prostate cancer. However, in other studies, a direct correlation between prostate cancer and serum IGF-I could not be found (33, 34). IGF-II also plays a critical role in the autocrine and paracrine stimulation of prostatic neoplasms. IGF-II is involved in the autocrine control of proliferation of PC-3 and DU-145 androgen-independent prostate cancer cells (31, 35). Furthermore, Tennant *et al.* (36) reported that the expression of IGF-II mRNA in prostate cancer specimens was 30% higher than in benign prostatic epithelium. In other clinical studies, a significant correlation was found between elevated IGF-II levels and clinicopathological parameters such as Gleason score and extraprostatic invasion (37). Nickerson *et al.* (30) also found an up-regulation of mRNA levels for IGF-I and IGF-II in androgen-dependent LNCaP prostate cancers during progression to androgen-independent growth.

Our work shows that GHRH antagonist JV-1-38 can interfere with the synthesis of IGFs in androgen-sensitive and -insensitive prostate cancers xenografted into nude mice. Thus, JV-1-38 alone inhibited IGF-I levels in DU-145 and C4-2b prostate cancers, but did not suppress mRNA and protein levels of IGF-I in MDA-PCa-2b and LNCaP tumors. Treatment with JV-1-38 alone also decreased IGF-II levels in MDA-PCa-2b and C4-2b tumors, although no such suppression was observed in DU-145 and LNCaP prostate cancers. Moreover, JV-1-38 enhanced the inhibitory effects of castration on IGF-II protein and mRNA expression in the MDA-PCa-2b tumor model.

Previous work (8) presented evidence that GHRH antagonists can also alter the expression of VEGF, which is thought to be one of the critical factors in angiogenesis. Plasma VEGF concentrations in patients with metastatic prostate cancer are higher than in those with localized disease (38, 39), and autocrine stimulation involving VEGF and its receptor Flt-1 can be associated with a malignant phenotype (4). Stewart *et al.* (40) provided evidence that androgen deprivation therapy decreases VEGF levels and counteracts the ability of tumors to form blood vessels. The ability of castration to reduce VEGF levels is lost in advanced prostate cancer (41). In our study on the MDA-PCa-2b model, the tumor inhibition observed with the combined therapy based on castration and GHRH antagonist could be due in part to the inhibition of VEGF synthesis, which was observed both at the mRNA and protein levels. However, an inhibition of mRNA expression of VEGF did not occur in LNCaP tumors after 7 days of *in vivo* treatment with JV-1-38, castration, or their combination. Therapy with JV-1-38 alone in androgen-independent C4-2b and DU-145 tumors resulted in a suppression of VEGF synthesis, similar to that observed in previous studies in PC-3 tumors (8).

The production of PSA by LNCaP, MDA-PCa-2b, and C4-2b tumors allowed us to investigate the influence of therapy with GHRH-antagonist on PSA serum levels. PSA is considered the most useful clinical tool available for the diagnosis and staging of prostate cancer (42). Rekasi *et al.* (15) found a decrease in the PSA secretion index in an orthotopic LNCaP model after treatment with GHRH antagonists. However, the present work shows no changes of PSA secretion index after treatment with JV-1-38 in LNCaP, C4-2b, and MDA-PCa-2b prostate cancer models. These findings are of clinical importance, considering

that PSA levels are needed to evaluate the therapeutic response to treatment with GHRH antagonists.

The results of our study extend the existing findings on the inhibitory effects of GHRH antagonists on growth of prostate cancers and point to some of the possible mechanisms involved in the inhibitory action. GHRH antagonists inhibit tumor growth by a direct action apparently exerted through tumoral SV receptors for GHRH, resulting in a suppression of the autocrine/paracrine production of IGF-I, IGF-II, and VEGF. The interference with the action of tumoral GHRH also may be involved, although it was not investigated in this study. In earlier stages of prostate cancer, when tumor cells do not exclusively depend on the stimulation by growth factors, the treatment with GHRH antagonist alone cannot counteract the strong mitogenic effects of androgens. After castration, when the tumor cells acquire different mitogenic signals (26), GHRH antagonists strongly potentiate the effects of androgen ablation on tumor growth. In MDA-PCa-2b model, we found the suppression of the synthesis of tumoral IGF-I, IGF-II, and VEGF and a delay in the time to relapse. However the lack of inhibition of IGF-II and VEGF expression in the LNCaP model indicates that other unidentified mechanisms may contribute to the antiproliferative effects of JV-1-38. Androgen-independent prostate cancers are more dependent on autocrine or paracrine stimulation by growth factors (reviewed in ref. 26) and thus more susceptible to inhibition by GHRH antagonists. PSA secretion is not directly affected by GHRH antagonists, but its suppression reflects the inhibitory effect of GHRH antagonists on tumor growth.

In conclusion, this work demonstrates that, in androgen-dependent prostate cancers, GHRH antagonist JV-1-38 strongly

potentiates the effects of androgen deprivation and prolongs the time to relapse. In androgen-independent cancers, which no longer respond to androgen deprivation therapy, GHRH antagonists alone can suppress tumor growth. This inhibitory effect of GHRH antagonists is likely to be mediated by isoforms of GHRH receptors on prostate cancers. The possible mechanisms of action of GHRH antagonists include an interference with growth factors such as IGF-I, IGF-II, and VEGF. Our findings support the rationale for the clinical use of GHRH antagonists to enhance and extend the effects of androgen deprivation in management of prostate cancer and to delay the relapse. Thus, GHRH antagonists could be used together with LH-RH agonists or castration as the primary therapy of androgen-dependent prostate carcinoma as well as for the treatment of relapsed androgen-independent prostate cancer. Collectively, our work suggests that GHRH antagonists might provide new approaches to therapy of patients with androgen-dependent and -independent prostatic carcinomas.

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