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Inhibitory effects of antagonists of growth hormone-releasing hormone on growth and invasiveness of PC3 human prostate cancer

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Abstract

New approaches are needed to the therapy of advanced prostate cancer. This study determined the effect of growth hormone- releasing hormone (GHRH) antagonists, JMR-132 and JV-1-38 on growth of PC3 tumors as well as on angiogenesis and metastasis through the evaluation of various factors that contribute largely to the progression of prostate cancer. Human PC3 androgen-independent prostate cancer cells were injected subcutaneously into nude mice. The treatment with JMR-132 (10 μg/day) or JV-1-38 (20 μg/day) lasted 41 days. We also evaluated the effects of JMR-132 and JV-1-38 on proliferation, cell adhesion and migration in PC-3 cells *in vitro*. Several techniques (Western blot, reverse transcription polymerase chain reaction, immunohistochemistry, ELISA and zymography) were used to evaluate the expression levels of GHRH receptors and its splice variants, GHRH, vascular endothelial growth factor (VEGF), hypoxia inducible factor (HIF)-1α, metalloproteinases (MMPs)-2 and -9, β-catenin and E-cadherin. GHRH antagonists suppressed the proliferation of PC-3 cells *in vitro* and significantly inhibited growth of PC3 tumors. After treatment with these analogues, we found an increase in expression of GHRH receptor accompanied by a decrease of GHRH levels, a reduction in both VEGF and HIF-1α expression and in active forms of MMP-2 and MMP-9, a significant increase in levels of membrane-associated β-catenin and a significant decline in E-cadherin. These results support that the blockade of GHRH receptors can modulate elements involved in angiogenesis and metastasis. Consequently, GHRH antagonists could be considered as suitable candidates for therapeutic trials in the management of androgen-independent prostate cancer.

Key words: GHRH, GHRH antagonists, prostate cancer, angiogenesis, metastasis

Introduction

One in nine men over 65 years of age will develop an invasive prostatic cancer, which represents a major international health problem.[1] The type of treatment used depends on the stage of the disease; when it is detected early, prostate cancer can be successfully treated by radical prostatectomy, hormonal therapy, cryotherapy or chemotherapy. However, when tumor cells show a metastatic phenotype, current treatments are only palliative. In this regard, bone metastases, with an incidence of 85% in patients at advanced stages of the disease, are the most common cause of death.[2] Therapeutic strategies should be planned to transform prostate cancer from a fatal disease to a chronic condition.[3]

Antagonistic analogues of growth hormone-releasing hormone (GHRH) have been shown to inhibit the growth of various cancers, including prostatic cancer, through indirect and direct pathways.[4–9] Thus, GHRH antagonists can sup- press tumor growth indirectly by blocking GH release from the pituitary and consequently the hepatic production of insulin-like growth factor-I (IGF-I), which is an established mitogen for various cancers, is also inhibited. However, GHRH antagonists exert their main antitumor effects through direct mechanisms, by the inhibition of tumoral GHRH and IGF-I and or IGF-II, which serve as autocrine/ paracrine growth factors. Pituitary-type GHRH receptors and their splice variants (SVs) have been detected in different human cancers and various cancer cell lines.[10] The routes of intracellular signal transduction involved in the antiproliferative effects of GHRH antagonists have not been fully identified. GHRH antagonists, such as JV-1-38 and JMR-132, show very high binding affinities for tumor GHRH receptors.[11] Likewise, it should be taken into account that the combinations of GHRH analogues with other antitumor compounds, in some cases, have shown a nearly complete inhibition of tumor growth.[11]

Neovascularization, the process by which new blood vessels are formed from pre-existing

host vasculature, is an essential requirement for tumor growth and metastasis.[12] This complex process involves proteolysis of basement membrane, endothelial cell migration, proliferation and matrix remodeling. One of the most important angiogenic molecules is the vascular endothelial cell growth factor (VEGF), a potent and specific angiogenesis-related cytokine that is responsible for endothelial cell differentiation, migration, proliferation, tubular formation and vessel assembly. Together with that factor, IGF-I and IGF-II also contribute either alone or in combination to the action of metalloproteinases (MMPs), which results in the degradation of basement membrane in the microvessel walls and the formation of new blood vessels.[12] MMP-2 and MMP-9 belong to the subfamily of gelatinases and degrade fibrillar collagen (type IV) present in basement membranes, the first barrier to cancer invasion; these proteases are overexpressed in many cancers types including prostate cancer.[13–15]

Cadherins and catenins form adherens junctions, which are central mediators of cell-cell adhesion. Many studies show that alterations in cell-cell adhesion correlate with epithelial tumor progression and metastasis.[16,17] In prostate cancer, an increase in β-catenin expression levels correlates with disease progression.[18] This protein plays a relevant role in both cadherin-mediated adhesion and the Wnt signaling cascade. Once released from cadherin-complex, β-catenin behaves as a transcriptional modulator of many target genes including CD44, cyclin D and c-Myc.[19,20]

The aim of this study was to determine the effects of GHRH antagonists JMR-132 and JV-1-38 on the growth of PC3 human androgen-independent prostate tumors as well as on angiogenesis and metastasis through the evaluation of several factors that contribute largely to the progression of prostate cancer. The novelty of the study in relation with previous data on the effects of GHRH antagonists in prostate cancer[4,7–10] mainly resides in the

consideration of different aspects of tumor invasiveness leading to the observation of metastasis. The results extend the information on the mechanisms of action of GHRH antagonists and underline their potential usefulness in the therapy of this malignancy.

Material and Methods

Peptides

GHRH antagonists JV-1-38 and JMR-132 were synthesized in the laboratories of one of us (AVS). JV-1-38 and JMR-132 structures are [PhAc-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Har^{9}, Tyr(Me)^{10}, Abu^{15}, Nle^{27} and Har^{29}] human GHRH$_{1\text{-}29}$NH$_2$ and [PhAc^{0}-Tyr^{1}, D-Arg^{2}, Phe(4-Cl)^{6}, Ala^{8}, Har^{9}, Tyr(Me)^{10}, His^{11}, Abu^{15}, His^{20}, Nle^{27} and D-Arg^{28}, Har^{29}] human GHRH$_{1\text{-}29}$NH$_2$, respectively. Abu is α-aminobutyric acid, Har is homoarginine, Nle is norleucine, PhAc is phenylacetyl and Tyr(Me) is O-methyltyrosine.

Cell culture

The androgen-unresponsive cell line PC3 was obtained from the American Type Culture Collection (Manassas, VA) and may be related to recurrent prostate cancers that have achieved androgen independence. All culture media were supplemented with 1% penicillin/streptomycin/amphotericin B (Life Technologies, Barcelona, Spain). The culture was performed in a humidified 5% CO_2 environment at 37°C. After the cells reached 70–80% confluence, they were washed with PBS, detached with 0.25% trypsin/0.2% EDTA and seeded at 30,000–40,000 cells/cm^2. The culture medium was changed every 3 days.

Animals, xenografts and processing of tumors

Athymic male nude mice (nu/nu) 5–6 weeks old were obtained from Harlan (Oxon, UK) and maintained in microisolator units on a standard sterilizable diet. Mice were housed under humidity- and temperature-controlled conditions and the light/dark cycle was set at 12 hr intervals. Experimental procedures were carried out according to Spanish and European

Directives. For preparation of xenografts, PC3 cells were washed with PBS, detached with 0.25% trypsin/0.2% EDTA, centrifuged at 400*g* and resuspended in fresh medium at 5 x 10^7 cells/ml. The cell suspension was mixed with Matrigel (Becton Dickinson, Madrid, Spain) synthetic basement membrane (1:1, v/v) and then injected subcutaneously into the right flank of nude mice (5 x 10^6 cells/mouse). The experiment was started when the tumors had grown to ~75 mm^3. Animals were randomly divided into three treatment groups: group 1 (10 mice), control and vehicle solution; group 2 (six mice), GHRH antagonist JMR-132 injected s.c. once a day at a dose of 10 μg/animal and group 3 (six mice), GHRH antagonist JV-1-38, injected s.c. every day at a dose of 20 μg/animal. Tumor volume (length x width x height x 0.5236)[21] and body weight were measured twice a week. The experiment was ended on day 41. After mice were anaesthetized with halothane, tumors were dissected and cleaned. Tumor specimens were divided into three approximately equal portions: one portion was processed for immunohistochemistry (10% formalin fixed and paraffin embedded) and the other portions were frozen in liquid nitrogen and maintained at -80ºC for further experiments. The development of metastases in the whole skeletal apparatus was monitored at the end of the experiment by radiography using a Faxitron cabinet X-ray system (Faxitron Bioptics, Tucson, AZ). The semi- quantitation scoring method was formulated as 0, no lesions; 1, minor changes; 2, small lesions; 3, significant lesions in bone or lung; 4, significant lesions in bone and lung. The results represent the average scores from two observers. The median value for each group was 3.15 ± 0.27 for control, 1.18 ± 0.60 for JMR-132 and 0.75 ± 0.47 for JV-1-38. There were statistical differences among the control and treated groups ($p < 0.001$).

Isolation of cell lysates, membranes and nuclei

To obtain tissue lysates, tumor specimens were homogenized in 1 M Tris–HCl (pH 7.6)

containing 1% Nonidet P40, 150 mM NaCl, 2 mM ortovanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml pepstatin and then rotated for 30 min in a cold room. The extract was cleared by centrifugation at 15,000*g*, for 30 min, at 4ºC. The isolation of membranes and nuclei from tumor tissue was performed as described previously.[22]

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from tumors by using Tri^{R} Reagent (Sigma, Alcobendas, Madrid) according to the manufacturer's instruction. Two micrograms of total RNA were reverse-transcribed into cDNA by means of Moloney murine leukemia virus reverse transcriptase, according to the manufacture's guidelines (Life Technologies). Reverse transcriptions were also performed without enzyme to rule out genomic DNA contamination. Primers were chosen with the assistance of the computer program Primer3 v.0.4.0. Gene specific primers for human GHRH and β-actin were as follows: GHRH: 5'- AATTGGAGAGCTCCTGGTG-3' (sense), 5'-CCAGTTGCATTTTGGCTACA-3' (antisense) and β-actin: 5^{0}-AGAAGGAT TCCTATGTGGGCG-3' (sense), 5'-CATGTCGTCCCAGTTGGTGAC-3' (antisense). The number of cycles was determined in preliminary experiments to be within the exponential range of PCR amplification. Negative controls with water instead of cDNA were run in parallel to exclude contamination. PCR-conditions were as follows: denaturation at 94ºC for 5 min, followed by 94ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min by 40 cycles for GHRH and 25 cycles for β-actin and then a final cycle of 10 min at 72ºC. PCR products were subjected to electrophoresis on a 2% agarose gel, stained with GelRed^{TM} nucleic acid gel stain (Biotium, Hayward, CA) and visualized under ultraviolet light.

Western blot

Proteins from cell lysate extracts (30 μg) were denatured by heating. Then, they were resolved by 10% SDS-PAGE and blotted onto a BioTrace^{TM} nitrocellulose membrane (Pall

Corp., Alcobendas, Spain) overnight in 50 mM Tris–HCl, 380 mM glycin, 0.1% SDS and 20% methanol. Antibodies against GHRH-R (batch number: JH-2321/5) and SV_1 (batch number: JH2317/5) were raised in the laboratory of one of us (AVS). Rabbit anti-pGHRHR (1:4,000), anti-SVs (1:4,000), anti-MMP-9 (Abcam, Cambridge, UK; 1:2,000), anti-MMP-2 (Abcam; 1:2,000), mouse anti-E-cadherin (Becton Dickinson; 1:5,000), anti-β-catenin (Becton Dickinson; 1:2,000) or anti-GHRH (Abbiotec, San Diego, CA; 1:500) antibodies were added followed by incubation for 1 hr at room temperature. After treatment with secondary antiserum (1:4,000) for 1 hr at room temperature, signals were detected with enhanced chemiluminescence reagent (Thermo Scientific, Walltham, MA) using b-actin antibody (Merck, Madrid, Spain) in loading control.

Determination of VEGF

VEGF levels were determined in tumor homogenates (15 μg) by ELISA (human VEGF DuoSet, R&D Systems, Madrid, Spain) according to the manufacturer's instructions. Data were normalized to the protein concentration in each sample.

Gelatin zymography

Zimography assays were carried out as described previously.[14] Briefly, the samples (6 lg of protein) were subjected to 10% SDS-PAGE with 0.1% (w/v) gelatin (Sigma) as the substrate. After staining, the activity of MMP-2 and MMP-9 was semiquantitatively determined by densitometry.

Immunohistochemistry

Serial sections, 5-μm-thick, were deparaffinized in xylene and rehydrated using graded ethanol concentrations. To retrieve the antigen, the sections were hydrated and placed in a glass jar containing 10 mM sodium citrate buffer, pH 6.0 and heated in a pressure cooker for 2 min. The endogenous peroxidase activity was inhibited by incubation with 3% hydro-

gen peroxide for 20 min at room temperature. After rinsing in Tris-buffered saline (TBS), the slides were incubated with blocking solution (3% normal donkey serum plus 0.05% Triton in TBS) for 45 min to prevent nonspecific binding of the first antibody. Afterwards, the sections were incubated over-night at 4ºC with the primary antibodies: SV1-SV2-SV4 (1:2,000); pGHRH-R (1:2,000); MMP-2 (1:100); MMP-9 (1:200), hypoxia inducible factor (HIF)-1α (1:100) and CD34 (1:100) (Abcam); E-cadherin (1:500) (Becton Dickinson) in the blocking solution diluted 1:9. Then, the sections were washed in TBS and incubated for 20 min with biotinylated link universal antibody (Dako, Barcelona, Spain). After an extensive wash in TBS, detection was made by the conventional labeled-streptavidin-biotin method (LSAB-kit, Dako), except for CD34 since an anti-rat-biotinylated antibody was used. The peroxidase activity was detected using the glucose oxidase-DAB-nickel intensification method. Sections were dehydrated, cleared in xylene and mounted in DePex (Probus, Barcelona, Spain). To assess the specificity of immunoreaction, negative and positive controls were used. Sections of samples identically processed, but not incubated with the primary antibodies, were used as negative controls. As positive controls, sections of skin, rat adrenal gland and kidney were processed with the same antibody.

Proliferation assays

PC-3 (2 x 10^5) cells were grown in 6-well plates. After 24 hr, the culture medium was removed and replaced with RPMI-1640 medium containing 0% FBS and 1% antibiotic/antimycotic (penicillin/streptomycin/amphotericin B) for 16 hr. Then, cells were treated with 0.1 μM JMR-132 or JV-1-38 for 24 hr. Cells were pulsed with 10 μM bromodeoxyuridine (BrdU) in the last 30 min of incubation. After incubation, the cells were washed with PBS, fixed with ice-cold absolute ethanol and stored at -20ºC for 30 min. Fixative was removed by centrifugation and the cell pellets were washed with PBS. DNA was partially

denatured by incubation with 1 M HCl for 30 min at room temperature and then the cells were washed three times with PBS containing 0.05% Tween- 20 (pH 7.4) and 0.1 BSA. Cells were incubated with 20 μl of anti-BrdU monoclonal antibody with FITC (Beckton Dickinson) in the dark for another 30 min. The cells were stained with propidium iodide (PI) staining solution (50 μg/ml PI, 10 μg/ml RNase and 1 x PBS) before flow cytometric analysis. The amount of DNA distribution in the difference phases of the cell cycle was analyzed with the use of the Cyflogic program (Version 1.2.1.).

Cell adhesion assay

Concentrated type-I collagen solution was diluted in 10 mM glacial acetic acid and coated onto 96-well plates for 1 hr at 37ºC. Plates were washed twice with PBS (pH 7.4). Cells were harvested with 0.25% trypsin/0.2% EDTA and collected by centrifugation. They were resuspended in RPMI medium/ 0.1% (w/v) BSA (pH 7.4) and treated with 0.1 μM JMR-132 or JV-1-38 for 30 min. Then, cells plated at 2.5 x 10^4 cells per 100 μl. The assay was terminated at indicated time intervals by aspiration of the wells. Cell adhesion was quantified by adding 1 mg/ml of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) followed by 1 hr incubation. Isopropanol (50 μl) was added to each well to dissolve the formazan precipitates and absorbance at 540 nm with a reference wavelength at 630 nm was reported.

Wound-healing assay

PC3 cells were incubated in 24-well plates and a small wound area was made with a scraper in the confluent monolayer. Afterwards, cells were incubated in the absence or presence of 0.1 μM JMR-132 or JV-1-38. Four representative fields of each wound were captured using a Nikon Diaphot 300 inverted microscopy at different times (0–24 hr). Wound areas of untreated samples were averaged and assigned a value of 100%.

Data analysis

Quantification of band densities was performed using Quantitive One Program (Bio-Rad, Alcobendas, Spain). Data were subjected to one-way ANOVA and differences were determined by Bonferroni's multiple comparison test. Each experiment was repeated at least three times. Data are shown as the means of individual experiments and presented as the mean ± SE; $p < 0.05$ was considered statistically significant.

Results

Effect of GHRH antagonists JMR-132 and JV-1-38 on growth of xenografted PC3 human prostate cancer cells

Treatment with GHRH antagonists, JMR-132 (10 μg/day) and JV-1-38 (20 μg/day) was performed after injection of PC3 cells in nude mice and further formation of tumors. Final tumor volume measurements revealed that JMR-132 significantly inhibited tumor growth by 58% (319.3 ± 115 mm^3) and JV-1-38 by 70% (224.3 ± 88 mm^3) after 41 days of treatment, as compared with the control group which measured 750.2 ± 74.56 mm^3 (Figs. 1*a* and 1*b*). Interestingly, after treatment with JMR-132 or JV-1-38, two and one tumor masses, respectively, had almost entirely regressed, respectively. At the end of the experiment, no significant differences in body weights were observed between groups, indicating that treatment with GHRH antagonists was not toxic to tumor-bearing animals.

Effect of GHRH antagonists on metastatic potential of PC3 cells

Antimetastatic potential of GHRH antagonists was evaluated. The injection of 5 x 10^6 PC3 cells into the flank of nude mice resulted in the formation of metastases in several sites such as the spine (Fig. 1*c*). We obtained total body radiographies of all mice and analyzed them by digital scan to evaluate the reduction in development of metastases. The metastasis was considered positive when there was at least one osteoblastic zone or metastatic nodules in lungs

and/or soft tissues of the mouse. Control group showed 80% of mice with metastases, while treatment with GRHR antagonists JMR-132 and JV-1-38 reduced the number of osteoblastic lesions and/or metastatic nodules by 33 and 40%, respectively, per mouse. Histological study of spine affected by metastases showed the presence of tumor cells in the bone marrow (Figs. 1*d* and 1*e*). Interestingly, none of the mice from treated groups exhibited lung metastases.

Expression of GHRH and GHRH receptors

The expression of GHRH both in control conditions and after treatment with GHRH antagonists was analyzed by immunohistochemistry (Fig. 2*a*), RT-PCR (Fig. 2*b*) and Western blot (Fig. 2*c*) assays. The histological examination of hematoxylin-eosin stained tumor sections showed that these masses were surrounded by a capsule of connective tissue (Fig. 2*a*). In their inner, different zones were distinguished, one of them presented big tumor cells with polygonal outline intermingled with smaller cells associated to infiltration zones and nearby to blood vessels. The ground substance was scarce and collagen fiber bundles could be observed in some areas. This pattern was also shown by xenografts from mice treated with either JV-1-38 or JMR-132. Interestingly, tumor masses of treated groups showed a more loose arrangement than that of control group. The immunoreactivity for the GHRH peptide was lower in treated groups than that in control group. After GHRH antagonist-treatments both mRNA (Fig. 2*b*) and peptide (Fig. 2*c*) levels of GHRH were significantly decreased by about 35–40% and 50–70%, respectively. To assess whether GHRH expression was accompanied by changes on GHRH receptor levels, the expression of these receptors was evaluated in all the groups. The reactivity observed to anti GHRH receptor and SVs antibodies was the same independently of the antibody used; only a few cells were positive. In control groups, the number of positive cells was lower than that in

antagonist groups and the JMR-132 group showed the highest number of positive cells (Fig. 2*a*). It should be noted that endothelial cells in tumor masses presented high immunoreactivity for both GHRH receptors and GHRH.

Expression of angiogenic factors VEGF, HIF-1α and CD34

We observed in the tumor extraction process that tumor masses exhibited less blood supply than control animals after treatment with GHRH antagonists, JMR-132 and JV-1-38. Figure 3*a* shows a representative tumor from the control group and groups treated with JMR-132 and JV-1-38. To determine whether these tumors presented increased angio- genesis and its possible variations, we checked VEGF levels by an ELISA assay. VEGF expression showed a significant decrease of 40% in both groups treated with GHRH antagonists (Fig. 3*b*). In addition, we studied the expression of CD34, a hematopoietic marker of stem cells that recognizes a protein present in endothelial cells. Control groups showed a low number of CD34-immunolabeled neoformed capillaries; antagonist JMR-132 led to the highest number of blood vessels and JV-1-38 group also presented a higher number of blood vessels than control group (data not shown). Faced on these results, we decided to evaluate the hypoxic state of the tumors. For this purpose, we used a specific antibody against HIF-1α (Fig. 3*c*). Immunohistochemical studies revealed a decrease in the number of immunopositive cells for this factor in the tumors treated with both GHRH antagonists.

Expression and activity of metalloproteinases 2 and 9

The expression of metalloproteinases MMP-2 and MMP-9 in tumors of groups treated with GHRH antagonists, JMR-132 and JV-1-38, and the control group was analyzed by immunodetection techniques (Figs. 4*a* and 4*b*). The immunohistochemistry analysis (Fig. 4*a*) revealed that tumors from control group showed a high expression of MMP-2 that was reduced in both groups treated with each antagonist. However, MMP-9 expression was

lower in the control group than in treated mice. By means of Western blotting analysis, we detected bands at 92 kDa for MMP-9 and 70 kDa for MMP-2 (Fig. 4*b*). Densitometry of the bands showed that there was a significant decrease of MMP-9 (50%) and MMP-2 (56%) after treatment with JMR-132 as compared with control group. Treatment with JV-1-38 resulted in a significant decrease in the expression of MMP-9 protein (48%) and MMP-2 protein (91%) as compared with the control group.

The activity of both gelatinases was assessed by zymography assays (Fig. 4*c*). Latent forms of MMP-9 (95 kDa) and MMP-2 (72 kDa) and active forms of MMP-9 (88 kDa) and MMP-2 (65 kDa) were detected. The densitometric analysis showed that the activity of the latent form of MMP-9 increased in the group treated with GHRH antagonist JMR-132 by about 50% ($p < 0.001$) and in the group treated with JV-1-38 by about 25% as compared with the control group. By contrast, the active-MMP-9 form decreased significantly by 45–59% in the groups treated with both antagonists as compared with the control. The latent form of MMP-2 showed a significant decrease of 38% in the group treated with antagonist JMR-132, while the active form of MMP-2 was significantly diminished (48%) after treatment with both GHRH antagonists.

Expression of β-catenin and E-cadherin

To determine the expression of certain proteins involved in cell-cell adhesion, the levels of β-catenin (95 kDa) and E-cadherin (120 kDa) levels were evaluated. The expression of β-catenin was assessed in membranes and nuclei (Fig. 5*a*). Levels of this membrane-associated protein were significantly increased in membranes from tumors treated with GHRH antagonists, JMR-132 (by about 40%) and JV-1-38 (by about 75%). However, expression of β-catenin was significantly diminished in nuclei from groups treated with

JMR-132 (by 46%) and JV-1-38 (by 18%) as compared with the controls.

Densitometric analysis showed that E-cadherin levels were significantly decreased in tumor lysates from groups treated with JMR-132 (by 35%) and JV-1-38 (by 87%) (Fig. 5*b*). Immunohistochemical studies revealed that there was a fewer number of immunopositive cells for E-cadherin in treated groups as compared with those in the control group. E-cadherin immunolabeling was localized in plasma membrane of the positive cells (Fig. 5*c*).

Proliferation, cell adhesion and migration assays

The effect of GHRH antagonists, JMR-132 (0.1 μM) and JV- 1-38 (0.1 μM) on the proliferation, cell adhesion and migration of prostate cancer PC3 cells were assessed by BrdU incorporation, adhesion to type-I-collagen and recovery of monolayer wounds assays, respectively. Treatment with the GHRH antagonists, JMR-132 and JV-1-38 for 24 hr significantly decreased cell proliferation by 18–42% in PC3 cells as compared with control conditions (Fig. 6*a*). To investigate whether the increased membrane association of b-catenin leads to corresponding increases in cell adhesion *in vitro*, we incubated PC3 cells in the absence or presence of GHRH antagonists on a collagen plate. PC3 cells rapidly adhered to collagen basement in a time-dependent manner. Treatment with JMR-132 resulted in a significant increase of cell adhesion in PC3 cells. However, JV-1-38-treated cells showed an adhesion pattern similar to that of control cells (Fig. 6*b*). For evaluation of cell migration we performed wound healing assays (Fig. 6*c*). After 24-hr incubation, the cells treated with the GRHRH antagonists showed a lower migration capability (44–53% of wound healing) than that of control cells (20% of wound healing).

Discussion

In this work, we evaluated the effects of two GHRH antagonists, JMR-132 and JV-1-38. Previously, JV-1-38 had been used in studies on the inhibition of cancer cell growth

including non- small cell lung carcinomas, gastroenteropancreatic and prostatic cancer cell lines.[10,23,24] We used this antagonist JV-1-38 at the dose of 20 μg/day because a previous report indicated that it had no effect at lower doses.[25] JMR-132, a more recent and highly potent GHRH antagonist, has been shown to inhibit growth of human prostate, breast, lung and ovarian cancer cell lines and breast carcinomas.[7–9,11,26–30] Our results are in agreement with previous studies,[11] which observed a significant reduction of tumor volume. Neither of the analogs had toxic effects or affected the weight of experimental animals as described previously.[11,23] Interestingly, after treatment with GHRH antagonists JMR-132 and JV-1-38 two and one PC3 tumor masses, respectively, had almost entirely regressed. This could be due to the blockade of binding sites for GHRH. The treatment of PC3 cells with JMR- 132 and JV-1-38 also prevented the proliferation of cultured cells as previously reported.[7]

The main effects of the GHRH antagonists are exerted directly on tumors.[31] When GHRH receptors were blocked with the antagonists JMR-132 and JV-1-38, we found a decrease of both mRNA and GHRH peptide levels in PC3 tumors. This result is in accord with recent findings indicating that the analogs exert their effect on the expression of GHRH at the transcriptional level, which in turn implies the suppression of growth and proliferation of tumor cells by blocking the action of tumoral GHRH.[31] A recent report described that the inhibitory effect produced by JMR-132 involves inactivation of the PI3K/Akt/mTOR and Raf/MEK/ ERK pathways and the reduction in GHRH produced by PC3 cells.[7] Furthermore, pretreatment of PC-3 cells with the GHRH antagonist JMR-132 prevented the activation of ERK by GHRH.[7] Moreover, treatment with either GHRH analogue caused an increase in the expression of GHRH receptors in tumor. Faced with a decline of GHRH, the tumor appears to respond by an increase in the binding sites for the peptide. Blockade of GHRH receptor may be responsible for the almost complete tumor regression in the case of some tumors

treated with GHRH antagonists JMR-132 and JV-1-38.

Prostate carcinoma is a urological malignancy where the process of angiogenesis may play a relevant role in the development and progression of the tumor. Tumor cells produce or induce the synthesis of a large number of angiogenic factors by other cells to achieve a functional vasculature.[32] Several studies have shown that VEGF is closely correlated with neovascularization and prognosis in many solid tumors. Thus, an increased expression of VEGF in prostate cancer[32] as well as a positive correlation between VEGF and Gleason score, tumor grade and microvessel density (MVD) has been observed.[27–35] In our study, at first glance, the blood supply in tumors treated with GHRH antagonists seemed to be diminished compared with the untreated group. When we assessed $VEGF_{165}$ and HIF-1α levels in PC3 tumors, it was observed that the treatment with GHRH antagonists JMR-132 and JV-1- 38 significantly decreased both proangiogenic factors. A similar inhibition of VEGF expression by JV-1-38 has been observed in DU-145 human androgen-independent prostate cancer cell line.[36] Conversely, when we evaluated MVD by measuring CD34 levels, we found augmented levels of this protein after treatment with GHRH antagonists. Similar results have been reported by Pavlakis *et al*.[37] who showed a negative correlation between MVD and CD34 in human breast carcinomas.

The interrelationship of angiogenic growth factors and matrix metalloproteinases (MMPs) in human prostate carcinoma has been described previously. A significant correlation between the expression of MMP-9, MMP-2 and VEGF has been observed in cell lines as well as in tissue specimens of prostate cancer.[38,39] Overactive MMPs contribute to an almost complete loss of the basement membrane proteins in most cancers including prostate carcinomas.[40] Present *in vitro* studies showed that GHRH antagonists, JMR-132 and JV-1-38 decreased migration in PC3 human androgen-independent prostate cancer cells.

In this regard, similar effects of the GHRH antagonist, MIA-602 have been reported for glioblastoma and breast and ovarian cancer cells.[41] Moreover, it has been described that the invasion and the motility of prostate tumor cells were increased by MMP-2 and MMP-9.[42] Interestingly, in our study, GHRH antagonists affected MMP-2 and MMP-9 differently. We observed that the expression of both active and latent MMP-2 isoforms was decreased after treatment with JMR-132 and JV-1-38. How- ever, latent MMP-9 isoform was increased and active MMP-9 isoform was diminished after GHRH analogues-treatment. This result could indicate that GHRH antagonists might control elements involved in the proteolytic activation of MMP-9.[43] In this respect, it seems that MMP-26, which is capable of activating pro-MMP-9 by cleavage of the proenzyme, may be a biochemical mechanism contributing to invasion of human carcinoma cell *in vivo*.[40] Further studies should be performed in order to clarify whether GHRH antagonists affect either the expression or the activity of MMP-26.

Our research on an aggressive stage of prostate cancer was completed with preliminary studies on the assessment of levels of b-catenin and E-cadherin. The levels of plasma membrane b-catenin were increased after treatment with GHRH antagonists. In this regard, expression of adherent junction proteins is often decreased in tumors and reconstitution of functional adherent junctions can revert to the noninvasive phenotype of cancer cells.[44] Present *in vitro* studies revealed that GHRH antagonists, JMR-132 and JV-1-38 decreased cell adhesion in PC3 human androgen-independent prostate cancer cells. Likewise, it has been described that b- catenin leads to the disruption of cell to cell contacts in DU- 145 human androgen-independent prostate cancer cells.[44]

Furthermore, we found that E-cadherin levels were decreased in groups treated with GHRH antagonists. Thus, it has been observed in MDCK cells that E-cadherin is not essential to maintain cell-cell adhesion, but is important only for the establishment of cell to cell

contacts.[45] Moreover, E-cadherin does not regulate cell motility and invasion in DU145 human androgen-independent prostate cancer cells.[44] In addition, the down-regulation of E-cadherin levels in MCF10A breast cancer cells reduces cell migration and invasion.[46]

However, β-catenin has also an important role in Wnt-signaling and cancer cell proliferation which is thought to be independent of its cadherin function.[47] In prostate cancer, β-catenin levels have been shown to correlate with disease progression, whereas activation of β-catenin signaling and subsequent localization in the nucleus may increase cell proliferation through activation of transcription.[18,20] In our study, the expression of β-catenin in cell nuclei was significantly decreased after treatment with GHRH antagonists JMR-132 and JV-1-38. Consequently, the reduction of these levels and a possible subsequent inactivation of β-catenin/TCF transcription complex would in turn down-regulate many target genes including CD44, cyclin D1 and c-Myc.[19]

Androgen-independent prostate cancer cells injected subcutaneously into nude mice generated a higher number of metastases in control animals as compared with the groups treated with GHRH antagonists. When osteoblastic lesions and pulmonary nodules were monitored by radiography, tumor cells were found only in the bone marrow of affected spinal column from control animals and none of the mice in the treated groups exhibited lung metastases. Taken together, all results reveal the aggressive and invasive capabilities of androgen-independent prostate cancer cells and the antimetastatic effects of GHRH antagonists JMR-132 and JV-1-38.

In conclusion, this study sheds more light on the inhibitory action of GHRH antagonists on the growth of PC3 androgen-independent human prostate cancers. Our most important finding is that GHRH antagonists can produce a reduction in the expression of angiogenic and metastatic factors such as VEGF, HIF-1α, MMPs and nuclear β-catenin as well as an

increase in the expression of membrane β-catenin. Consequently, GHRH antagonists could be considered for the development of new therapies for advanced androgen-independent or castration-resistant prostate cancer.

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Figure Legends

Figure 1. (*a*,*b*) Effect of GHRH antagonists JMR-132 (10 μg/day) (*a*) and JV-1-38 (20 μg/day) (*b*) s.c. on growth of s.c. PC3 human androgen- independent prostate cancer in nude mice (six mice per group). Treatment was started when tumors had grown to ~75 mm^3 and lasted for 41 days. Data in each bar are the means ± SE. **p* < 0.05; ***p* < 0.01; ****p* < 0.001 *vs*. control (10 mice). (*c*–*e*) Development of bone metastasis; (*c*) Representative X-ray images of whole body of an animal from control group. The right panel shows the typical aspect of osteoblastic lesions (white arrows) observed in nude mice subcutaneously inoculated with human PC3 cells. (*d*) Spinal metastasis (black arrows) of a mouse from control group. (*e*) Tumor cells (black arrows) in the bone marrow from bone metastases.

Figure 2. Effect of GHRH antagonists JMR-132 (10 μg/day) and JV-1-38 (20 μg/day) on GHRH and GHRH receptor expression (six mice per group). (*a*) Hematoxylin and eosin images (*a'–c'*) and immunoexpression of GHRH (*d'–f'*), pGHRHR (*g'–i'*) and SVs (*j'–l'*) were observed in histological sections from PC3 tumors. For all figures, original magnification x300. (*b*) GHRH mRNA and (*c*) protein levels were assessed by RT-PCR and Western blot assays, respectively. Expression levels were normalized with those for β-actin. Data in each bar are the means ± SE. **p* < 0.05; ***p* < 0.01; ****p* < 0.001 *vs*. control (10 mice).

Figure 3. Effect of GHRH antagonists JMR-132 (10 μg/day) and JV- 1-38 (20 μg/day) on tumor blood supply (*a*) and on the expression of both proangiogenic factors VEGF (*b*) and HIF-1α (*c*). Six mice were used in each group. A representative tumor sample from each group after 41 days-treatment is shown. Data in each bar are the means ± SE. **, *p* < 0.01 *vs*. control (10 mice).

Figure 4. Effect of GHRH antagonists JMR-132 (10 μg/day) and JV-1-38 (20 μg/day) on the expression (*a*, *b*) and activity (*c*) of metalloproteinases MMP-2 and MMP-9 in PC3 tumors. Six mice were used in each group. (*a*) Immunolabeling to MMP-9 (*a'–c'*) and MMP-2 (*d'–f'*) antibodies. For all figures, original

magnification x300. (*b*) MMPs protein levels and (*c*) their gelatinolitic activities as mean Western blot and zimography studies were carried out. Expression levels were normalized with those for β-actin. Data in each bar are the means ± SE. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs*. control (10 mice).

Figure 5. Effect of GHRH antagonists JMR-132 (10 μg/day) and JV- 1-38 (20 μg/day) on the expression levels of β-catenin (*a*) and E- cadherin (*b*) as well as on E-cadherin localization (*c*) in PC3 tumors. Expression levels were normalized with those for β-actin. Data in each bar are the means ± SE. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs*. control (10 mice).

Figure 6. Effects of GHRH antagonists JMR-132 (0.1 μM) and JV-1-38 (0.1 μM) on the cell proliferation (*a*), cell adhesion (*b*) and migration (*c*) of PC3 prostate cancer cells. The effects of GHRH antagonists were assessed as described under Material and Methods by BrdU incorporation, adhesion to type-I-collagen and recovery of monolayer wounds assays, respectively. Results are the means ± SE of 5–7 experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs*. corresponding control.

Figure 1

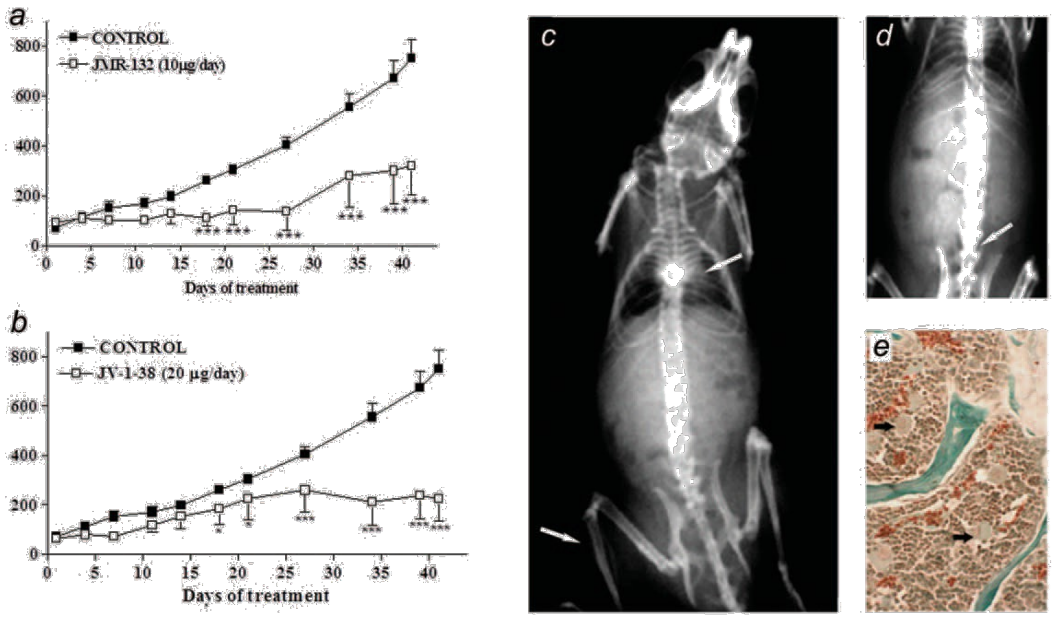

a
CONTROL
JVIR-132 (10µg/day)
Days of treatment
b
CONTROL
JV-1-38 (20 µg/day)
Days of treatment
c
d
e

Figure 2

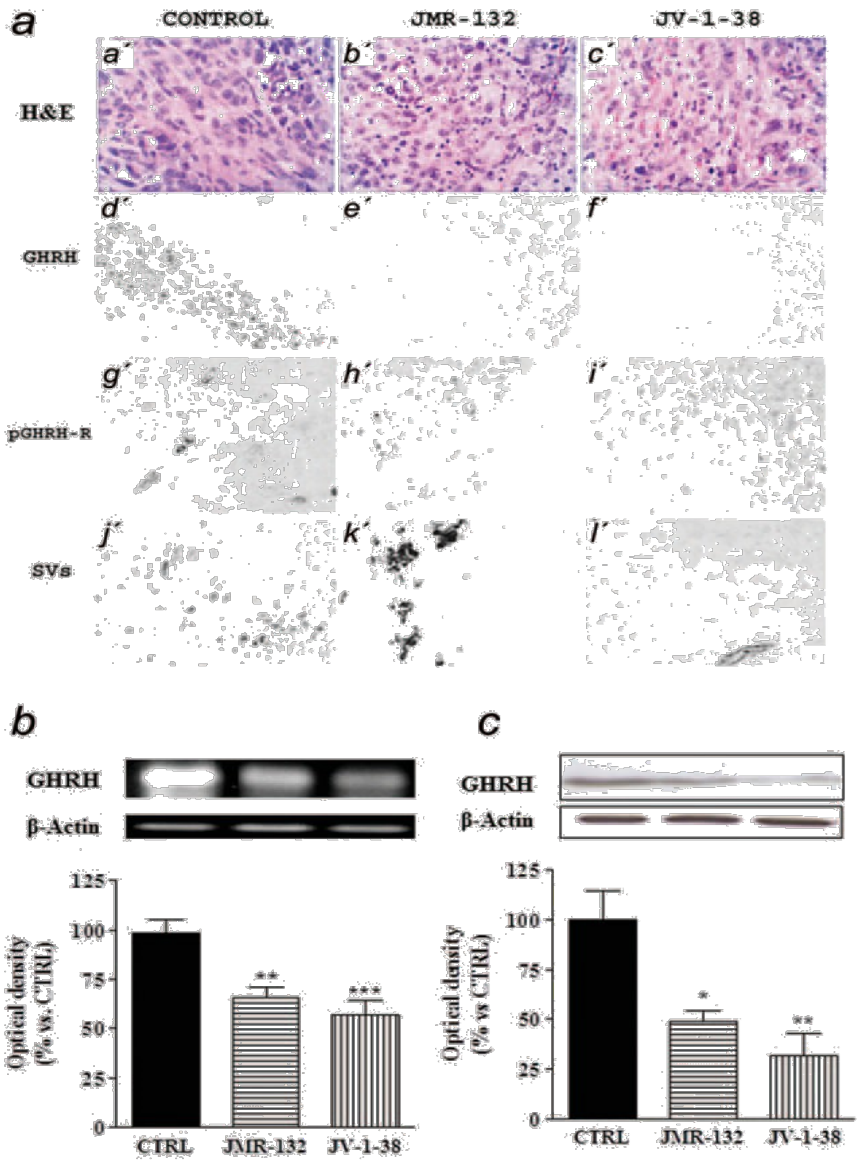

a
CONTROL
JMR-132
JV-1-38
a´
b´
c´
H&E
d´
e´
f´
GHRH
g´
h´
i´
pGHRH-R
j´
k´
l´
SVs
b
GHRH
β-Actin
Optical density (% vs. CTRL)
125
100
75
50
25
0
**
***
CTRL
JMR-132
JV-1-38
c
GHRH
β-Actin
Optical density (% vs CTRL)
125
100
75
50
25
0
*
**
CTRL
JMR-132
JV-1-38

Figure 3

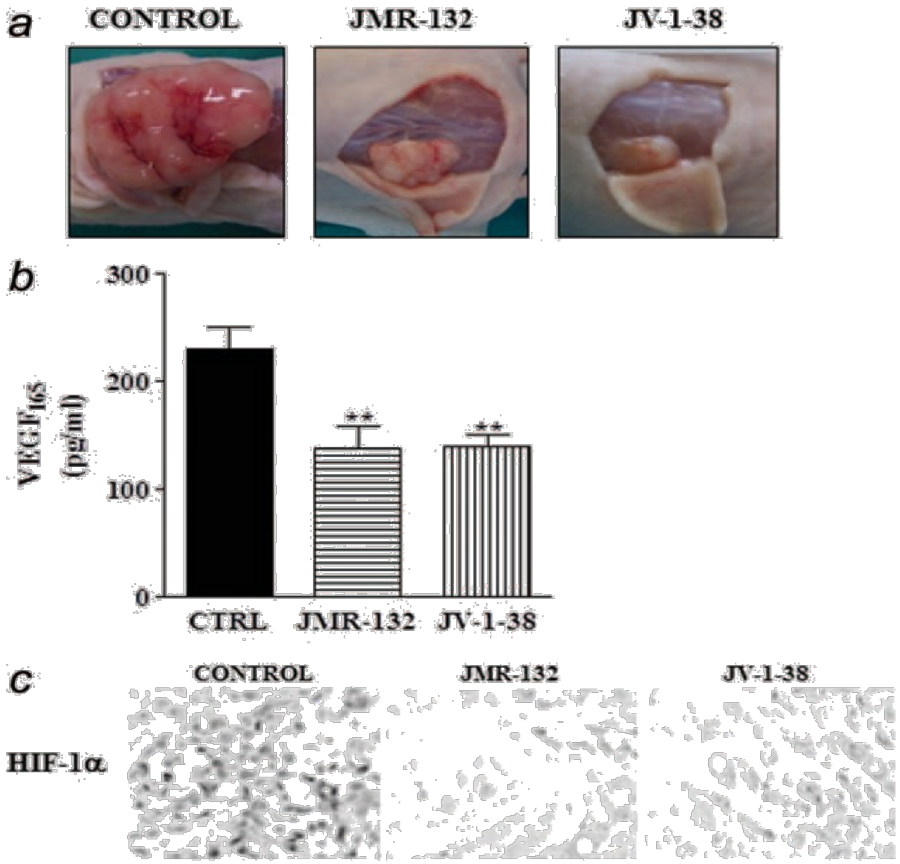

a
CONTROL
JMR-132
JV-1-38
b
300
200
100
0
VEGF165 (pg/ml)
**
**
CTRL
JMR-132
JV-1-38
c
CONTROL
JMR-132
JV-1-38
HIF-1α

Figure 4

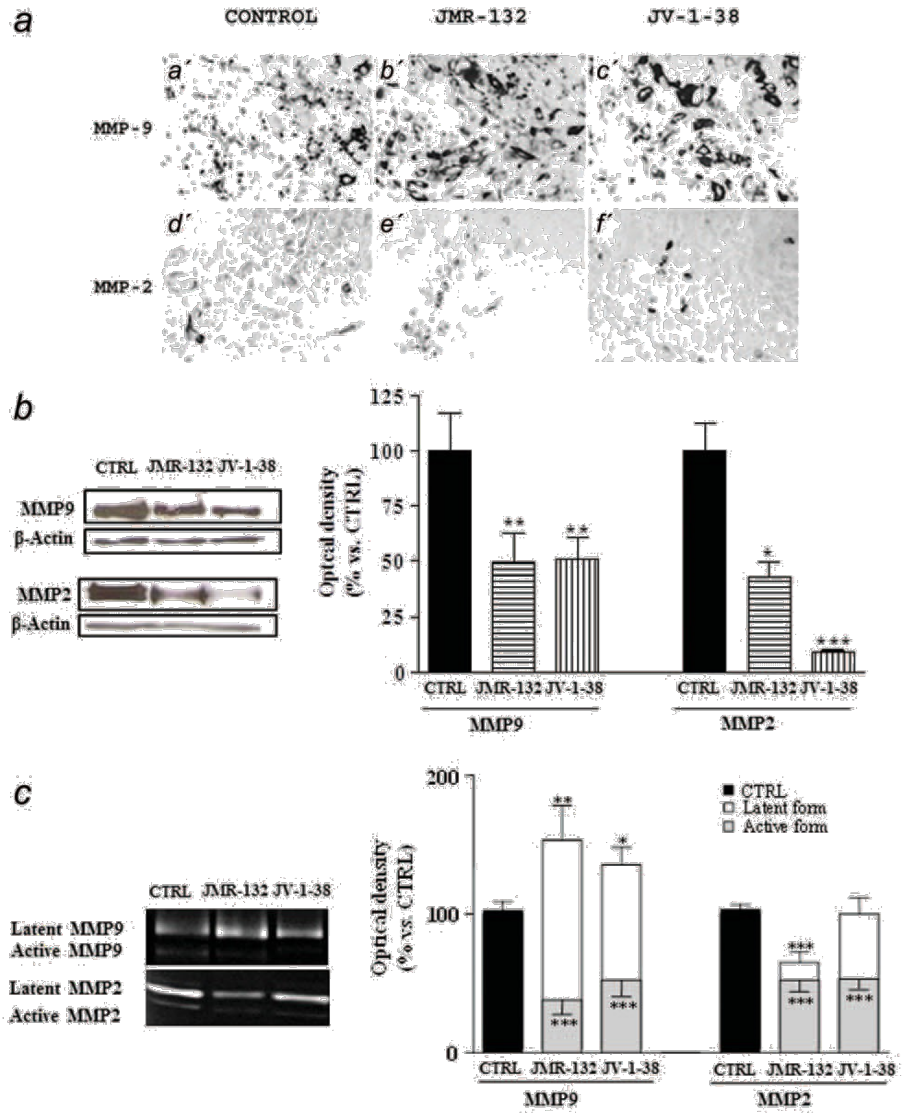

a
CONTROL
JMR-132
JV-1-38
a´
b´
c´
MMP-9
d´
e´
f´
MMP-2
b
CTRL JMR-132 JV-1-38
MMP9
β-Actin
MMP2
β-Actin
Optical density (% vs. CTRL)
125
100
75
50
25
0
**
**
*
***
CTRL JMR-132 JV-1-38
CTRL JMR-132 JV-1-38
MMP9
MMP2
c
CTRL JMR-132 JV-1-38
Latent MMP9
Active MMP9
Latent MMP2
Active MMP2
Optical density (% vs. CTRL)
200
100
0
CTRL
Latent form
Active form
**
*
***
***
***
***
CTRL JMR-132 JV-1-38
CTRL JMR-132 JV-1-38
MMP9
MMP2

Figure 5

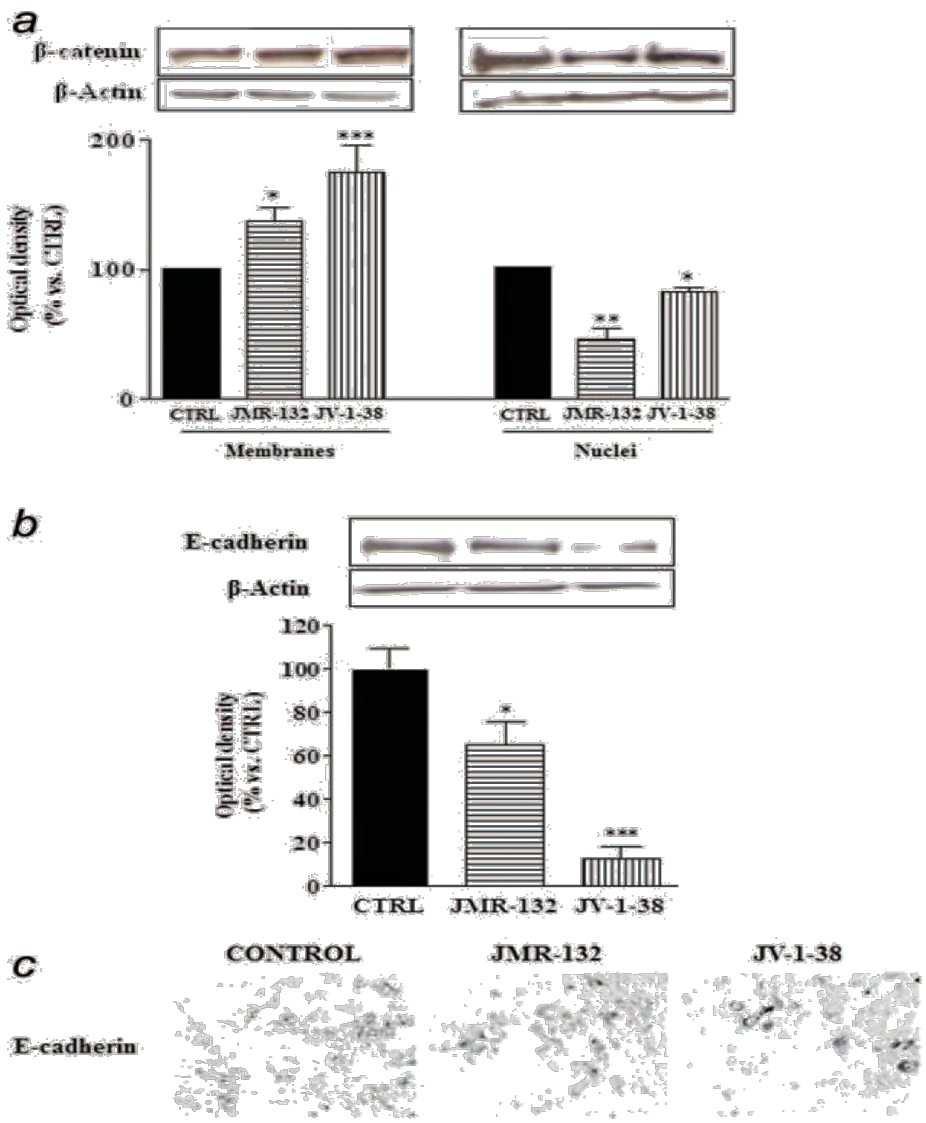
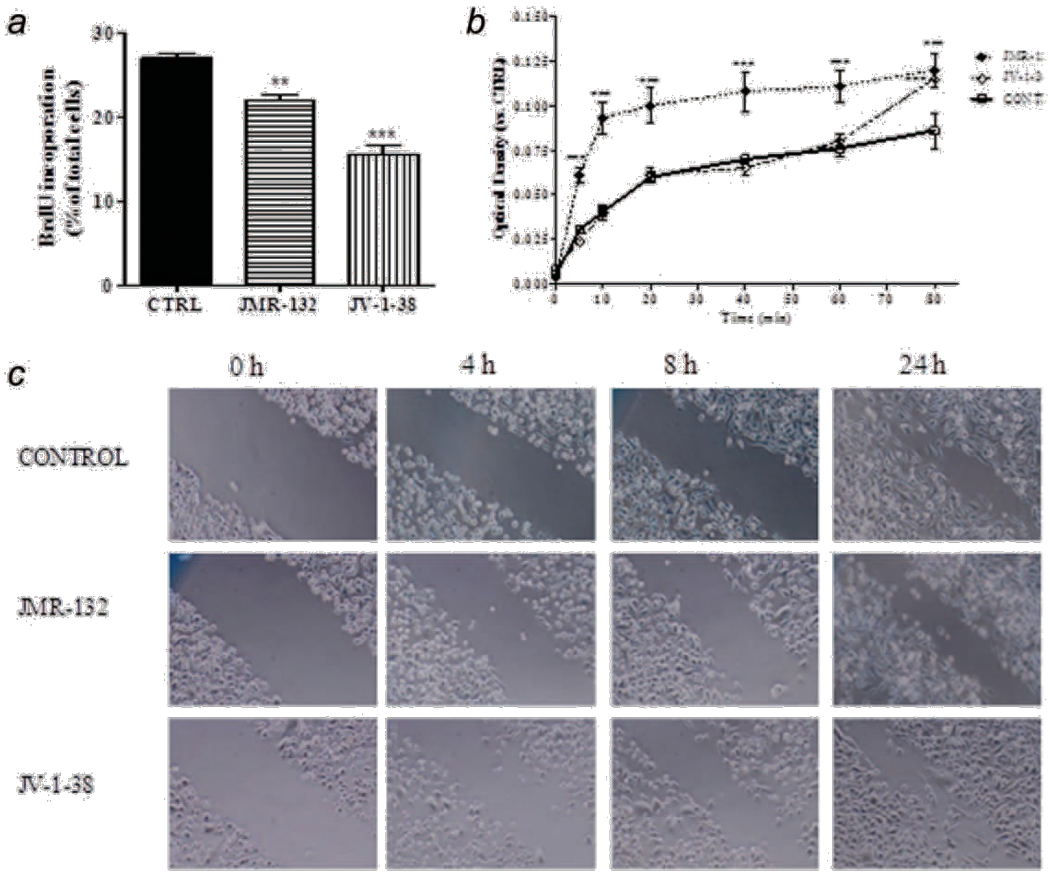

a
β-catenin
β-Actin
Optical density (% vs. CTRL)
200
100
0
*
***
**
*
CTRL
JMR-132
JV-1-38
CTRL
JMR-132
JV-1-38
Membranes
Nuclei
b
E-cadherin
β-Actin
Optical density (% vs. CTRL)
120
100
80
60
40
20
0
*
***
CTRL
JMR-132
JV-1-38
c
CONTROL
JMR-132
JV-1-38
E-cadherin

Figure 6


a
BrdU incorporation (% of total cells)
30
20
10
0
**
***
CTRL
JMR-132
JV-1-38
b
Optical Density (vs. CTRL)
Time (min)
CONT
c
0 h
4 h
8 h
24 h
CONTROL
JMR-132
JV-1-38