Inhibitory Effect of Antagonists of Bombesin and Growth Hormone-Releasing Hormone on Orthotopic and Intraosseous Growth and Invasiveness of PC-3 Human Prostate Cancer in Nude Mice

Anton Stangelberger,1,2 Andrew V. Schally,1,2 Jozsef L. Varga,1,2 Marta Zaranidi,1,2 Karoly Szepeshazi,1,2 Patricia Armatis,1,2 and Gabor Halmos1,2

1Endocrine, Polypeptide and Cancer Institute, Veterans Affairs Medical Center and 2Section of Experimental Medicine, Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana

ABSTRACT

Purpose: To determine whether antagonists of growth hormone-releasing peptide (GHRH) and bombesin/gastrin-releasing peptide (BN/GRP) can inhibit the orthotopic and metastatic growth of PC-3 human androgen-independent prostate cancers.

Experimental Design: The effects of administration of GHRH antagonist MZ-J-7-118, BN/GRP antagonist RC-3940-II, and their combination on the growth and metastatic spread of PC-3 tumors implanted orthotopically into nude mice were evaluated. The efficacy of this treatment on PC-3 tumors implanted intratibially and s.c. was also determined.

Results: Treatment with MZ-J-7-118, RC-3940-II, or their combination significantly inhibited the growth of PC-3 tumors implanted orthotopically, intraosseously, and s.c. The combination of the two antagonists had the greatest effect, inhibiting orthotopic tumor growth by 77%, intraosseously implanted tumors by 86%, and s.c. tumors by 86%. The therapy with BN/GRP and GHRH antagonists, especially in combination, also reduced the local tumor spread and distant metastases in animals bearing orthotopic tumors. Combination therapy was likewise the most effective in reducing the incidence and severity of tibial osteolytic lesions and pathologic fractures in intraosseously implanted tumors. High-affinity binding sites for BN/GRP and GHRH were found in s.c. and orthotopic PC-3 tumor samples, MZ-J-7-118, RC-3940-II, and the combination of both compounds inhibited in vitro growth of PC-3 cells.

Conclusions: Our findings show the efficacy of BN/GRP antagonists and GHRH antagonists for the treatment of advanced prostate cancer in preclinical metastatic models. As BN/GRP antagonists are already in clinical trials and GHRH antagonists are effective in androgen-independent prostate cancer models, these analogues could be considered for the management of advanced prostate carcinoma.

INTRODUCTION

Prostate cancer is the most common noncutaneous malignant tumor and the second leading cause of cancer-related deaths among men in the Western world (1). About 70% of all newly diagnosed prostate cancers occur in men over age 65 years (2). Many patients with organ-confined disease can be cured by radical prostatectomy or radiation therapy, but ~30% of prostate cancer patients who undergo radical prostatectomy show a recurrence of prostate-specific antigen elevation and some will ultimately develop disseminated disease (3). Bone is the most common site of metastasis, other than lymph nodes, in prostate cancer patients (4, 5). Androgen deprivation, achieved by treatment with luteinizing hormone-releasing hormone agonists alone or in combination with anti-androgens, induces a remission in 80% to 90% of men with advanced prostate cancer, but a relapse is observed after a median time of 12 to 33 months (6).

To date, no efficacious therapy exists for patients who relapse the first-line hormonal treatment based on androgen deprivation. Most chemotherapeutic regimens provide essentially only a minor benefit with respect to survival or quality of life, although new chemotherapy combinations may provide a survival advantage (7, 8). Consequently, the present methods of treatment of relapsed prostate cancer must be improved.

New therapeutic approaches are being developed based on recent advances in the understanding of the role of neuropeptides and growth factors in the progression of prostate cancer (9–13). Bombesin/gastrin-releasing peptides (BN/GRP) are produced in prostatic, breast, and pancreatic cancers and small cell lung carcinoma and act as autocrine/paracrine growth factors (10). It has been proposed that the secretion of BN/GRP by neuroendocrine cells might be responsible for prostate cancer progression, androgen independence, and poor prognosis (12). BN/GRP was also shown to increase the proliferation and invasiveness of androgen-independent prostate cancer (14, 15). Specific binding sites for BN/GRP are present in androgen-independent cell lines (16) and in surgical specimens of prostate cancer (17, 18). BN/GRP antagonists, such as RC-3095 or RC-3940-II, were developed for therapeutic use, in view of the tumor stimulatory effect of BN/GRP and the high incidence of their receptors in various human cancers (11, 17, 19). These compounds were shown to inhibit various experimental BN/GRP receptor-positive cancers, including that of prostate, and are currently undergoing clinical trials (9–11, 20). Antiproliferative effects of BN/GRP
antagonists are thought to be mediated by the blockade of mitogenic stimuli of BN/GRP and the down-regulation of receptors for BN/GRP and epidermal growth factor (9, 11, 20).

Various potent antagonistic analogues of growth hormone-releasing hormone (GHRH) were also synthesized (21) for clinical applications in the field of cancers dependent on GHRH and insulin-like growth factor (IGF-I and -II. It was shown that GHRH antagonists inhibit a large variety of human tumors xenografted into nude mice, including androgen-independent prostate cancers (9–11, 20–22). The effects of GHRH antagonists are in part exerted indirectly through the inhibition of the secretion of pituitary growth hormone and the resulting reduction in levels of serum IGF-I, which is mainly of hepatic origin (21). However, recent evidence indicates that direct inhibitory effects of GHRH antagonists on the cancers are even more important for tumor growth inhibition than IGF-I suppression (11, 23). GHRH is produced by various human tumors, including prostate cancer, and seems to exert an autocrine/paracrine stimulatory effect on tumors (11, 23–26). The expression of GHRH and truncated splice variants of GHRH receptors was shown in surgical specimens from patients with locally advanced prostate cancer (26) and in LNCap, MDA-PCa-2h, C4-2h, PC-3, and DU-145 human prostate cancer lines (22, 24, 25, 27). GHRH antagonists seem to act through splice variant receptors and decrease synthesis of IGF-I, IGF-II, and vascular endothelial growth factor (9, 11, 20, 22, 23, 28, 29).

Because metastases constitute the main cause of mortality in prostate cancer patients, it is essential to develop treatment regimens that inhibit effectively the growth of metastatic prostate cancer. Nude mouse models are useful for the investigation of the metastatic behavior and intraosseous growth as well as for the assessment of the effects of antimetastatic therapies (5, 30–32). GHRH antagonists have been shown to inhibit the orthotopic growth and metastatic behavior of CAKI-1 human renal cell carcinoma and MDA-MB-435 human breast cancer models in nude mice (28, 32).

This study was done to investigate the effect of antagonists of BN/GRP and GHRH on the growth and metastatic spread of human androgen-independent PC-3 prostate cancer xenografted s.c., orthotopically, and intraosseously into nude mice.

MATERIALS AND METHODS

Peptides and Reagents. GHRH antagonist MZ-J-7-118 was synthesized in our laboratory by solid-phase methods (33), its chemical structure being [CH3-(CH2)6-CO-Tyr1, d-Arg2, Phe(4-Cl)6, Ala8, His9, Tyr(ET)10, His11, Abu15, Nle17, d-Arg18, Har19]hGHRH(1-29)NH2, where Phe(4-Cl) is 4-chlorophenylalanine, Abu is α-aminobutyric acid, Nle is norleucine, and Har is homogargin. MZ-J-7-118 belongs to the class of d-Arg2-substituted antagonistic analogues of human (h) GHRH(1-29)NH2 as the earlier GHRH antagonists such as MZ-4-71, MZ-5-156, JV-1-36, and JV-1-38 used in previous oncolgic and endocrinologic studies (9–11, 20–24, 28–29, 32, 33). Other amino acid replacements as well as the N-acyl moiety are aimed to enhance the potency of MZ-J-7-118 compared with earlier antagonists. The BN/GRP antagonist RC-3940-II, with the structure of [Hca6,Leu13][CH2(N)-Tac14]BN(6-14) based on the sequence of the bombesin and containing a reduced peptide bond at its COOH terminus, was also made in our laboratory as described (ref. 19; Hca is desamino-phenylalanine and Tac is thiazolidine-4-carboxylic acid). For daily injection, the compounds were dissolved in 0.1% DMSO in 10% aqueous propylene glycol solution.

Cell Line and Animals. The PC-3 human androgen-independent prostate cancer cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in culture as described (16).

Male athymic (Ncr nu/nu) nude mice, ~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-hour light/12-hour dark schedule and fed autoclaved standard chow and water ad libitum.

All experiments were done in accordance with institutional guidelines of animal care.

In vivo Studies. Experiment 1: Orthotopic Tumor Growth. Nude mice were implanted orthotopically with PC-3 tumors to observe the orthotopic growth and development of metastases. Tumor tissue, harvested aseptically from donor animals after 10 weeks of s.c. growth, was cleaned of connective tissue and necrotic parts, weighed, mechanically minced, suspended in RPMI 1640, and pushed through a medium mesh tissue sieve. The tissue suspension was centrifuged for 10 minutes at 1,000 rpm; the supernatants were then removed and the pellets were resuspended in RPMI 1640 at a final concentration of 500 mg tumor tissue/mL. Each mouse received 0.02 mL of the suspension (corresponding to 10 mg of tumor tissue) injected orthotopically with a 25-gauge needle. After 1 week, animals were randomly assigned into the following groups: control 11 mice, GHRH antagonist MZ-J-7-118 (5 μg/d) 8 mice, BN/GRP antagonist RC-3940-II (10 μg/d) 8 mice, and combination of MZ-J-7-118 (5 μg/d) and RC-3940-II (10 μg/d) 8 mice. Antagonists of GHRH and BN/GRP were given by daily s.c. injections. Controls received 100 μL/d of s.c. injected vehicle solution containing 0.1% DMSO and 10% propylene glycol in water. Treatment was started 1 week after the implantation of orthotopic tumors and lasted for 28 days.

After both the first and second weeks, one animal from the control group was sacrificed by cutting the abdominal aorta under isoflurane (Abbott Laboratories, Chicago, IL) anesthesia and investigated for tumor growth and visible metastasis. The experiment was ended on day 28 when three control animals died and others became moribund. Tumors or tumor compartments of all animals were carefully excised, weighed, and snap frozen for further investigations. All mice were investigated for presence and spread of tumors, with special attention to those organs that are afflicted by prostate cancer metastases, such as bone, liver, lungs, intestines, kidneys, and lymph nodes, as well as to occurrence of carcinosis peritonei. Liver, heart, lungs, kidneys, spleen, testicles, prostate, and seminal vesicles were carefully removed and weighed. Samples of metastases were checked histologically to prove the origin of prostate cancer.

Experiment 2: Intraosseous and s.c. Tumor Growth. PC-3 cells (5 × 105) from cell culture suspended in PBS were injected with a 25-gauge needle into the medulla of the right tibia of 40 nude mice. At the same time, PC-3 tumor pieces of ~1 mm3 were implanted s.c. into the animals by trocar injection.
Two weeks after tumor injection, when the s.c. tumors measured ~50 mm³, the animals were divided randomly into three different treatment groups and one control group. Each group contained eight animals. Animals in treatment groups received GHRH antagonist MZ-J-7-118 (5 µg/d), BN/GRP antagonist RC-3940-II (10 µg/d), or a combination of the two agents. Antagonists of GHRH and BN/GRP were given by daily s.c. injections. Controls received 100 µL/d of s.c. injected vehicle solution containing 0.1% DMSO and 10% propylene glycol in water. Treatment was started 2 weeks after the implantation of intraosseous and s.c. tumors. S.c. tumor volumes and body weights were recorded weekly. The experiment was ended when the animals became visibly moribund and the size of the tumor in the right limb caused problems in walking.

The animals were sacrificed on day 30 and investigated for tumor growth, and a complete necropsy was done. Hind limbs with macroscopically visible tumors were cut off at the proximal third of the femur, measured, and photographed. Subsequently, the tumor-bearing legs were imaged by a RMX-625 X-ray Unit (Raytheon Medical Systems, Melrose Park, IL). S.c. tumors were excised, weighed, snap frozen, and stored at −70°C for receptor analysis.

**Histologic Investigation.** Legs with intraosseous tumors as well as macroscopically altered tissues of bone and visceral organs were fixed in 10% neutral phosphate-buffered formalin and investigated histologically to evaluate tumor infiltration. Legs were cross-sectioned at two to three sites, and bones were decalcified overnight in Cal-EX Decalcifying Solution (Fisher Scientific, Pittsburgh, PA). The specimens were embedded in Tissue Path Paraplast X-tra embedding medium (Fisher Scientific, Pittsburgh, PA). S.c. tumors were excised, weighed, snap frozen, and stored at −70°C for receptor analysis.

**Receptor Binding Assay for BN/GRP and GHRH Receptors.** Binding characteristics of BN/GRP and GHRH receptors on membrane preparations from orthotopic and s.c. PC-3 tumors from the control group were determined by ligand competition assays using radiolabeled Tyr⁴-bombesin and GHRH antagonist JV-1-42. The preparation of tumor membrane fractions and receptor binding studies was described previously (17, 26, 28).

Receptor binding affinity (IC₅₀) of GHRH antagonist MZ-J-7-118 to membranes of s.c. grown PC-3 tumors from the control group was also measured in displacement experiments based on the competitive inhibition of [¹²⁵I]JV-1-42 binding by various concentrations (10⁻¹² to 10⁻⁶ mol/L) of MZ-J-7-118 as described (28). IC₅₀ is defined as the dose of MZ-J-7-118 causing 50% inhibition of the specific binding of [¹²⁵I]JV-1-42.

**In vitro Proliferation Assay.** PC-3 prostatic carcinoma cells were seeded into 96-well micro plates (Falcon, Becton Dickinson and Co., Lincoln Park, NJ). After a recovery period of 24 hours, the culture medium was removed and replaced with serum-free medium, composed of DMEM/F-12 Ham medium (1:1), 0.4% bovine serum albumin, and 1 mmol/L pyruvate, containing the test compounds dissolved in 0.1% DMSO. The test compounds included GHRH antagonist MZ-J-7-118, BN/GRP antagonist RC-3940-II, and their combination. Each treatment was done in octuplicate wells and the experiments were repeated three to four times.

**Crystal Violet Assay.** After 93 hours, the medium was removed and 1% glutaraldehyde was added for 15 minutes to fix the cells. The glutaraldehyde was then removed, replaced with PBS, and kept at 4°C until staining. The PBS was then decanted from the plates, replaced with an aqueous solution of crystal violet (0.02% Sigma, St. Louis, MO), and incubated for 30 minutes. After the plates were decanted and washed to remove the noninternalized stain, the crystal violet was extracted with 70% ethanol. The absorbance at 600 nm of each well was measured using a Beckman Coulter, Inc. (Palo Alto, CA) plate reader. Results were calculated as %T/C, where T is A₆₀₀ nm of treated cultures and C is A₆₀₀ nm of control cultures (medium plus vehicle).

**Statistical Analysis.** Data are expressed as mean ± SE. Differences between values were evaluated by one-way ANOVA followed by Fisher-LSD multiple comparison test, P < 0.05 being considered significant.

**RESULTS**

**Experiment 1: Tumor Progression and Survival of Animals Bearing Orthotopic PC-3 Tumor Xenografts.** At the end of both the first and second weeks of the experiment, one animal in the control group was sacrificed to observe the stages of tumor progression. Orthotopic PC-3 tumors grew very aggressively. Thus, after the first week (2 weeks following tumor implantation), a large tumor weighing 1,087 mg, with a volume of 511.82 mm³, was found in a control animal with abdominal propagation and lymph node metastases. At present, no symptoms of distress could be observed. The tumor formed a solid compartment, which involved the prostate, bladder, seminal vesicles, and local lymph nodes as well as parts of the ileum in contact with the primary tumor. After the second week (3 weeks after tumor implantation), metastatic lymph nodes in the para-aortal area and tumor spread to the colon were found. After 3 weeks, the animals of the control group started to lose body weight. One animal that died at that time had a large primary tumor weighing 1,754 mg, with a volume of 923.63 mm³, and had metastatic spread to the para-aortal lymph nodes, seminal vesicles, colon, and bladder. The experiment was ended after 4 weeks of treatment because of the increasing number of moribund animals especially in the control group. In total, two animals were sacrificed and three died in the control group. However, only one animal each died in groups treated with single agents and none in the group receiving combination therapy with BN/GRP and GHRH antagonist.

The body weights of some animals decreased by >20% by the end of the experiment probably due to the advanced tumor growth, this effect being most marked in the control group (Table 1). However, the final mean body weights of the treated groups were not significantly different from the control group.

**Inhibition of Orthotopic Tumor Growth and Metastases by GHRH and BN/GRP Antagonists.** In mice treated with RC-3940-II, MZ-J-7-118, and their combination, there was a significant decrease in tumor volume and tumor weight at the end of the experiment compared with controls (Table 1). The greatest inhibition of tumor growth was in the animals that received a combination therapy. The tumor burden was also significantly decreased in all treatment groups, the combination therapy again producing the strongest effect (Table 1).
Table 1  Effect of BN/GRP antagonist RC-3940-II, GHRH antagonist MZ-J-7-118, and their combination on tumor growth, body weight, and survival of nude mice bearing orthotopic PC-3 androgen-independent prostate cancer

<table>
<thead>
<tr>
<th>Treatment groups and the number of evaluable animals</th>
<th>Final tumor volume, mm³ (± % inhibition)</th>
<th>Tumor weight, mg (% inhibition)</th>
<th>Tumor burden, mg/g body weight (% inhibition)</th>
<th>No. (%) of mice with &gt;20% decrease in body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)*</td>
<td>631.64 ± 157.34</td>
<td>1,046.50 ± 220.51</td>
<td>45.88 ± 10.77</td>
<td>4 (67)</td>
</tr>
<tr>
<td>RC-3940-II (n = 7)</td>
<td>228.33 ± 77.13</td>
<td>354.63 ± 113.28 (66)</td>
<td>14.90 ± 5.67 (68)</td>
<td>0</td>
</tr>
<tr>
<td>MZ-J-7-118 (n = 7)</td>
<td>200.88 ± 82.51</td>
<td>321.4 ± 132.09 (69)</td>
<td>18.90 ± 7.15 (59)</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Combination of both analogues (n = 8)</td>
<td>144.25 ± 50.91</td>
<td>305.96 ± 103.56 (71)</td>
<td>11.49 ± 3.82 (75)</td>
<td>1 (12.5)</td>
</tr>
</tbody>
</table>

*The control group originally had 11 animals, but one animal was sacrificed after both the first and second weeks. In addition, 1 animal died in week 3 and 2 died in week 4.

These analogues also strongly inhibited the prostate cancer metastases. The incidence and size of metastases were investigated at the time of necropsy. Treatment with GHRH and BN/GRP antagonist did not affect the number of local metastases but reduced their sizes. The incidence and size of distant metastases were remarkably reduced by all treatments. Figure 1 shows a representative animal from the control group with extensive tumor invasion and metastases in the lower abdominal organs. Animals that received treatment had visibly less intra-abdominal spread of the tumor. The animals from the control group showed the most widespread metastases in number and size (Table 2). All six control mice with metastatic lesions had macroscopically enlarged regional and distant lymph nodes, mainly para-aortal or axillar, and metastatic lesions on the intestines. Two mice had kidney metastases. The metastases in the treated groups were much smaller and occurred less frequently. In the group treated with BN/GRP antagonist, distant lymph node metastases in 4 of 7 (57%) animals and intestinal metastases in 3 of 7 (43%) animals were found. In the group that received GHRH antagonist, the tumors spread to distant lymph nodes in 5 of 7 (71%) mice but to intestines in only 2 (29%) animals. In the group treated with the combination of GHRH and BN/GRP antagonist, 3 of 8 (38%) animals had metastases in distant lymph nodes and 1 animal had metastatic involvement of the ileum. The primary tumors and metastases in the combination group also were much smaller than in other groups, and it was possible to separate the seminal vesicles and the bladder in three animals, indicating that the grade of tumor progression to the adherent organs like seminal vesicles, bladder, or ileum was lower. In all other groups, the tumor compartment involved the prostate, bladder, seminal vesicles, and, in some cases, the adherent intestines. Histologic examination showed that, in two control animals, the tumor infiltration reached the fibrous capsule of the kidney. No metastases were found in bones, liver, lung, or spleen, demonstrating that, after 5 weeks of orthotopic growth of PC-3 tumors, only a few distant metastases occurred.

Experiment 2: Intraosseous and s.c. Implantation of PC-3 Xenografts. Growth of Tumors after Intraosseous Implantation. Two weeks after tumor implantation, we observed a swelling of the hind limbs of some animals and the treatment was started. After 3 weeks of treatment, animals in the control group became visibly moribund, lost body weight, and showed the first signs of staggering of their hind limb movement due to tumor growth.

One animal each died in the control, BN/GRP, and GHRH antagonist groups during the last week of treatment, but no deaths occurred among animals that received combined treatment. At the end of the experiment, when most of the limb tumors were macroscopically visible (Fig. 2C and D), the volumes of tumor-bearing hind limbs in treated groups were significantly smaller compared with controls. The final tumor volumes were as follows: 576.03 ± 113.97 mm³ in the control group, 221.27 ± 99.70 mm³ in the group treated with RC-3940-II (P < 0.05), 290.02 ± 90.44 mm³ in the group that received MZ-J-7-118 (P < 0.05), and 86.81 ± 35.75 mm³ in the combination treatment group (RC-3940-II and MZ-J-7-118; P < 0.001), corresponding to a tumor inhibition of 62%, 50%, and 86%, respectively. We were not able to measure the tumor weight of intraosseously implanted PC-3 tumors because the tumor tissue could not be separated from bone and muscle tissue. The differences in weights between the limbs were not significant, because the legs bearing the tumor had a smaller amount of muscle than the intact legs.

At the end of the experiment, the right legs of the animals were investigated by X-ray and histology. Osteolytic lesions, such as partial or complete bone dissolution and pathologic fractures, were observed in all groups. However, X-ray showed that the extent of osteolytic lesions and the number of pathologic fractures were much smaller in the animals that received the combined treatment with both antagonists compared with the control group (Fig. 2A and B). Treatment with single agents also decreased the bone lesions but less strongly than the combined
treatment (data not shown). Histologic analysis showed that after intraosseous injection, in most cases, the tumor penetrated the bone cortex of the tibia and infiltrated the muscles surrounding the bones (Fig. 3A). The bones were often irregular, fragmented parts being mixed with newly formed trabecules (Fig. 3B). The tibia was completely surrounded by the tumor, with very little muscle tissue remaining especially in the control animals. In some legs, parts of the bone disappeared due to invasive osteolytic tumor growth. Some samples showed tumor cells in the lymph vessels (Fig. 3C).

**Growth of s.c. Tumors.** To compare the efficacy of our analogues for the inhibition of osseous and s.c. tumor growth, we also implanted PC-3 tumor xenografts s.c. into nude mice bearing intraosseously injected PC-3 cells. At the end of the experiment, tumor volumes and tumor weights of s.c. PC-3 tumors in treated animals were significantly smaller compared with controls (Fig. 4). Average tumor volumes and tumor weights were $1,289.62 \pm 519.36 \text{ mm}^3$ and $1,828.66 \pm 687.76 \text{ mg}$ in controls. Animals treated with RC-3940-II showed a final tumor volume of $427.33 \pm 120.91 \text{ mm}^3$ ($P < 0.05$) and a final tumor weight of $572.29 \pm 164.21 \text{ mg}$ ($P < 0.05$), corresponding to a 69% tumor inhibition. Final tumor volume and weight of the group that received MZ-J-7-118 were $565.13 \pm 123.77 \text{ mm}^3$ ($P < 0.05$) and $810.57 \pm 146.03 \text{ mg}$ ($P < 0.05$), corresponding to ~56% to 58% inhibition. The strongest antiproliferative effect was caused by combined therapy with both compounds, resulting in a final tumor volume of $224.03 \pm 102.31 \text{ mm}^3$ ($P < 0.01$; 86% inhibition) and a final tumor weight of $376.32 \pm 162.07 \text{ mg}$ ($P < 0.01$; 79% tumor suppression). Tumor volume doubling time was $7.94 \pm 2.56$ days in controls and was extended nonsignificantly by RC-3940-II, MZ-J-7-118, and their combination to $10.23 \pm 0.85$, $8.72 \pm 0.75$, and $13.20 \pm 1.28$ days, respectively.

**Binding Assay for BN/GRP and GHRH Receptors.** Specific receptors for BN/GRP and GHRH were detected on membrane fractions of control tumors. In s.c. and orthotopically grown PC-3 tumors from the control group, radiolabeled Tyr4-bombesin was bound to a single class of specific, high-affinity binding sites ($K_d = 1.33 \pm 0.11$ and $1.15 \pm 0.13 \text{ nmol/L}$, respectively), with a mean maximal binding capacity (maximum

### Table 2  Effect of BN/GRP antagonist RC-3940 II, GHRH antagonist MZ-J-7-118, and their combination on tumor spread after orthotopic implantation of PC-3 prostate cancer in nude mice: Location of the metastases and number and percentage of animals that had metastases at local and distant sites at the end of the experiment

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Total number of animals</th>
<th>Location of metastases</th>
<th>Local lymph nodes</th>
<th>Distant lymph nodes</th>
<th>Intestines</th>
<th>Prostate/seminal vesicles</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>6 (100)</td>
<td>6 (100)</td>
<td>6 (100)</td>
<td>6 (100)</td>
<td>2 (33)</td>
<td></td>
</tr>
<tr>
<td>RC-3940-II</td>
<td>7</td>
<td>6 (86)</td>
<td>4 (57)</td>
<td>3 (43)</td>
<td>7 (100)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MZ-J-7-118</td>
<td>7</td>
<td>7 (100)</td>
<td>5 (71)</td>
<td>2 (29)</td>
<td>7 (100)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Combination of both analogues</td>
<td>8</td>
<td>8 (100)</td>
<td>3 (38)</td>
<td>1 (12.5)</td>
<td>8 (100)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2  A. X-rays show tibias of nude mice of control group 6 weeks after PC-3 cell injection into the bone marrow cavity. B. X-rays of legs of nude mice after 4 weeks of treatment with a combination of BN/GRP antagonist RC-3940-II (10 \text{ mg/d}) s.c. and GHRH antagonist MZ-J-7-118 (5 \text{ mg/d}) s.c. 6 weeks after intraosseous injection of PC-3 cells. C. representative leg of a mouse from the control group 6 weeks after implantation of PC-3 cells. The tumor rapidly replaced the bone marrow cavity and invaded through the bone shaft and into the muscle adjacent to the bone. D. representative leg of an animal that received combined treatment with BN/GRP antagonist RC-3940-II and GHRH antagonist MZ-J-7-118. In most of the animals of this group, the evasion of intraosseously injected PC-3 tumor cells was markedly lower compared with control, and leg circumferences at the end of the experiment were significantly smaller than in controls.
number of binding sites) of 547.6 ± 16.3 and 481.3 ± 67.2 fmol/mg membrane protein, respectively. In s.c. and orthotopically grown PC-3 tumors from the control group, radiolabeled GHRH antagonist JV-1-42 was bound to a single class of specific, high-affinity binding sites (Kd = 0.57 ± 0.03 and 0.69 ± 0.22 nmol/L, respectively), with a mean maximal binding capacity (maximum number of binding sites) of 152.1 ± 33.5 and 128.1 ± 1 fmol/mg membrane protein, respectively. In competitive displacement experiments on s.c. grown PC-3 tumors, GHRH antagonist MZ-J-7-118 was able to displace the [125I]JV-1-42 radioligand with an IC50 of 0.15 nmol/L.

**In vitro Proliferation Studies.** To evaluate the direct antiproliferative effect of GHRH antagonist MZ-J-7-118, BN/GRP antagonist RC-3940-II, and their combination on PC-3 cells in vitro, we used the crystal violet assay. At the concentrations of 1 and 3 μmol/L and following an exposure of 71 hours, MZ-J-7-118 decreased the growth of PC-3 cells by 34% and 38%, respectively (P < 0.001), and after 118 hours by 25% and 80% (P < 0.001), respectively. RC-3940-II at a concentration of 1 and 3 μmol/L and after an exposure of 71 hours reduced in vitro PC-3 cell growth by 11% (P < 0.05) and 17% (P < 0.001), respectively. After 118 hours of exposure, RC-3940-II at 1 and 3 μmol/L decreased PC-3 cell growth by 15% and 25%, respectively (P < 0.001). Treatment with the combination of MZ-J-7-118 and RC-3940-II at equimolar doses of 1 or 3 μmol/L of each antagonist diminished PC-3 cell growth by 23% and 55%, respectively (P < 0.001) after 71 hours of exposure. After an exposure of 118 hours to both compounds at 1 or 3 μmol/L concentration, PC-3 cell growth was decreased by 30% and 78% (P < 0.001), respectively.

**DISCUSSION**

The high mortality and morbidity associated with prostate carcinoma is due mainly to the fact that most patients already have metastatic disease at the time of diagnosis. More than 75% of patients with advanced prostate cancer have bone metastases (4). Because of the limitations of currently available therapeutic modalities for the treatment of metastatic prostate cancer, it is necessary to explore novel options of therapy based on new insights into the mechanisms of metastatic tumor growth.

It has been suggested that epidermal growth factor, hepatocyte growth factor/scatter factor, IGF, and osteoblast-derived growth factors may support the proliferation of prostate cancer in the bone (34). Local production of IGF-I and II, epidermal growth factor, vascular endothelial growth factor, basic fibroblast growth factor, and keratinocyte growth factor also aberrantly stimulate the androgen receptor pathway.

Fig. 3 Histologic appearance of PC-3 human prostate cancers injected into tibiae of nude mice (H&E staining). A, low-power overview of a control tumor destroying cortical bone and propagating into muscle tissue (×120). B, bone fragments surrounded by tumor tissue from the group treated with combination of BN/GRP and GHRH antagonists. There are several osteoclasts (arrows) alongside irregular bone trabecules. In addition, a proliferation of fibroblasts and osteoblasts can be seen (×250). C, presence of cancer cells in large numbers in highly dilated lymph vessels (arrowheads) in the muscle tissue of the leg of a control mouse (×250).

![Fig 4](image-url)

**Fig 4** Effect of GHRH antagonist MZ-J-7-118 (5 μg/d) s.c., BN/GRP antagonist RC-3940-II (10 μg/d) s.c., and their combination on growth of s.c. PC-3 human androgen-independent prostate cancer in nude mice. Treatment was started when tumors had grown to 46-53 mm3 and lasted for 30 days. Bars, SE. *, P < 0.05, versus control; **, P < 0.01, versus control.
(35, 36), contributing to the androgen-independent growth of prostate cancer cells. Therefore, some strategies for the management of advanced-stage prostate cancer could be based on agents that block the production and/or effects of these local growth factors.

Antagonists of BN/GRP and GHRH suppress the growth of human experimental prostate cancers and other malignancies by mechanisms that include direct antiproliferative (cytostatic) effects on the cancer cells (16, 23) as well as the inhibition of various autocrine/paracrine and endocrine growth factors (9–11, 21). In our present study, we showed the direct cytostatic effect of BN/GRP and GHRH antagonists on PC-3 cells cultured in vitro.

Previously, we reported that antagonists of BN/GRP and GHRH block the synthesis and/or actions of locally produced growth factors, such as GHRH, BN/GRP, IGF-I, IGF-II, and vascular endothelial growth factor, and down-regulate the expression of receptors for GRP, epidermal growth factor, and IGF-I in PC-3, DU-145, and C4-2b androgen-independent as well as LNCaP and MDA-PCa-2b androgen-sensitive prostate cancer models (9–11, 20–23). In the MDA-MB-468 human breast cancer model, GHRH antagonists inhibit the expression of stromal IGF-I, which is a known paracrine growth factor for breast cancer cells (37). Thus, the ability of GHRH antagonists to alter the environment, which surrounds the malignant cells, could also contribute to their antiproliferative effect. In prostate cancer cells growing orthotopically or intraosseously, the microenvironment surrounding the cells mimics more closely the situation found in patients than in previously tested s.c. models. The ability of the microenvironment to supply the prostate cancer cells with growth factors could contribute to their favorable growth in orthotopic sites and their metastases to the bone. Some therapeutic agents that were effective in s.c. xenograft models in nude mice worked poorly in humans and vice versa (38). The lack of predictive value of the s.c. xenograft model is due mainly to the essentially nonmetastatic growth compared with naturally occurring tumors in humans, which almost invariably metastasize (38, 39). New experimental models that more accurately reflect the different stages of disease are necessary to speed up the development of new therapies for metastatic prostate cancer (39).

Consequently, one of the objectives of this study was to extend previous investigations carried out on s.c. xenografts of PC-3 prostate cancer and to test the effectiveness of BN/GRP and GHRH antagonists in orthotopic and metastatic models of this cell line. The PC-3 cell line was originally isolated from a bone metastasis of a patient with advanced prostate cancer (40). It is insensitive to androgens, does not produce prostate-specific antigen, and grows more aggressively in vivo compared with other frequently used experimental prostate cancer cell lines. Local tumors produced following s.c. injection of PC-3 cells in nude mice seldom metastasize to the bone or other organs (31). However, when PC-3 cells are inoculated orthotopically, local tumors are readily produced, which can metastasize to the lymph nodes (41–44). The orthotopic, metastatic PC-3 model has been used for testing the antimetastatic effects of various therapeutic agents (42–44).

In contrast to the osteolytic bone lesions caused by PC-3 cells injected into tibiae of nude mice, bone metastases in patients with prostate cancer are osteoblastic rather than osteolytic. Thus, PC-3 represents only one aspect of human bone metastases and may be inappropriate to represent the clinical situation of prostate cancer bone metastasis. However, the PC-3 bone model due to its bone metastatic origin and its aggressive growth has been used in previous studies to investigate bone metastatic mechanisms and therapeutic agents (30, 45). PC-3 cells injected into the tibia rapidly replace bone marrow in the bone channel and invade through the bone shaft and into the muscle adjacent to the bone (30). Thus, it was found that disodiumetidronate, an osteoclast inhibitor employed for the treatment of bone metastasis, failed to reduce the incidence, size, or number of bone metastases in a PC-3 model (45).

Because GHRH and BN/GRP antagonists are also being tested in other cancers causing osteolytic bone metastases such as breast and lung cancer, the inhibition of osteoblastic lesions in bone may be an important advance in cancer therapy.

For our initial evaluation of BN/GRP and GHRH antagonists in a bone metastatic model, we chose the PC-3 cell line, because most of our previous studies with these compounds on s.c. tumors in nude mice have been carried out on PC-3. Compared with other cell lines developed for bone metastatic models of prostate cancer such as C4-2 and MDA-PCa-2b that show osteoblastic bone lesions, PC-3 grows more aggressively, and its osteoblastic lesions in mouse tibiae can be detected by conventional X-rays. Further studies with other cell lines developed for evaluation of prostate cancer, including C4-2 or MDA-PCa-2b, will be done soon.

In our study, we found an extensive tumor growth in the hind limbs into which we injected PC-3 tumor cells. Visible tumors in the hind limbs could be observed 3 weeks after the injection. To compare simultaneously the effects of therapy on the s.c. and intraosseous tumor growth, PC-3 cancers were implanted s.c. and intraosseously into the same animals. The effect on orthotopic tumor growth and metastatic spread was observed in a separate experiment. Previously, the s.c. PC-3 model was used for the assessment of antitumor effects and mechanisms of BN/GRP-antagonists and of earlier, less potent GHRH antagonists, such as MZ-4-71, MZ-5-156, and JV-1-38 (9, 11, 20, 21, 23). The GHRH antagonist employed in the present study, MZ-J-7-118, was developed recently in our laboratory. MZ-J-7-118 shows increased antitumor potency and is used at a dose of 5 µg/d, whereas earlier antagonists MZ-4-71, MZ-5-156, and JV-1-38 were given at doses of 20 to 40 µg/d (9, 11, 20–23).

Our results show that bombesin antagonist RC-3940-II and GHRH antagonist MZ-J-7-118 exhibit a strong inhibitory effect at all three tumor sites. The combination therapy with BN/GRP and GHRH antagonist inhibited orthotopic tumor growth by 77%, hind limb tumors by 86%, and s.c. tumors by 86%. The invasion of the orthotopically implanted tumors to adjacent organs in the lower abdominal cavity was also powerfully suppressed by the analogues, especially in the animals receiving the combination treatment. The growth of intratibially injected PC-3 cells, serving as a model for established bone metastases, was likewise inhibited. Signs of osteolytic actions like pathologic fractures, or in some cases even complete dissolution of the bone structure, were observed less frequently in treated groups. The present study clearly shows that the BN/GRP and
GHRH antagonists inhibit PC-3 tumor growth at sites such as in the prostate and bone.

The receptors of GHRH and BN/GRP were investigated by binding assays in orthotopic and s.c. tumor tissue from the control animals. Our results show that both types of receptors are highly expressed and that their affinity and concentration are not significantly different in s.c. and orthotopically grown PC-3 tumors. High-affinity binding sites and the expression of mRNA for BN/GRP receptors (16, 46) and GHRH receptors (24, 25) have been described previously in PC-3 cells and tumors. In view of promising findings presented here, similar studies should be carried out on other androgen-independent orthotopic and metastatic models, some of which produce prostate-specific antigen.

Previous studies (20, 23, 47) and our present findings with BN/GRP and GHRH antagonists show that combination therapies that interfere with several endocrine and local autocrine and paracrine growth factors lead to a greater inhibition of tumor growth than single agents. Although combined treatment with BN/GRP and GHRH antagonist in this study was not significantly more effective than single agents, combined therapy with both compounds augmented the antiproliferative effects in orthotopic, intraosseous, and s.c. tumors. Because in this preclinical study in nude mice BN/GRP and GHRH antagonists, especially in combination, inhibited primary orthotopic tumor growth and metastatic spread as well as the growth of established bone metastases, these analogues could provide a new nontoxic therapeutic modality for patients with advanced prostate cancer. Our work suggests the merit of further studies with antagonists of BN/GRP and GHRH in prostate cancers.

REFERENCES
Inhibitory Effect of Antagonists of Bombesin and Growth Hormone-Releasing Hormone on Orthotopic and Intraosseous Growth and Invasiveness of PC-3 Human Prostate Cancer in Nude Mice

Anton Stangelberger, Andrew V. Schally, Jozsef L. Varga, et al.


Updated version Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/11/1/49

Cited articles This article cites 43 articles, 12 of which you can access for free at: http://clincancerres.aacrjournals.org/content/11/1/49.full#ref-list-1

Citing articles This article has been cited by 6 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/11/1/49.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.