Combination of GHRH antagonists and docetaxel shows experimental effectiveness for the treatment of triple-negative breast cancers

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Abstract. In preclinical studies, antagonists of growth hormone-releasing hormone (GHRH) have demonstrated inhibitory effects on the growth of various types of cancers expressing the pituitary type of GHRH receptors (pGHRH-R) and/or its active splice variant 1 (SV1). In this study, we investigated the effectiveness of the treatment of MDA-MB-231 human triple-negative breast cancer (TNBC) with GHRH antagonist JMR-132 alone or in combination with docetaxel. Receptor expression in the MDA-MB-231 human breast cancer cell line was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). Cell viability assays were performed on MDA-MB-231 cells treated with JMR-132, docetaxel or in combination. For studies *in vivo,* a subcutaneous nude mouse xenograft model was used. JMR-132 was administered s.c. at a dose of 10 μg/day and docetaxel at a dose of 10 mg/kg i.p. given on day 1 and 5. Similar regimens were used for the combination of both substances. At the end of the experiment, an mRNA-based human cancer pathway array including 84 major genes was performed on the tumor tissue of mice treated with JMR-132 to elucidate the mechanism of action of GHRH antagonists *in vivo*. The *in vitro* proliferation studies revealed that JMR-132 and docetaxel decreased the cell viability in a dose-dependent manner. The combination of both treatments produced a significantly greater inhibition of cell viability compared to the single agents. Treatment of nude mice bearing MDA-MB-231 xenografts with JMR-132 and docetaxel significantly ($p<0.05$) inhibited tumor growth by 46 and 50%, respectively. Treatment with the combination of JMR-132 and docetaxel led to an inhibition of tumor volume by 71.6% ($p<0.001$). Polymerase chain reaction array analysis revealed that JMR-132 interacts with signal transduction pathways involved in proliferation, apoptosis and angiogenesis. Our results suggest that GHRH antagonists in combination with taxanes may enhance the efficacy of treatment for patients with TNBC expressing the SV1 and/or the pGHRH receptor.

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Introduction

Breast cancer is the most frequently diagnosed cancer in women. Triple-negative breast cancer (TNBC) is a distinct subtype lacking the expression of receptors for estrogen (ER), progesterone (PR) and HER2/neu. Systemic treatment options are limited to chemotherapy. TNBC accounts for 10-17% of all breast cancers and is burdened by a poor prognosis (1-3). Thus, the development of novel therapeutic strategies is of paramount importance. Growth hormone-releasing hormone (GHRH) is a peptide hormone found in the hypothalamus (4), which stimulates growth hormone secretion from the pituitary gland and thus IGF-1 secretion by the liver. Various antagonists of GHRH were synthesized in our laboratory including JMR-132. GHRH antagonists suppress the release of GH from the pituitary resulting in an inhibition of IGF-I production by the liver. However, recent studies have shown an ectopic expression of GHRH and its receptors in various benign tissues (5-7), but more importantly in various types of cancers suggesting that GHRH acts as a local tumor growth factor via an autocrine/paracrine loop in these tissues (8-20).

Several preclinical *in vitro* studies where the hypothalamic/pituitary/hepatic axis is clearly excluded, underlined this hypothesis of an autocrine/paracrine function. Additional studies demonstrated the existence of several splice variants

of the pituitary GHRH receptor (pGHRH-R) of which splice variant 1 (SV 1) is a fully functional receptor (21-24).

GHRH receptors and its splice variants are present in surgical specimens of breast cancers. Our group demonstrated recently, that the pGHRH-R is expressed in 25% and the SV1 receptor in 70% of TNBC tissue samples. Additionally, 80% of these samples expressed GHRH, suggesting the presence of an autocrine/paracrine growth stimulatory loop (25). In HCC1806 human TNBC cells, MAP-kinases ERK-1/2 were activated by treatment with GHRH, and GHRH stimulated proliferation of MDA-MB-231 human breast cancer cell lines through activation of the MAP-kinase phosphorylation pathway via Ras/Raf/MEK/ERK (26,27).

Taxanes such as paclitaxel and docetaxel have been observed to impinge upon several signaling pathways to bring about cell cycle arrest and apoptosis. Some of the most common changes after treatment are Bcl-2 phosphorylation and the activation of mitotic spindle assembly checkpoint (28,29). This class of substances is now well established as one of the most efficacious for the treatment of early and metastatic breast cancer of all molecular subtypes.

We previously demonstrated that combined treatment with GHRH antagonist JMR-132 and docetaxel led to a potentiation of the antiproliferative effect of both compounds in human MX-1 breast cancers xenografted into nude mice (30).

To further explore this novel promising treatment approach, the combination of a GHRH antagonist and a taxane was investigated in *in vitro* and *in vivo* models of TNBC, given the dismal prognosis of this subgroup of breast cancers.

Materials and methods

Peptides and chemicals. The GHRH antagonist JMR-132 was synthesized in our laboratory. For daily injection, JMR-132 was dissolved in 0.1% dimethyl sulfoxide (DMSO) in 10% aqueous propylene glycol solution (vehicle solution). For *in vitro* experiments, JMR-132 was dissolved in DMSO and diluted further with PBS to the used concentration not exceeding 0.1% DMSO. Docetaxel (DOC) ready-to-use vials from Sanofi-Aventis were used for the experiments and diluted according to the manufacturer's instructions.

Cell lines and animals. The triple negative breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37˚C and 5% CO_2 atmosphere.

Female athymic nude mice (Ncr *nu/nu*) (5-6 weeks old) were obtained from the National Cancer Institute (NCI, Bethesda, MD, USA). The animals were housed in sterile cages under laminar flow hoods in a temperature-controlled room with a 12-h light/12-h dark schedule. They were fed autoclaved chow and water *ad libitum*. The Institutional Animal Care and Use Committee reviewed the protocols of the animal experiments and granted full approval.

Reverse transcription and RT-PCR. Total RNA was isolated and DNase-treated using the Macherey-Nagel NucleoSpin® kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). The yield and the quality of RNA samples were determined spectrophotometrically using 260 nm, and 260/280 and 260/230 nm ratio. Two micrograms of RNA from the sample was reverse transcribed into cDNA by QuantiTect reverse transcription kit (Qiagen, Hilden, Germany) in a final volume of 40 μl. We evaluated the mRNA expression of human pituitary GHRH receptor (pGHRH-R) and SV1 of GHRH-R as previously reported (31). The probe and primers for pGHRH-R and sense and antisense specific primers for SV1 were described previously (23).

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

Cell viability assays. For cell viability studies, 5,000 cells/well were seeded in a 96-well plate in 100 μl medium. After 24 h, the culture medium was replaced by JMR-132 at different concentrations (0.1, 1, 10 μM) and in other studies by JMR-132 (5 μM), DOC (1.28 or 0.635 nM) or the combination of JMR-132 and DOC at different concentrations. The cells were then incubated for 72 h in a humidified atmosphere at 37˚C. The effect of the agents on cell proliferation was evaluated using the MTS assay (CellTiter 96 Aqueous One Solution cell proliferation assay; Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Absorbance was measured at 550 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. All experiments were performed in hexaplicates and repeated 3 times.

In vivo experiments. For the animal experiments, $10x10^6$ MDA-MB-231 cells/mouse were injected into the flanks of 4 female nude mice. The resulting tumors were harvested and minced into ~3-mm^3 pieces and transplanted into both flanks of each female nude mouse using a mini trocar. The experiment was initiated when the MDA-MB-231 tumors had reached a volume of ~110 mm^3. Mice were divided into 4 experimental groups: control (animals n=10; tumors n=15), JMR-132 (10 μg/day) (animals n=8; tumors n=10), DOC (10 mg/kg on day 1 and 5, i.p.) (animals n=6; tumors n=10), and a combination of DOC (10 mg/kg on day 1 and 5, i.p.) and JMR-132 (10 μg/day) (animals n=8, tumors n=13). The weight of the animals was measured weekly.

Tumor volume (length x width x height x 0.5236) was measured weekly, and tumor doubling time was calculated at the end of the study using the formula: Study duration/(log final tumor volume - log initial tumor volume)/log2.

At the end of the experiment, mice were sacrificed under anesthesia, tumors were excised, snap frozen at -70˚C for further experiments and a full necropsy was performed.

Human cancer PathwayFinder PCR array. Quantitative mRNA expression analysis of 84 genes representative of the major biological pathways involved in transformation and tumorigenesis was performed using the Human Cancer PathwayFinder™ RT^2 Profiler™ PCR array (PAHS-033A; Qiagen, Valencia, CA, USA). RNA was extracted and DNAse-treated using the Macherey-Nagel NucleoSpin kit from 6 representative tumors grown in the nude mice (3 tumors from the control and 3 tumors from the animals treated with JMR-132). The yield and the quality of RNA samples, synthesis of cDNA and real-time RT-PCT arrays were performed as previously described (32). Real-time PCR reactions were performed using the iCycler iQ™ Real-Time PCR detection system. All genes represented by the array showed a single peak on the melting curve characteristic to the specific products. Data analysis of gene expression was performed as previously described (9). Briefly, using Excel-based PCR array data analysis software provided by the manufacturer, fold-changes in gene expression were calculated using the ΔΔCt method, and 5 stably expressed housekeeping genes (B2M, HPRT1, RPL13A, GAPDH and ACTB) were used for normalization of the results.

Statistical analysis. Data are expressed as means ± SE. One-way ANOVA followed by Bonferroni t-test or a two-tailed Student's t-test was used where appropriate, and significance was accepted at $p<0.05$.

Results

Receptor expression for pGHRH-R and SV1. Expression of mRNA of SV1 was detected in the MDA-MB-231 cell line (Ct value 32.6) by real-time RT-PCR. However the expression of the pGHRH-R could not be determined. For the positive control (pituitary) the Ct value for pGHRH-R was 20.5 and for SV1, 33.3.

Effect of GHRH antagonist JMR-132 and DOC on the viability of MDA-MB-231 human breast cancer cells in vitro. In the first experiment, we determined the effect of GHRH antagonist JMR-132 at different concentrations on the MDA-MB-231 cell line in the proliferation assays. A significant ($p<0.05$) dose-dependent inhibition of cell viability was noted when compared to the controls. In the second experiment, we evaluated the combination of JMR-132 and DOC and demonstrated a significant ($p<0.05$) dose-dependent inhibition of cell viability at different concentrations of DOC. The combination of both substances led to an even greater inhibition of cell viability compared to treatment with the single agents. This effect became statistically significant at a combination dose of 5 μM JMR-132 and 1.28 nM DOC (Figs. 1 and 2).

Effect of GHRH antagonist JMR-132 and DOC on the growth of MDA-MB-231 human breast cancer cell xenografted tumors in nude mice. The tumor volume was significantly ($p<0.05$) reduced by 46% following treatment with JMR-132 at a dose of 10 μg/day s.c., when compared to the controls at the end of the study (Fig. 3). Additionally, JMR-132 significantly ($p<0.05$) increased the tumor doubling time (Table I). When nude mice bearing MDA-MB-231 tumors received DOC as a monotherapy, tumor growth was inhibited by 50% ($p<0.05$, Fig. 3) as compared to the control group, and the tumor doubling time was significantly prolonged compared to the controls ($p<0.05$, Table I).

Combined treatment with JMR-132 and DOC resulted in significant tumor inhibition ($p<0.001$) after 14 days, and the inhibition remained significant until the end of the experiment when compared to the controls. A significant reduction in tumor volume by 70% was achieved in this group (Fig. 3). The combination treatment of DOC and JMR-132 was significantly more potent ($p<0.05$) with respect to inhibition of the tumor volume when compared to the monotherapy groups. The tumor doubling time was also significantly ($p<0.05$) extended when compared to the other groups (Fig. 3 and Table I).

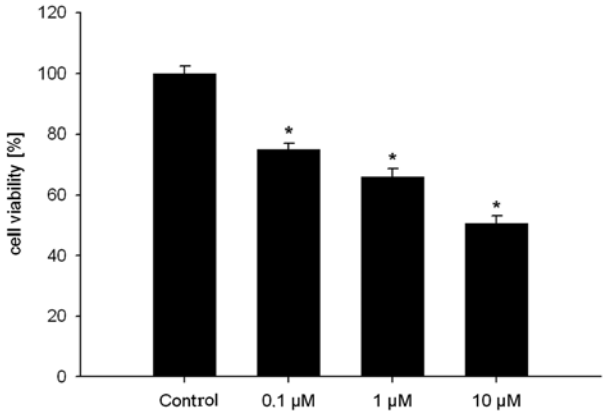

Figure 1. Proliferation assays of MDA-MB-231 cells treated with different concentrations of the GHRH antagonist JMR-132. Error bars, SE; *$p<0.05$ compared to control.

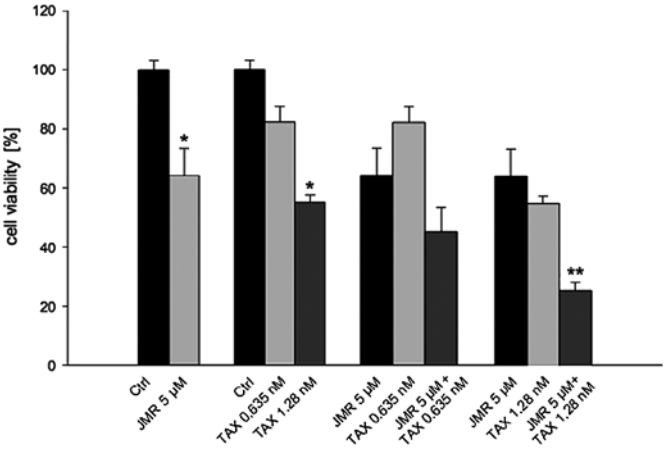

Figure 2. *In vitro* proliferation assay of MDA-MB-231 cells. Cells were treated with the GHRH antagonist JMR-132 and/or docetaxel (TAX) at different concentrations. Error bars, SE; *$p<0.05$ compared to black bars; **$p<0,05$ compared to both bars.

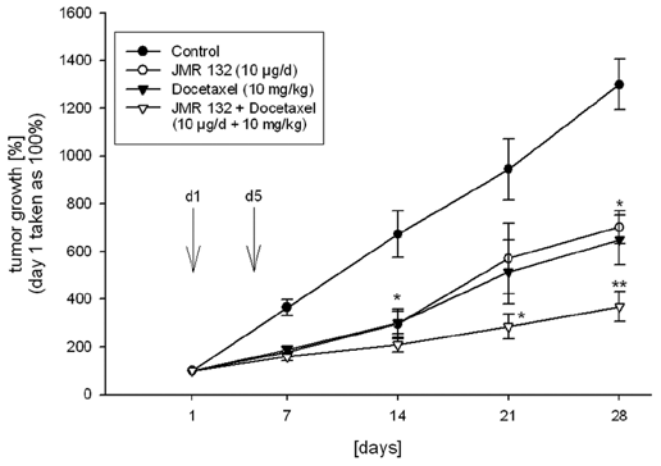

Figure 3. Inhibition of tumor growth *in vivo*. Error bars, SE. Arrows indicate i.p. injections of docetaxel (DOC) (10 mg/kg). JMR-132 was administered s.c. daily, docetaxel was administered i.p. *Day 14: ctrl vs. JMR-132, DOC, JMR-132 + DOC; p<0.05, respectively. *Day 21: ctrl vs. JMR-132 + DOC; p<0.05. *Day 28: ctrl vs. JMR-132, DOC; p<0.05, respectively. **Day 28: JMR-132 + DOC vs. all other groups; p<0.05.

Toxicity. No signs of drug-induced toxicity were observed in any of the mouse groups, as reflected by no differences in body and organ weights between the treated groups and the control animals.

Effect of GHRH antagonist JMR-132 on expression of genes related to apoptosis, angiogenesis and metastasis. The PCR array used contains 84 unique genes related to signal transduction, cell cycle and apoptosis. All genes represented by the array showed a single peak on the melting curve characteristic to the specific products. Fifteen genes in the MDA-MB-231 xenografts showed a significant change in mRNA expression after treatment with JMR-132 compared to the controls (Table II). Upregulated genes were found to belong to the TNF ligand family FASLG, LTA, TNFSF10, CD70, TNFSF8 (5.28-, 10.56-, 2.00-, 9.19-, 7.46-fold increases; p<0.05 for all). Significant transcriptional suppression of angiogenesis-related gene ANGPT1 (2.93-fold decrease, p<0.05) was observed. Significant alterations in transcriptional gene expression involved in apoptosis or cell death were detected for BCL2 (3.48-fold decrease, p<0.05), and HRK, CASP14, CASP4, CASP5, DAPK, CIDEA (14.93-, 14.93-, 2.3-, 10.53-, 3.03-, 9.17-fold increases, p<0.05 for all). Changes in the mRNA levels for molecules related to invasion and metastasis were found for MMP2 (2.07-fold decrease, p<0.05) and TWIST1 (2.73-fold increase, p<0.05).

Discussion

Novel therapeutic strategies for the treatment of many types of cancers are based on agents targeting cell signaling pathways that interacting with the cell cycle, apoptosis, proliferation or angiogenesis. New therapeutic agents such as trastuzumab and lapatinib have been clinically used with great success for years.

Previously, we found that the pGHRH-R is expressed in 25% and SV1 in 70% of TNBC tissue samples, and in 80% of these samples GHRH is expressed (25). Various findings suggest that GHRH acts as a growth factor in GHRH receptor-positive malignancies, which may represent an attractive therapeutic target for antagonists of GHRH (10-13,15-19).

Table I. Tumor doubling time calculated at the end of the study.

	Tumor growth (%) on day 28	Tumor doubling times (days)
Control	1,299±106.96	7.90±0.47
JMR-132	701±69.8	10.26±0.52[b]
Docetaxel	649±103.26	12.32±1.86[b]
JMR-132 + docetaxel	369±62.51[a]	19.52±1.02[c]

Tumor doubling time was calculated at the end of the study using the formula: Study duration/(log final tumor volume - log initial tumor volume)/log2. Values are the means ± SE. [a]JMR-132 + docetaxel vs. all other groups; p<0.05. [b]JMR-132 or docetaxel vs. control; p<0.05 [c]JMR-132 + docetaxel vs. all other groups; p<0.05.

Since chemotherapy is the only therapeutic option for TNBC, GHRH antagonists could provide a novel targeted approach for this subgroup of breast cancers.

In the present study we demonstrated the presence of SV1 but not pGHRH-R in the human triple-negative breast cancer cell line MDA-MB-231 and the antitumoral effect of the GHRH antagonist JMR-132. This finding provides strong evidence for the important role of SV1 as a functional receptor. Treatment with JMR-132 significantly inhibited cell proliferation in a dose-dependent manner in MDA-MB-231 human breast cancers *in vitro*. JMR also demonstrated antitumor activity *in vivo*. The *in vivo* study also revealed that GHRH antagonist JMR-132 interacts with various signal transduction pathways involved in proliferation, apoptosis and angiogenesis. In TNBC, the MAPK and the AKT signal transduction pathways have been found to be significantly overactivated (33,34). These two pathways induce a variety of cellular events, of which many are connected to proliferation and resistance to apoptosis. Notably, targeting the GHRH signal transduction pathway also resulted in the suppression of genes inducing proliferation and resistance to apoptosis. Thus, targeting the GHRH-receptor may also result in a synergistic effect in combination with novel antitumor agents, such as AKT and ERK inhibitors and not only with chemotherapeutic agents as demonstrated in our study.

Docetaxel is a chemotherapeutic agent frequently used in the treatment of breast cancers. Furthermore, taxanes have been favorably used in combination with monoclonal antibodies and small-molecule agents such as trastuzumab or lapatinib. Therefore, taxane could be likely candidates for combination therapy with GHRH antagonists. Accordingly, the present study showed that a combination of DOC with JMR-132 potentiated the antitumor effect *in vitro* and *in vivo* as compared to each substance alone.

In conclusion, this investigation demonstrated that GHRH antagonists are effective for the treatment of SV1-positive TNBC and can be combined with taxane-based chemotherapy. As in our study and various other studies, GHRH antagonists

Table II. Modulated genes with at least a 2-fold change relative to the untreated control after treatment of MDA-MB-231 xenografts with the GHRH antagonist JMR-132.

Name of gene	Gene symbol	Accession no.	Fold change[a]
Angiopoietin 1	ANGPT1	NM_001146	**-2.93**
Antigen identified by monoclonal antibody Ki-67	MKI67	NM_002417	-2.69
B-cell CLL/lymphoma 2	BCL2	NM_000633	**-3.48**
Baculoviral IAP repeat containing 8	BIRC8	NM_033341	4.29
Bcl2-like 10	BCL2L10	NM_020396	3.03
Breast cancer 1, early onset	BRCA1	NM_007294	-2.73
Caspase 14	CASP14	NM_012114	**14.93**
Caspase 4	CASP4	NM_001225	**2.30**
Caspase 5	CASP5	NM_004347	**10.53**
CD40 ligand	CD40LG	NM_000074	5.66
CD70 molecule	CD70	NM_001252	**9.19**
Cell death-inducing DFFA-like effector a	CIDEA	NM_001279	**9.17**
Cullin 1	CUL1	NM_003592	-2.18
Cyclin B2	CCNB2	NM_004701	-2.04
Cyclin D2	CCND2	NM_001759	12.77
Death-associated protein kinase 1	DAPK1	NM_004938	**3.03**
Fas ligand (TNF superfamily, member 6)	FASLG	NM_000639	**5.28**
Fibroblast growth factor receptor 2	FGFR2	NM_000141	7.21
Granzyme A	GZMA	NM_006144	**3.14**
Harakiri, BCL2 interacting protein	HRK	NM_003806	**14.93**
Integrin, β 3	ITGB3	NM_000212	-3.87
Interleukin 8	IL8	NM_000584	3.13
Lymphotoxin α (TNF superfamily, member 1)	LTA	NM_000595	**10.56**
MAD2 mitotic arrest deficient-like 1 (yeast)	MAD2L1	NM_002358	-3.08
Matrix metallopeptidase 1	MMP1	NM_002421	4.44
Matrix metallopeptidase 2	MMP2	NM_004530	**-2.07**
Platelet-derived growth factor β polypeptide	PDGFB	NM_002608	2.55
Serpin peptidase inhibitor, clade B (ovalbumin), member 5	SERPINB5	NM_002639	-2.56
TEK tyrosine kinase, endothelial	TEK	NM_000459	2.54
Tumor necrosis factor	TNF	NM_000594	6.28
Tumor necrosis factor	TNF	NM_000594	16.00
Tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	NM_003810	**2.00**
Tumor necrosis factor (ligand) superfamily, member 8	TNFSF8	NM_001244	**7.46**
Tumor necrosis factor receptor superfamily, member 9	TNFRSF9	NM_001561	9.19
Tumor protein p53	TP53	NM_000546	-2.19
Twist homolog 1	TWIST1	NM_000474	**2.73**

Multiple genes related to cell proliferation, apoptosis, cell cycle, angiogenesis, invasion and metastasis were evaluated for expression using real-time PCR via RT^2 Profiler™ PCR array system. The table lists the genes of interest evaluated and their fold increase or decrease in MDA-MB-231 human breast cancer xenografts treated with GHRH antagonist JMR-132 (10 μg/day). Data represent the fold differences in the expression of individual genes between the JMR-132-treated group and the controls. [a]Positive values indicate upregulation of individual genes, while negative values indicate downregulation. Three experiments were run for each study group. The data were evaluated by two-tailed Student's t-test. Bold print depicts significant changes ($p<0.05$).

displayed no toxic side effects. Thus, they should be assessed in combination with taxanes in phase I trials for the treatment of patients with TNBC expressing the SV1 and/or pGHRH receptor.

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