Effects of antagonists of luteinizing hormone- and growth hormone-releasing hormone on experimental benign prostatic hyperplasia and prostate cancer

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1. INTRODUCTION

1.1 Benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a progressive hyperplasia of prostatic glandular and stromal tissues. BPH is an age-related disease and is present in 20% of 40-y-old men and in 70% of 60-y-old men. Currently, there is no completely effective treatment for BPH. Medical therapies consist of α-adrenergic blockers, which lower adrenergic tone, and 5α-reductase inhibitors, which decrease levels of dihydrotestosterone (DHT). In some patients surgery, mostly transurethral resection of the prostate, is the only effective intervention. New therapies are clearly needed.

Despite the enormous burden of BPH on public health, its pathogenesis is incompletely understood. Hyperplastic growth in BPH has been ascribed to an imbalance between androgen/estrogen signaling, tissue remodeling in the aging prostate, chronic inflammation, stem cell defects, overexpression of stromal and epithelial growth factors, hypoxia, epithelial–mesenchymal transition, and other obscure factors.

However, an emerging body of evidence suggests that inflammation may play a key role in the development and progression of BPH. Clinically, several cross-sectional studies have proposed that a relationship exists between the presence of inflammatory infiltrates and an increase in prostate volume. A minor correlation was observed between presence of prostatic inflammation and lower urinary tract symptoms (LUTS). Proinflammatory cytokines such as IL-2, IL-6, IL-8, IL-15, IL-17, and IFNγ were found to be overexpressed in surgical specimens of BPH. Various growth factors such as FGF-2, FGF-7, IGF-I, IGF-II, TGFb, and VEGF, are also involved in the pathogenesis of BPH.

1.2 Prostate cancer

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

1.3 Antagonists of growth hormone-releasing hormone (GHRH) and luteinizing hormone-releasing hormone (LHRH)

Hypothalamic neurohormones growth hormone-releasing hormone (GHRH) and luteinizing hormone-releasing hormone (LHRH) are also produced by various extrahypothalamic sources, can modulate cell proliferation in many tissues including prostate, especially in malignancies.

LHRH agonists provide the preferred primary treatment for advanced androgen-dependent prostate cancer. However, LHRH-antagonists would be more desirable because of their immediate hormone suppression through a blockade of pituitary LHRH receptors (LHRH-R) thereby avoiding the ''flare-up'' effect seen with the LHRH agonists. A powerful LHRH antagonist, Cetrorelix synthesized in our laboratory was shown to inhibit the growth of experimental prostate cancers by suppressing LH and sex steroid secretion, inducing apoptosis and decreasing LHRH, epidermal growth factor (EGF) and EGF-R as well as IGF-II expression in tumors. The inhibitory effect of Cetrorelix on serum LH, mRNA for LHRH-R, and LHRH-R protein in the pituitary, was greater than that of Decapeptyl. Cetrorelix and other LHRH antagonists are used clinically for the treatment of benign prostate hyperplasia, leiomyomas, and endometriosis as well as in assisted reproductive technology for *in vitro* fertilization and embryo transfer. Cetrorelix is also under clinical investigation for oncological uses since it exerts anti-proliferative effects on human experimental prostatic, breast, and ovarian cancers. Cetrorelix is effective clinically in patients with advanced prostate cancer as evidenced by reduction in serum PSA, regression of metastatic lesions and rapid improvement of disease related symptoms, for example, bone pain, paresthesia and paraplegia. The prostate is a highly hormone-sensitive organ, which is primarily under the control of the pituitary-gonadal axis. There is also evidence that growth factors, such as IGF-I and II, EGF, FGF-2, VEGF and others and some neurohormones such as LHRH and GHRH can affect the function and growth of this gland.

The hypothalamic neuropeptide GHRH stimulates the secretion of growth hormone (GH) from the anterior pituitary gland upon binding to its receptors (GHRH-R). In turn, GH stimulates the production of insulin-like growth factor 1 (IGF-1), a major anabolic growth factor and a potent mitogen for many cancers. GHRH and its pituitary type receptor as well as its truncated receptor splice variants (SV) are expressed in various normal human tissues including prostate, kidney, lung, and liver and on many human cancer cell lines and tumors. Pituitary type GHRH-R and SV1 appear to mediate effects of GHRH and its antagonists on

tumors. GHRH itself acts as an autocrine/paracrine growth factor in human cancers, including prostate.

In order to develop new therapies for cancer, our laboratory has synthesized GHRH antagonists with high antiproliferative activity in numerous experimental cancer models. The inhibitory effect of these analogues is exerted in part by indirect endocrine mechanisms through the suppression of GHRH-evoked release of GH from the pituitary, which in turn results in the inhibition of the hepatic production of IGF-I. Direct mechanisms involved in the main antitumor effects of GHRH antagonists appear to be based on blocking the action of autocrine GHRH on tumors and inhibition of autocrine IGF-1/2. GHRH antagonists inhibit the growth of androgen-independent human prostatic cancers and also numerous other cancers xenografted into nude mice and suppress tumoral growth factors EGF, FGF-2, IGF-1, IGF-2 and VEGF-A. Recent studies also indicate that GHRH antagonists reduce generation of reactive oxygen species, which cause damage to prostatic stroma and epithelium.

LHRH antagonists including cetrorelix, ozarelix cause marked and protracted improvement in LUTS, reduction in prostate volume and increase in urinary peak flow rate in men with BPH. Russo *et al* showed that ganirelix counteracts experimental detrusor overactivity in female rats. This suggests that LHRH-R regulates bladder function and supports reports of beneficial effects of LHRH receptor blockade in LUTS patients.

1.4. Role of p53 and p21 in prostate cancer

The tumor suppressor gene p53 is mutated in about half of all human cancers. p53 appears to play an important role in sensing and repairing DNA damage, inhibiting the cell cycle to allow DNA repair, and inducing apoptosis to eliminate severely damaged cells. The multifunctional p53 protein, which can act as a transcriptional activator or repressor, is induced by DNA damage, and interacts with proteins involved in DNA replication and repair. Mutant p53 (mt-p53) is preferentially expressed in hormone-refractory and metastatic prostate cancer. A poor response to chemotherapy is clearly associated with mutations in the p53 gene.

The cyclin-dependent kinase (CDK) inhibitor p21 is involved in p53-mediated growth arrest and has been identified as a key factor for the regulation of cell growth. Recent studies also indicate to an important anti-apoptotic and pro-survival role of p21 in various cancers including prostatic, colorectal, breast as well as in renal cell carcinomas and melanomas. An increased expression of p21 was found to be associated with androgen independent prostate cancer. In clinical studies, p21 expression was identified as an indicator of poor survival in prostate cancer patients.

2. AIMS OF OUR STUDIES

2.1. Experimental benign prostatic hyperplasia studies

2.1.1. To show presence of GHRH-R and LHRH-R on rat prostates

2.1.2. To investigate effects of:

- LHRH antagonist cetrorelix

- GHRH antagonists JMR-132, MIA-313 and MIA-459

- combination of LHRH antagonist cetrorelix and GHRH antagonist JMR-132on androgen-induced model of BPH

2.1.3. To explore mechanisms of action of antagonists of LHRH and GHRH, and their combination in experimental BPH

2.2. Human prostate cancer xenograft studies

2.2.1. To investigate the minimum effective dose and dose-response relationship of potent GHRH antagonist MZ-J-7-138 for the treatment of androgen-independent PC-3 prostate cancer *in vivo*

2.2.2. To assess effect of GHRH antagonist MZ-J-7-138 on tumoral IGF-II and VEGF in PC-3 xenografts in view of evidence from earlier studies that tumor inhibition by GHRH antagonists affects multiple tumoral growth factors and their signaling

2.2.3. To investigate inhibitory effects of GHRH antagonist MZ-J-7-138 on growth of androgen independent human experimental prostate cancer DU-145 and androgen sensitive MDA-PCa-2b

2.2.4. To evaluate the effect of GHRH antagonist MZ-J-7-138 on apoptotic mechanisms including p53, and p21 in human experimental prostate cancer xenografts such as androgen independent PC-3 and DU-145 lines expressing mutant p53 and androgen sensitive MDA-PCa-2b line expressing wilde type p53

3. MATERIALS AND METHODS

3.1. Peptides and reagents

The GHRH antagonists JMR-132, MIA-313, MIA-459 and MZ-J-7-138 were synthesized by solid-phase methodology. The LHRH antagonist Cetrorelix originally synthesized in our laboratory by solid-phase methods was made by Aeterna-Zentaris. In our rat BPH model, testosterone enanthate (TE), corn oil vehicle and 5α-reductase 2 (5AR2) inhibitor finasteride were used.

3.2 Animals

In our BPH studies adult male Wistar rats were used. Rats were allowed standard laboratory diet and tap water *ad libitum*. For xenograft studies, approximately 5–6 weeks old male athymic (Ncr nu/nu) nude mice were obtained and fed autoclaved chow and water *ad libitum*. Rodents were housed in a climate-controlled, environment with a 12-h light/dark cycle.

3.3. *In vivo* experimental models

3.3.1. Testosterone-induced model of BPH

The fact that in our preliminary studies we found that our target receptors GHRH-R and LHRH-R are exclusively expressed in the rat prostatic epithelium supports the rationale for using a testosterone-induced model of BPH with predominant epithelial hyperplasia.

In our *in vivo* experiments on BPH, after 7 days acclimatization, rats were randomly divided into experimental groups and one negative control group of ten animals each. BPH was induced in experimental groups by daily subcutaneous injection in the right flank of long acting testosterone enanthate (2mg/day), dissolved in corn oil from Day -28 to Day 0 (induction phase). Negative control animals received subcutaneous injections of corn oil alone on the same schedule.

3.3.1.A. Investigation of effects of LHRH antagonist Cetrorelix

Experimental groups consisted of: (1) TE only, (2) TE/ Cetrorelix 0.625 mg/kg, (3) TE/Cetrorelix 1.25 mg/kg, and (4) TE/Cetrorelix 12.5mg/kg body weight. TE only positive control animals were injected with mannitol instead of Cetrorelix on the same schedule.

3.3.1.B. Investigation of effects of GHRH antagonist

Experimental groups consisted of (1) TE only, (2) TE/finasteride (0.1 mg·kg−1·d−1), (3) TE/JMR-132 40 μg/d, (4) TE/MIA-313 20 μg/d, and (5) TE/ MIA-459 20 μg/d. TE-only positive control animals received 0.1% DMSO in 10% aqueous propylene glycol solution instead of finasteride or GHRH antagonists on the same schedule.

3.3.1.C. Investigation of effects of combination of GHRH antagonist and LHRH antagonist

Experimental groups consisted of: (1) TE only, (2) TE/finasteride 0.1 mg/kg/day, (3) TE/JMR-132 40 μg/day, (4) TE/Cetrorelix 0.625 mg/kg and (5) TE/JMR-132 40 μg/day and Cetrorelix 0.625 mg/kg. TE-only positive control animals received 0.1% DMSO in 10% aqueous propylene glycol solution instead of finasteride or GHRH antagonists and mannitol instead of Cetrorelix on the same schedule.

Venous blood samples were collected before the experiment and on the last day of the experiment (day 42). Serum was separated by centrifugation (10 min at 1000 rpm) and stored

at -80°C. Rats were weighed and sacrificed under anesthesia on the morning of day 42; whole prostates were immediately removed, weighed and snap frozen. Alternate prostrate lobes were immersed in phosphate-buffered 10% formalin (pH 7.4) and embedded in paraffin for histological analysis.

3.3.2. Tumor models

3.3.2.A. Cell cultures

Human androgen-independent (PC-3 and DU-145) and human androgen sensitive (MDA-PCa-2b) prostate cancer cell lines were used.

3.3.2.B. Tumor xenograft model

In our *in vivo* tumor xenograft assays, 1.5 million of PC-3, DU-145 or MDA-PSa-2b cells were injected s.c. at each flank to three donor animals. Tumor tissue was harvested aseptically from donor animals. Nude mice were xenografted subcutaneously with 3 mm^3 pieces of respective tumor tissue using a trocar needle. When tumors had grown to a mean volume of approximately 30-75 mm3, the animals were randomly assigned to treatment groups and control (n = 8 to 9).

Tumor volumes (length x width x height x 0.5236) and body weights were recorded every week. At the end of the experiments, mice were anaesthetized with pentobarbital and sacrificed by cutting the abdominal aorta. Tumors were carefully excised, weighed, snap frozen and stored at -80 °C for further investigations. Blood was collected and a complete necropsy was performed of all animals. Liver, heart, lungs, kidneys, spleen, testicles, prostate and seminal vesicles were carefully removed and weighed.

3.4. Histological procedures and morphological analyses

Serial 5 µm-thick sections from each fixed tissue specimen were prepared, mounted on glass slides, and stained with hematoxyline-eosin for morphological analysis. The sections were analyzed with a Nikon Eclipse 90i microscope with a built-in digital camera. The mitotic and apoptotic cells in the ventral prostate from three animals in each group were counted in 10 random fields at 40x objective magnification from three different individual ventral prostate sections.

3.5. Immunohistochemical staining

Serial 4-µm sections of rat ventral prostates were used for immunoperoxidase staining following standard protocols. Antibodies to GHRH receptor in 1:1,000 dilution, to LHRH-R in 1:5 dilution, and to androgen receptor (AR) in 1:20 dilution were added to the slides and incubated for 30 min at room temperature. Positive reaction for GHRH-R and LHRH-R appeared as orange-brown granules, whereas nuclear localization of AR appeared black.

3.6. Total DNA Isolation.

To quantify the cellular content of rat prostates, total DNA was prepared from 20 mg of ventral prostate tissue for each sample using the DNeasy Blood and Tissue kit (Qiagen). Five prostate samples from each group were analyzed. The yield and purity of DNA was determined according to manufacturer's instructions.

3.7. Total RNA Isolation and cDNA sythesis.

Total RNA was isolated from 30 mg of prostate tissue for each sample using the NucleoSpin kit (Macherey-Nagel). Three prostate samples from each group were analyzed. The yield and quality of total RNA was determined spectrophotometrically. Two micrograms of RNA with a final volume of 40 µl were reverse transcribed into cDNA with the QuantiTect Reverse Transcription Kit using the Veriti 96-well Thermal Cycler.

3.8. Quantitative real-time RT-PCR

We evaluated the mRNA expression of rat and human target genes using specific probes and primers. All real-time PCR reactions were performed in the iCycler iQ Real-Time PCR Detection System. The relative gene expression ratios were calculated using Pfaffl's method.

3.9. RT^2 Profiler PCR Array

Rat Growth Factor, Inflammatory Cytokines/Receptors, and Signal Transduction Real-Time PCR Arrays were used to examine the mRNA levels of 252 genes related to growth factors, inflammatory cytokines, and signal transduction. Fold-changes in gene expression were calculated using the ΔΔCt method. Normalization was performed using five housekeeping genes on the arrays.

3.10. Western-blot

Rat prostate tissue and tumor tissue were homogenized. Protein was isolated with NucleoSpin Kit and sonicated. Protein lysates were adjusted to equal concentrations and processed by standard Western blot method.

3.11. Radioimmunoassay (RIA) and ELISA

For our rat BPH studies, we used commercial immunoassay kits to determine GH, LH, DHT, IGF-1, and PSA levels in serum. All immunoassays were done according to manufacturers' instructions.

For our human PC-3 prostatic cancer studies, human VEGF and IGF-II levels were determined by RIA in tumor tissue homogenates.

3.12. Ligand competition assays

Receptors for GHRH and LHRH on rat prostate tissues and receptors for GHRH on human prostate cancer xenograft tumor tissues from the experimental groups were characterized by the ligand competition assay.

3.13. Statistical Analysis.

For statistical evaluation, SigmaStat 3.0 software (Sytat Software) was used. Results are expressed as means ± SEM. One-way ANOVA followed by Bonferroni t test, Student–Newman–Keuls test, or a two-tailed Student's t test was used where appropriate, and significance was accepted at $P < 0.05$.

4. MOST SIGNIFICANT RESULTS

4.1. Effects of LHRH antagonist cetrorelix on experimental benign prostatic hyperplasia (Study 1)

4.1.1. Effect of LHRH antagonist Cetrorelix on rat prostate weight

Corn oil-injected control prostates (negative control) weighed 264.8±9.6 mg/100 g rat; while in TE controls prostates were enlarged by 40.52% to 372.1±25.3 mg/100 g rat (p<0.001). Cetrorelix pamoate 0.625 mg/kg significantly lowered prostate weights by 17.88% (p=0.02). This decrease was similar to that obtained with 1.25 mg/kg Cetrorelix (18.65 % reduction [p=0.01]); further reduction occured with 12.5 mg/kg Cetrorelix (35.17% reduction [p<0.001]).

4.1.2. Effect of Cetrorelix 0.625 mg/ kg on expression of inflammatory cytokine/growth factor mRNA

Treatment with Cetrorelix significantly downregulated cytokines IFN-γ, IL-1α, IL-3, IL-4, IL-5, IL-6, IL-13, IL-15, IL-17β and LTA. Among chemokines and chemokine receptors, expression of C5, CCL25, SPP1, CCR4, CCR9 and BLR-1/CXCR5 was significantly decreased by Cetrorelix.. Levels of FGF-2, FGF-7, FGF-8 and FGF-14 were significantly reduced by Cetrorelix. Levels of mRNA for TGF-β superfamily members, TGF-β1 and BMP-7, showed decrease after Cetrorelix. The level of VEGF-A was lower after Cetrorelix.

Evaluating our PCR array data, we used real-time RT-PCR to analyze selected proinflammatory and growth factor genes. Treatment with Cetrorelix 0.625 mg/kg significantly decreased expression of EGF, TGF-β1, TGF-β2, FGF-2, FGF-7 , VEGF-A, IL-1β and IL-6.

4.1.3. Effect of Cetrorelix 0.625 mg/kg on expression of LHRH-R, LHRH, AR and 5α-reductase 2 in rat prostate

mRNA for LHRH-R and LHRH-R protein and its LHRH ligand were detected in rat prostate. There were no significant changes in the mRNA and the protein levels of LHRH-R after treatment with Cetrorelix. Expression of LHRH was elevated after Cetrorelix; this is significant compared to control. Cetrorelix significantly downregulated mRNA for AR and AR protein levels Cetrorelix significantly lowered 5α-reductase 2 mRNA and protein levels.

4.1.4. Binding assay for LHRH receptors in rat prostate

Receptor analyses revealed a single class of high affinity binding sites for LHRH in rat prostate.

4.2. Effects of GHRH antagonists on experimental benign prostatic hyperplasia (Study 2)

4.2.1. Immunohistochemical confirmation of the expression of GHRH receptor protein.

Immunohistochemical analyses revealed that expression of GHRH-R is confined to the cytoplasm and luminal membrane of prostatic acinar cells in rat.

4.2.2. Reduction of prostate size by GHRH antagonists.

Corn oil-injected control prostates weighed 234.9 ± 16.7 mg/100 g rat; while in TE controls prostates were enlarged by 55.5% to 365.4 ± 20.3 mg/100 g rat ($P < 0.001$). GHRH antagonists JMR-132 at 40µg/day, MIA-313 at 20µg/day, and MIA-459 at 20µg/day significantly lowered prostate weights by 17.8%, 17.0% and 21.4%, respectively compared to TE controls ($P < 0.05$). These reductions in prostate weight were superior to that obtained with finasteride 0.1 mg/kg/day (nonsignificant 14.43 % reduction). In addition, GHRH antagonists significantly decreased prostatic DNA content as well.

4.2.3. GHRH antagonists suppress proinflammatory IL-1β, NF-κβ and COX-2.

Prostatic IL-1β, NF-κβ/p65 (RelA), and COX-2 protein levels were significantly increased after TE treatment compared to control, while GHRH antagonists JMR-132, MIA-313, and MIA-459 and finasteride significantly reduced their levels.

4.2.4. GHRH antagonists inhibit cell division and induce apoptosis.

Apoptotic cell numbers were higher in the groups treated with GHRH antagonists MIA-313, MIA-459, and finasteride, but the differences from TE treated controls were not statistically significant. The expression of mRNA for Bax was elevated after treatment with all three GHRH antagonists or finasteride, while that of Bcl-2 was decreased after treatment with JMR-132, MIA-313 and MIA-459. GHRH antagonists JMR-132 and MIA-459 significantly reduced PCNA protein.

4.3. Effects of combination of antagonist of LHRH with antagonist of GHRH on experimental benign prostatic hyperplasia (Study 3).

4.3.1. Immunohistochemical confirmation of the expression of GHRH-R and LHRH-R protein.

Immunohistochemical analyses revealed that expression of both GHRH-R and LHRH-R is limited to the cytoplasm and luminal membrane of rat prostatic acinar cells.

4.3.2. Reduction of prostate size by GHRH antagonist JMR-132, LHRH antagonist cetrorelix and their combination.

Control prostates weighed 248.0±10.7 mg/100 g rat BW; while in TE controls prostates were enlarged by 48.2% to 367.5±15.9 mg/100 g rat BW. GHRH antagonist JMR-132 at 40µg/day, LHRH antagonist cetrorelix at 0.625 mg/kg, and their combination significantly lowered prostate weights by 18.6%, 21.3% and 30.3%, respectively compared to TE controls. This reduction of prostate weight was superior to that obtained with finasteride 0.1 mg/kg/day (nonsignificant 13.8 % reduction). In addition, JMR-132, cetrorelix and combinations decreased prostatic DNA content.

4.3.3. Effect of GHRH antagonist JMR-132, LHRH antagonist cetrorelix and their combination on 5AR2, α1A-AR, AR, PSA and STEAP.

GHRH antagonist JMR-132 lowered protein levels of 5AR2. LHRH antagonist cetrorelix caused a decrease in α_{1A}-AR protein levels. Protein levels of prostatic AR were significantly increased in TE-induced BPH; among treatments only JMR-132 alone resulted in a significant increase in AR protein. Prostatic PSA protein was lowered significantly after TE treatment by 62% compared with control, whereas combination of GHRH/LHRH antagonists significantly reduced PSA levels by 83%. Prostatic STEAP protein expression was significantly decreased by JMR-132 and combination therapy by 74% and 96%, respectively.

4.3.4. Combination of GHRH and LHRH antagonists suppresses IL-1β, NF-κβ and COX-2

The expression prostatic IL-1β protein was significantly decreased by the combination by 57%. JMR-132, cetrorelix and combination treatment also significantly lowered prostatic NF-κβ/p65 protein levels by 48%, 37% and 54%, respectively. Prostatic COX-2 protein was significantly lowered after JMR-132 and combination treatment by 79% and 97 %, respectively.

4.3.5. Combination of GHRH and LHRH antagonists inhibit cell division and induce apoptosis.

Morphologic evaluation on H&E slides revealed that JMR-132, cetrorelix, their combination and finasteride significantly decreased the sizes of average epithelial areas in the ventral prostate. The number of mitoses was significantly reduced in all groups compared to TE treated BPH controls. Apoptotic cell numbers were higher in the groups treated with JMR-132, cetrorelix and their combination and finasteride, but the differences from TE treated controls were not statistically significant.

The combination of GHRH and LHRH antagonists reduced PCNA protein by 58%. We observed transcriptional downregulation of Bcl-2 after treatment with JMR-132, cetrorelix, and combination. Bax mRNA expression was elevated after treatment with finasteride and combination. Transcriptional suppression of p53 was found after treatment with finasteride, JMR-132, cetrorelix and combination.

4.4 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 (Study 4)

4.4.1. Effect of GHRH antagonist MZ-J-7-138 on the growth of PC-3 human androgen independent prostate cancer in nude mice

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%. The tumor doubling time increased from 6.2 days in control group to 12.4 days by the treatment with 10µg/day.

4.4.2. Effect of GHRH antagonist MZ-J-7-138 on the expression of IGF-II and VEGF

Administration of 10 µg/day MZ-J-7-138 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.

4.4.3. Binding assays for GHRH receptors in PC-3 tumors

Binding studies demonstrated the presence of a single class of specific, high affinity binding sites for GHRH receptor in the membrane preparation of PC-3 tumors.

4.5. Inhibitory effects of antagonists of growth hormone releasing hormone on experimental prostate cancers are associated with upregulation of wild-type p53 and decrease in p21 and mutant p53 proteins (Study 5)

4.5.1. Experiment 1: Effect of GHRH antagonist MZ-J-7-138 on the growth of PC-3 human androgen independent prostate cancers in nude mice

Treatment with MZ-J-7-138 significantly inhibited PC-3 tumor growth after four weeks of treatment. The final tumor inhibition was 77%, as compared to controls (p<0.01). This

antiproliferative effect is also reflected by final tumor weights (a 66% inhibition). The tumor volume doubling time was similarly significantly extended by MZ-J-7-138 compared to controls.

4.5.2. Experiment 2: Effects of GHRH antagonist MZ-J-7-138 on the growth of DU-145 human androgen independent prostate cancers in nude mice

MZ-J-7-138 significantly suppressed the proliferation of s.c. implanted DU-145 xenografts after 6 weeks of treatment and led to a 66% inhibition of tumor volume. This tumor inhibition is also reflected by tumor weights, which were lower by 62% in animals treated with GHRH antagonist MZ-J-7-138 compared to controls. Tumor volume doubling time was extended from 10.6 to 25.2 days.

4.5.3. Experiment 3: Effects of GHRH antagonist MZ-J-7-138 and LHRH antagonist Cetrorelix on the growth of MDA-PCa-2b human androgen sensitive prostate cancer in nude mice

An even greater inhibition of tumor growth than in PC-3 and DU-145 tumors was observed in mice bearing MDA-PCa-2b cancers that received a combination therapy of MZ-J-7-138 and Cetrorelix. Significant tumor inhibition was found in all treated groups and combined therapy with MZ-J-7-138 and Cetrorelix caused the greatest inhibition. Final tumor weights of all treated groups, were also significantly smaller as compared to controls, the combination of MZ-J-7-138 and Cetrorelix causing the greatest inhibition. Tumor volume doubling time (TDT) was also significantly extended in animals treated with Cetrorelix alone and with combination of GHRH antagonist and Cetrorelix as compared to controls.

4.5.4. GHRH Receptor-binding studies

Binding studies demonstrated the presence of a single class of specific, high-affinity binding sites for GHRH receptor in all 3 human prostate cancer models investigated.

4.5.5. Effects of GHRH antagonist MZ-J-7-138 and LHRH antagonist Cetrorelix on the expression of p53 tumor suppressor protein

Mutant p53 in PC-3 and DU-145 was significantly decreased by treatment with GHRH antagonist. Wild type p53 in MDA-PCa-2b tumors was significantly up regulated by treatment with Cetrorelix and an increase of wt-p53 was observed in MDA-PCa-2b tumors after treatment with MZ-J-7-138 and the combination of MZ-J-7-138 and Cetrorelix.

4.5.6. Effects of GHRH antagonist MZ-J-7-138 and LHRH antagonist Cetrorelix on p21 protein expression

Treatment of PC-3 and DU-145 prostate cancers with GHRH antagonist MZ-J-7-138 significantly inhibited the expression of p21 protein levels. In MDA-PCa-2b tumors treated

with the same GHRH antagonist or Cetrorelix, the decrease in p21 protein expression did not reach statistical significance as compared to controls due to the high standard error.

5. DISCUSSION

5.1. Effects of LHRH antagonist cetrorelix on experimental benign prostatic hyperplasia (Study 1)

Clinical data have demonstrated that therapy with LHRH antagonist Cetrorelix resulted in long-lasting improvement in LUTS. This improvement, including reduction in prostate volume and increase in urinary peak flow rate, appears to be superior to that produced by α-blockers or 5α-reductase inhibitors. Low doses of Cetrorelix used in recent clinical trials cause only a partial suppression of pituitary-gonadal axis and testosterone levels.

In the present study, we have shown that Cetrorelix significantly reduced prostate weights by 18% in non-castrating doses of 0.625 mg/kg. Histological observations in our rat model of BPH revealed marked hyperplastic morphological changes in the prostates of testosterone-treated BPH animals, while treatment with a low dose of Cetrorelix (0.625 mg/kg) caused an involution of these hyperplastic changes resulting in a morphology similar to that of normal animals.

The presence of LHRH and LHRH-R in rat prostate was demonstrated by real-time PCR and Western blot. Furthermore, ligand competition assay detected specific high affinity receptors for LHRH in rat prostate. Cetrorelix 0.625 mg/kg significantly lowered prostatic AR and 5α-reductase 2 levels, however, serum DHT and LH were only slightly decreased. Changes in serum PSA were not significant after treatment with Cetrorelix at 0.625 mg/kg. Moreover, the expression of LHRH and LHRH-R and direct antiproliferative effects of LHRH and its analogs have been demonstrated in many malignant human tumors. Recently we showed that Cetrorelix inhibits the proliferation of human prostate epithelial BPH-1 cell line *in vitro*. These findings suggest that low doses of Cetrorelix did not impair gonadal function in rats as was also shown by experimental and clinical findings. Prostate shrinkage is a result of direct inhibitory effects of Cetrorelix exerted through prostatic LHRH receptors, implies the presence of an LHRH-based autocrine regulatory system.

The real-time PCR arrays showed that several proinflammatory cytokines and growth factors were upregulated in control animals with induced BPH and markedly downregulated in Cetrorelix-treated animals. These insulin-like, transforming and fibroblast growth factors and downstream effector molecules as well as a variety of interleukins, can lead to abnormal stromal and epithelial prostate cell growth. Low doses of Cetrorelix caused a marked

reduction in proinflammatory cytokines mRNA levels. These cytokines are part of an inflammatory network in BPH including several growth factors. IFN-γ, produced by infiltrating T-cells, is a natural antagonist of growth inhibiting TGF-β; FGF-2 stimulates growth. IFN-γ also stimulates IL-15, thereby augmenting increased influx of T-lymphocytes. These T-cells further produce lymphokines such as IL-4 and IL-13, facilitating formation of active androgens and estrogens by inducing 3β-hydroxydehydrogenase/isomerase. T-cell derived IL-17 fine tunes immune response, stimulating IL-6, IL-8 and IL-1α and β.

In summary, our wide-range analysis of gene expression in the prostate of rats with testosterone-induced BPH revealed the transcriptional activation of several genes including those for proinflammatory interleukins, chemokines and prostatic growth factors. The expression of these genes was suppressed in Cetrorelix-treated animals. These findings suggest that Cetrorelix exerts its beneficial effects on BPH by suppressing proinflammatory cytokines and growth factors at the transcriptional level.

The results of this study indicate that the reduction in prostate volume could be due to direct inhibitory effects of Cetrorelix exerted through prostatic LHRH receptors as well as transcriptional suppression of proinflammatory cytokines and growth factors. These findings shed light on the mechanism of action of LHRH antagonists in BPH and also suggest a role for LHRH as a locally acting growth factor in BPH. It is possible that LHRH antagonists could be clinically used for therapy of BPH in combination with other agents.

5.2. Effects of GHRH antagonists on experimental benign prostatic hyperplasia (Study 2)

The main finding of our study is that GHRH antagonists JMR-132, MIA-313 and MIA-459 reduce prostate size in an experimental model of BPH. In addition to prostate shrinkage in rats, multiple factors related to growth and inflammation, which are crucial in the pathogenesis and progression of BPH, were markedly reduced by treatment with GHRH antagonists. The expression of GHRH, GHRH-R and its splice variant SV1 in rat prostate was demonstrated by Western blot. Furthermore, ligand competition assay detected specific high affinity receptors for GHRH in rat prostate and immunohistochemical analyses revealed that this expression of GHRH-R is confined to luminal epithelial cells of the rat prostate. Changes in serum GH, IGF-1, DHT, and PSA were not significant after treatment with GHRH antagonists. Recently we showed that GHRH antagonists inhibit the proliferation of human prostate epithelial BPH-1 cell line *in vitro*. These findings strongly suggest that prostate shrinkage is a result of direct inhibitory effects of GHRH antagonists exerted through prostatic GHRH receptors, not involving the GH/IGF-1 axis. The demonstration of the co-

expression of GHRH and its receptors in rat prostate supports the hypothesis that GHRH produced locally in the prostate could act in an autocrine/paracrine manner through an interaction with the GHRH receptors. The presence of this pathway, which is disrupted by GHRH antagonists, provides a mechanistic explanation for the antiproliferative effects of such antagonists in prostate cell growth in culture and in nude mice xenograft models of prostate cancer. Our data also imply that GHRH could be involved in the pathogenesis of BPH.

Real-time PCR arrays showed that several growth factors were upregulated in TE-induced BPH control rats and markedly downregulated in animals treated with GHRH antagonists. Growth factors are regulatory peptides that govern the response of cells to injury and mediate the highly coordinated processes of cell growth, differentiation, and apoptosis. Among them there are many polypeptides which use autocrine or paracrine pathways to signal stromal and epithelial cells in the microenvironment. We confirmed by real-time RT-PCR that GHRH antagonists suppress transcriptional expression of IGF-2, TGF-α, TGF-β1 and-β2, EGF, FGF-2, VEGF-A and IL-1β. Several of these growth factors were reported to be involved in the pathogenesis of BPH.

Our observation of the transcriptional activation of inflammatory cytokines in the prostate of rats with induced BPH is consistent with clinical findings and with experimental findings in rats. We found that GHRH antagonists significantly lowered transcriptional expression of several cytokines. These cytokines are part of an inflammatory network in BPH including several growth factors; they promote T-lymphocyte infiltration and the subsequent inflammation progression associated with BPH.

We showed that treatment with TE results in elevated levels of IL-1β, NF-κβ and COX-2 protein in the rat prostate, while GHRH antagonists JMR-132, MIA-313, and MIA-459 caused a pronounced comparative decrease in IL-1β, NF-κβ and COX-2 protein levels. IL-1β, an inflammatory cytokine, causes NF-κβ activation in the mouse prostate. The NF- κβ family proteins, like NF-κβ/p65 (RelA) are inducible transcription factors that regulate the expression of hundreds of genes in immune response, angiogenesis, cell adhesion, proliferation, differentiation, and apoptosis. The activation of NF-κβ is one of the earliest events in chronic inflammation. COX-2, an inducible isoform of cyclooxygenase enzyme, is an early response gene upregulated by specific stimuli such as mitogens, growth factors, and a variety of cytokines including IL-1. Expression of COX-2, and COX-2-dependent prostanoid production induced by proinflammatory mediators are predominantly regulated by NF- κβ dependent gene transcription, suggesting a causal relationship between the lowered

NF-κβ/p65 levels, inhibition of COX-2 upregulation and decreased IL-1β production caused by GHRH antagonists. Overexpression of COX-2 in human BPH samples was reported, while GHRH antagonists were shown to lower levels of COX-2 in experimental lung cancer and prostate cancer. COX-2 was also shown to upregulate anti-apoptotic Bcl-2 with an associated decrease in apoptosis in prostate tissue.

All three GHRH antagonists were demonstrated to inhibit cell proliferation, elevate tumor suppressor p53, and lower PCNA levels in rat prostatic epithelium. We observed an increased expression of anti-apoptotic Bcl-2 in TE-induced BPH prostates. This overexpression of Bcl-2 corresponds to observations of Alonso-Magdalena *et al* in human BPH samples, which suggest that BPH is not a proliferative disease, but rather an accumulation of cells resistant to death. Our work shows that treatment with GHRH antagonists causes significant translational upregulation of proapoptotic Bax and suppression of anti-apoptotic Bcl-2 in rat prostates. The number of apoptotic cells in prostatic epithelium after GHRH antagonists was also increased, although this increase was not statistically significant. These propapoptotic effects of GHRH antagonists might be due to the significant suppression of prostatic COX-2 or to inhibition of both intrinsic and extrinsic pathways of p53 mediated apoptosis.

Analyzing transcriptional changes in signal transduction pathways with quantitative PCR arrays, we observed the involvement of the mitogenic, hedgehog, PI3/AKT and phospholipase C pathways and their downstream effectors. These may be responsible for transmitting beneficial effects of GHRH antagonists in experimental BPH. GHRH antagonists can strongly inhibit the proliferation rate of cancer cells through the inhibition of the MAPKs pathway.

Therapeutic effects of GHRH antagonists were superior to that of finasteride in many aspects of our study including prostatic shrinkage, suppression of growth factors and proinflammatory COX-2, as well as antiproliferative and proapoptotic effects. The adverse effects of finasteride may inculpate GHRH antagonists as an alternative medical therapy of BPH.

In summary, herein we demonstrated that GHRH antagonists JMR-132, MIA-313, and MIA-459 reduce prostate volume in an experimental BPH model. Our data suggest that this reduction in prostate volume is due to direct inhibitory effects of GHRH antagonists exerted through prostatic GHRH receptors as well as through transcriptional suppression of enumerated growth factors and proinflammatory cytokines. We also showed strong inhibition of proinflammatory IL-1β, NF-κβ, and COX-2. Antiapoptotic effects of these GHRH antagonists have also been demonstrated. These findings suggest mechanisms of action of

GHRH antagonists in BPH and also indicate a role for GHRH as a locally acting growth factor in BPH. It is possible that GHRH antagonists could be clinically useful for therapy of BPH alone or in combination with other agents.

5.3. Effects of combination of antagonist of LHRH with antagonist of GHRH on experimental benign prostatic hyperplasia (Study 3).

Our study shows that the combination of GHRH antagonist JMR-132 with LHRH antagonist cetrorelix augments prostate shrinkage and reduction in cellular content in experimental BPH. Similarly, multiple functional molecules potentially related to pathogenesis and progression of BPH, were markedly reduced by treatment with GHRH antagonist.

Immunohistochemical analyses revealed that this expression of GHRH-R and LHRH-R is confined to luminal epithelial cells. The expression of GHRH, LHRH and their receptors in rat prostate was demonstrated by Western blot. Previously, we reported that ligand competition assay detected specific high-affinity receptors for GHRH and LHRH in rat prostate. Serum GH, LH, IGF1, and DHT were not significantly affected by treatment with combination of GHRH and LHRH antagonist. Prostatic and serum PSA, a well-known tumor marker markedly declined after combination treatment. Levels of STEAP, a cell surface antigen expressed predominantly in prostate cancer and a potential target for immunotherapy in various solid tumors, were radically lowered after combination treatment.

Recently we showed that antagonists of GHRH and LHRH inhibit the proliferation of the human prostate epithelial BPH-1 cell line *in vitro*. These findings strongly suggest that prostate shrinkage is a result of direct inhibitory effects of GHRH and LHRH antagonists exerted through their prostatic receptors, not involving the hypothalamic-pituitary axis. The demonstration of co-expression of GHRH-R or LHRH-R and their ligands in rat prostate supports the view that prostatic GHRH and LHRH could act in an autocrine/paracrine manner through interaction these receptors. This pathway, which is disrupted by antagonists of GHRH and LHRH, provides a mechanistic explanation for the antiproliferative effects of these antagonists on prostate cell growth in culture and in nude mouse xenograft models. Our data also imply that GHRH and LHRH could be involved in the pathogenesis of BPH.

We demonstrated that combination of JMR-132 and cetrorelix caused a pronounced decrease in IL-1β, NF-κβ/p65, and COX-2 protein levels and decreased phosphorylation of NF-κβ/p50. The role of IL-1β, NF-κβ/p65, and COX-2 in BPH were discussed above.

Our study showed that combination treatment markedly decreases the sizes of average epithelial areas in ventral prostate, prostatic DNA content and protein levels of proliferation

marker PCNA. Significant transcriptional downregulation of anti-apoptotic Bcl-2, and upregulation of pro-apoptotic Bax were observed.

The therapeutic effects of JMR-132 and cetrorelix combination were superior to those of finasteride on prostatic shrinkage and suppression of PSA, STEAP, IL-1β and COX-2 and so were its antiproliferative and proapoptotic effects. The adverse effects of finasteride may support the use of combination of GHRH and LHRH antagonists as an alternative medical therapy for BPH.

This study shows that combination of GHRH antagonist with LHRH antagonist potentiates reduction in prostate volume in an experimental BPH model. Our data indicate that shrinkage of prostate is induced by direct inhibitory action of GHRH and LHRH antagonists exerted through prostatic receptors as well as by strong suppression of PSA, STEAP, IL-1β, NF-κβ, and COX-2. Proapoptotic and antiproliferative effects of combination therapy were also demonstrated. Our findings shed light on the mechanisms of action of combinations of GHRH plus LHRH antagonists and also imply that GHRH and LHRH may serve as a local growth factor in BPH. Our study suggests that GHRH antagonists should be considered for further development of a therapy for BPH, possibly in combination with LHRH antagonists.

5.4. 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 (Study 4)

Treatment of relapsed androgen-independent prostate cancer remains a major challenge. New therapeutic modalities are being developed based on antagonists of LHRH, GHRH and targeted cytotoxic peptide analogs. 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. Some of these growth factors aberrantly stimulate the androgen receptor pathway 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. 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.

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*.

In this study, with the one of 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 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. 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 the present investigation, we confirmed the expression of GHRH-R by RT-PCR and radioligand binding studies. 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.

5.5. Inhibitory effects of antagonists of growth hormone releasing hormone on experimental prostate cancers are associated with upregulation of wild-type p53 and decrease in p21 and mutant p53 proteins (Study 5)

Mutations of the tumor suppressor gene p53 are among the most common genetic changes found in malignant tumors. Since p53 protein modulates cellular functions, such as gene transcription, DNA synthesis and repair, cell cycle arrest, and apoptosis, mutations in the p53 gene can abrogate these functions and may lead to genetic instability and progression to cancer.

Alterations of p53 are clearly associated with androgen independent prostate cancer and significant overexpression of mt-p53 is found in hormone-refractory and metastatic prostate cancer tissue.

Different growth characteristics *in vivo* and *in vitro* as well as different responses to the treatment with various anticancer agents observed in androgen independent prostate cancer models such as PC-3 and DU-145, compared to androgen sensitive models MDA-PCa-2b and LNCaP have been attributed to the status of p53 as the former models express mutant type and the latter wt p53. The present study demonstrates that inhibition of human experimental prostate cancers by GHRH antagonist MZ-J-7-138 and LHRH antagonist Cetrorelix involves a different effect on wt-p53 and mt-p53 protein levels. MZ-J-7-138 inhibited proliferation of PC-3 and DU-145 tumors inducing a decrease in the levels of mutant p53. MZ-J-7-138 also suppressed MDA-PCa-2b tumors with an upregulation of wt-p53. Cetrorelix also significantly increased the wt-p53 protein expression. Induction of the expression of pro-apoptotic wt-p53 protein may be involved in the inhibitory effect of GHRH and LHRH antagonists on MDA-PCa-2b tumor growth observed in our study. Our results are in accord with recent findings showing an increase of wt-p53 expression after treatment with GHRH antagonists in an experimental BPH model, and a decrease of mt-p53 expression after therapy with GHRH antagonists in DMS-153 small cell lung carcinomas. In MDA-PCa-2b, combined treatment with LHRH antagonist Cetrorelix and the GHRH antagonist MZ-J-7-138 resulted in a greater tumor inhibition. MZ-J-7-138 is the first GHRH antagonist that significantly decreased growth of MDA-PCa-2b human androgen sensitive prostate cancers without concomitant androgen deprivation. In previous studies using earlier and less potent GHRH antagonists, the androgen deprivation was required in order to achieve significant tumor inhibition of androgen sensitive experimental prostate cancers by GHRH antagonists. The presence of GHRH receptor and LHRH receptor on PC-3, DU-145 and MDA-PCa-2b human prostate cell lines was demonstrated previously.

GHRH antagonists inhibit the growth of various human experimental prostate cancers directly by blocking tumoral GHRH receptors and by decreasing the production of tumoral IGF-I and II. The intracellular signal transduction mechanisms leading to tumor inhibition following treatment with GHRH antagonists include changes in the expression of PKC isoforms, inhibition of the p42/44 MAPK (pERK1/2) – c-jun pathway, and Ca^{2+} entry into the cells which induces apoptosis.

In contrast to PC-3 and DU-145 tumors, the androgen sensitive MDA-PCa-2b prostate cancer model was found to express wt-p53. Treatment of MDA-PCa-2b tumors with MZ-J-7-138 led to a non-significant up-regulation of wt-p53.

Based on the role of p53 in the control of apoptosis after DNA damage, the p53 gene has been implicated as a major determinant of tumor responsiveness to cytotoxic therapies. Wild-type p53 protein can suppress tumorigenesis and promote apoptosis, acting as a transcriptional factor while mutant p53 proteins are linked to a number of pathophysiological processes and exert effects that are opposite to those of wild-type p53. The inhibition of anti-apoptotic mt-p53 by GHRH antagonists observed in PC-3 and DU-145 tumors may offer a strategy to increase chemosensitivity of advanced prostate cancers that respond poorly to chemotherapy and prevent further progression.

The present work, together with the results of several previous studies in our laboratory, indicates that GHRH antagonists are effective for the treatment of cancers with wt-p53 as well as mt-p53 status. GHRH antagonists could offer a distinct advantage for the treatment of cancers with mt-p53 status, since the conventional anticancer drugs, including DNA cross-linking agents, antimetabolites, and topo-isomerase I and II inhibitors, are less effective when mt-p53 is expressed. Recent investigations demonstrated, that inhibition of p53 function diminishes the androgen receptor-mediated signaling.

To shed more light on the downstream mechanisms of the p53 pathway in prostate cancers treated with GHRH antagonist we investigated the expression of the p53 downstream effector p21 (p21$^{WAF1/Cip1}$). Treatment with GHRH and LHRH antagonists invariably suppressed the p21 protein levels, regardless of whether the tumor models expressed wt- or mt-p53. This indicates that the antagonists possibly influence tumoral p21 levels by mechanisms independent of p53. Thus in MDA-PCa-2b model which contains wt-p53, an upregulation of wt-p53 levels after treatment with antagonists was accompanied by suppression of p21, even though wt-p53 is a well-known positive regulator of p21 expression. Nevertheless, in our experiments with PC-3 and DU-145 tumors expressing mt-p53, inhibition of mt-p53 levels by the GHRH antagonist was accompanied by decreased p21 levels. The possible mechanisms by which our antagonists could inhibit tumoral p21 levels in a p53-independent manner are the inhibition of tumoral MAPK (pERK1/2) and pAkt levels, since pERK1/2 and pAkt are positive regulators of p21 and GHRH antagonists inhibit pERK1/2 and pAkt levels in prostatic and lung cancers.

The inhibition of anti-apoptotic p21 levels by antagonists of GHRH and LHRH represents a very favorable molecular mode of action, suggesting that these antagonists could possibly

counteract the p21-upregulating effect of conventional chemotherapeutic agents and of radiotherapy, and act as chemotherapy or radiotherapy sensitizers when used with these treatment methods. Similarly it cannot be excluded that antagonists of GHRH and LHRH could exert some protective action against deleterious effects of radiation and chemotherapy. The decrease of p21 expression, in addition to the earlier described paracrine/autocrine growth factor blockade, could offer a multimodal approach to a new cancer therapy.

Tumor inhibition produced by GHRH antagonists *in vivo* could be partially explained by a repression of the defective p53 function in PC-3 and DU-145 and by the reduction in p21 protein expression. Further work with these analogs might lead to the development of new therapeutic modalities for patients with relapsed prostate cancer who no longer respond to conventional treatment.

6. NOVEL FINDINGS:

6.1. Experimental benign prostatic hyperplasia studies

- We showed expression of target receptors LHRH-R and GHRH-R and found a single class of high affinity binding sites for LHRH and GHRH in rat prostates
- We localized LHRH-R and GHRH-R on luminal membrane and apical cytoplasm of epithelial cells of rat prostates
- Treatment with LHRH antagonist cetrorelix caused significant reduction of prostate weights in experimental BPH in a dose-dependent manner
- Our findings suggest that the reduction in prostate volume could be due to direct inhibitory effects of cetrorelix exerted through prostatic LHRH receptors as well as transcriptional suppression of proinflammatory cytokines and growth factors
- We demonstrated that the GHRH antagonists JMR-132, MIA-313, and MIA-459 reduce prostate weigths and cellular content in experimental BPH
- Our data suggest that this reduction in prostate volume is caused by the direct inhibitory effects of GHRH antagonists exerted through prostatic GHRH receptors as well as by transcriptional suppression of enumerated growth factors and proinflammatory cytokines.
- We also showed strong inhibition of prostatic IL-1β, NF-κβ, and COX-2 after treatment with GHRH antagonists
- The proapoptotic effects of GHRH antagonists also have been demonstrated.

- We observed the involvement of the mitogenic, hedgehog, PI3/AKT, and phospholipase C pathways and their downstream effectors after treatment of experimental BPH with GHRH antagonists.
- We demonstrated that a combination of GHRH antagonist with LHRH antagonist potentiates reduction in prostate weight in experimental BPH.
- Our data indicate that shrinkage of prostate is induced by direct inhibitory action of GHRH and LHRH antagonists exerted through prostatic receptors.
- We found strong suppression of PSA, STEAP, IL-1β, NF-κβ, and COX-2 after treatment with combination of GHRH and LHRH antagonists.
- Proapoptotic and antiproliferative effects of combination therapy were also demonstrated.
- Our data imply that GHRH and LHRH may serve as a local growth factor in BPH

6.2. Human prostate cancer xenograft studies

- Our work indicates that GHRH antagonist MZ-J-7-138 is effective for the treatment of experimental prostatic cancers with wt-p53 (MDA-PCa-2b) as well as mt-p53 (PC-3 and DU-145) status.
- We showed that treatment with GHRH and LHRH antagonists invariably suppressed the p21 protein levels, regardless of whether the tumor models expressed wt- or mt-p53. This indicates that the antagonists possibly influence tumoral p21 levels by mechanisms independent of p53.
- We also demonstrated that GHRH antagonist MZ-J-7-138 inhibits growth of androgen-independent PC-3 prostate cancer in a dose-dependent manner.
- The reduction of growth factors VEGF and IGF-2 by this GHRH antagonist was evident at higher doses and after prolonged treatment.

7. LIST OF PUBLICATIONS

Impact factor of articles related to the dissertation: 23.456

Cumulative impact factor of all publications: articles: 79.027; abstracts: 117.95

Publications related to the dissertation

1. Peer-reviewed journal articles:

1. **Rick FG**, Schally AV, Block NL, Nadji M, Szepeshazi K, Zarandi M, Vidaurre I, Perez R, Halmos G, Szalontay L. Antagonists of growth hormone-releasing hormone (GHRH) reduce prostate size in experimental benign prostatic hyperplasia. *Proc Natl Acad Sci USA*. 2011 Mar 1;108(9):3755-60. **[IF: 9.771]** (2010)

2. **Rick FG**, Schally AV, Block NL, Halmos G, Perez R, Fernandez JB, Vidaurre I, Szalontay L. LHRH antagonist Cetrorelix reduces prostate size and gene expression of proinflammatory cytokines in a rat model of benign prostatic hyperplasia. *Prostate*. 2011 May 15;71(7):736-47. **[IF: 3.377]** (2010)

3. **Rick FG**, Szalontay L, Schally AV, Block NL, Nadji M, Szepeshazi K, Vidaurre I, Zarandi M, Kovacs M, Rekasi Z. Combining of antagonist of GHRH with antagonist of LHRH greatly improves BPH shrinkage. *J Urol*. 2012. Accepted for publication. **[IF: 3.862]** (2010)

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2. Citable abstracts:

1. **Rick FG**, Schally AV, Block NL, Szalontay L, Siejka A, Rincon R, Fensterle J, Engel J. Reduction in prostate size after treatment with Cetrorelix in a rat model of bening prostatic hyperplasia (BPH). *Urology*. 2009;74(4) S45. **[IF: 2.365]**

Further publications

1. Peer-reviewed journal articles:

1. Kanashiro-Takeuchi RM, Takeuchi LM, **Rick FG**, Dulce R, Treuer AV, Florea V, Rodrigues CO, Paulino EC, Hatzistergos KE, Selem SM, Gonzalez DR, Block NL, Schally AV, Hare JM. Activation of Growth Hormone Releasing Hormone (GHRH) Receptor Stimulates Cardiac Reverse Remodeling After Myocardial Infarction (MI). *Proc Natl Acad Sci USA*. 2011. Accepted for publication.   **[IF: 9.771]** (2010)

2. Pozsgai E, Schally AV, Hocsak E, Zarandi M, **Rick F**, Bellyei S. The effect of a novel antagonist of growth hormone releasing hormone on cell proliferation and on the key cell signaling pathways in nine different breast cancer cell lines. *Int J Oncol*. 2011 Oct;39(4):1025-32.   **[IF: 2.571]** (2010)

3. Papadia A, Schally AV, Halmos G, Varga JL, Seitz S, Buchholz S, **Rick FG**, Zarandi M, Bellyei S, Treszl A,. Lucci JA. Growth hormone releasing-hormone antagonist JMR-132 inhibits growth of ES-2 clear cell human ovarian cancer. *Horm Metab Res*. 2011 Oct;43(11):816-20.   **[IF: 2.414]** (2010)

4. Kovacs M, Schally AV, Hohla F, **Rick FG**, Pozsgai E, Szalontay L,. Varga JL, Zarandi M. A correlation of endocrine and anticancer effects of some antagonists of GHRH. *Peptides*. 2010 Oct;31(10):1839-46**.**   **[IF: 2.654]**

5. Pozsgai E, Schally AV, Varga JL, Halmos G, **Rick F**, Bellyei S. The inhibitory effect of a novel cytotoxic somatostatin analogue AN-162 on experimental glioblastoma. *Horm Metab Res*. 2010 Oct;42(11):781-6.   **[IF: 2.414]**

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9. Buchholz S, Seitz S, Schally AV, Engel JB, **Rick FG**, Szalontay L, Hohla F, Krishan A, Papadia A, Gaiser T, Ortmann O, Brockhoff G, Koster F. Triple negative breast cancers express receptors for LHRH and their growth can be inhibited by the LHRH antagonist Cetrorelix. *Int J Oncol*. 2009 Oct;35(4):789-96.   **[IF: 2.447]**

10. Treszl A, Schally AV, Seitz S, Szalontay L, **Rick FG**, Halmos G. Inhibition of Human Non-Small Cell Lung Cancers with a Targeted Cytotoxic Somatostatin Analog, AN-162. *Peptides*. 2009 Sep;30(9):1643-50.   **[IF: 2.705]**

11. Seitz S, Schally AV, Treszl A, Papadia A, **Rick F**, Szalontay L, Szepeshazi K, Ortmann O, Halmos G, Buchholz S. Preclinical evaluation of properties of new targeted-cytotoxic somatostatin analogue AN-162 [AEZS-124] and its effects on tumor growth inhibition. *Anticancer Drugs.* 2009 Aug;20(7):553-8. **[IF: 2.23]**

12. Hohla F, Schally AV, Szepeshazi K, Varga JL, Buchholz S, Koster F, Heinrich E, Halmos G, **Rick FG**, Kannadka C, Datz C, Kanashiro CA. Synergistic inhibition of growth of lung carcinomas by antagonists of growthhormone-releasing hormone in combination with docetaxel. *Proc Natl Acad Sci USA.* 2006 Sep 26;103(39):14513-8. **[IF: 9.643]**

8. ACKNOWLEDGEMENTS