

# 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

EVA POZSGAI<sup>1,2,3</sup>, ANDREW V. SCHALLY<sup>1,2</sup>, ENIKO HOCSAK<sup>3</sup>, MARTA ZARANDI<sup>1,2</sup>, FERENC RICK<sup>1,2,4</sup> and SZABOLCS BELYEI<sup>1,2,5</sup>

<sup>1</sup>Veterans Affairs Medical Center and South Florida Veterans Affairs Foundation for Research and Education; <sup>2</sup>Department of Pathology, University of Miami, Miller School of Medicine, Miami, FL 33125, USA; Departments of <sup>3</sup>Biochemistry and Medical Chemistry, <sup>4</sup>Anatomy, and <sup>5</sup>Oncology, University of Pécs, Pécs 7624, Hungary

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**Abstract.** Growth hormone releasing hormone (GHRH) antagonists have been developed for the treatment of various cancers. We investigated the effects of a novel GHRH antagonist, MIA-602, on nine breast cancer cell lines, differing in their expression for estrogen-, progesterone- and HER-2 receptors. We detected the presence of pituitary-type GHRH receptors (pGHRH-R) on 6 of the 9 breast cancer cell lines. The main splice variant of pGHRH-R, SV1, was found on all 9 cell lines. MTT assay showed that following treatment with MIA-602, cell viability decreased significantly in all 9 cell lines. The reduction in cell viability was greater in cells positive for both pGHRH-R and SV1, than in cells positive for only SV1, but the difference was not significant. Using Western blotting, we demonstrated that the levels of phospho-Akt, -GSK3 $\beta$  and -ERK1/2 decreased significantly following exposure to MIA-602 and the level of phospho-p38 increased after treatment. The reduction of the phosphorylated anti-apoptotic proteins was significantly greater in cells where both pGHRH-R and SV1 were present, than where only SV1 was expressed. In conclusion, our study shows that MIA-602 is effective against a wide range of breast cancer cells *in vitro*, independently of their receptor positivity, suggesting the potential use of GHRH antagonists also in the treatment of triple-negative breast cancer. The effect of MIA-602 was mediated nearly as well in tumors that expressed only the SV1 receptor compared to those in which both SV1 and pGHRH-R

were present, although a difference could be detected at the level of cell signaling.

## Introduction

Breast cancer is the most common malignancy in women and represents a leading cause of cancer-related deaths among the female patients worldwide (1-3). The great diversity of breast cancers in their histological and behavioral characteristics requires the application of tailored therapies. Effective targeted treatment for breast cancer positive for estrogen-, progesterone- and HER-2 receptors is currently available but satisfactory treatment modalities for triple-negative breast cancers still need to be developed (4,5).

Antagonistic analogues of growth hormone releasing-hormone (GHRH), developed in our laboratory, have been shown to inhibit the growth of various cancers (6-11), through indirect and direct pathways (6). Thus, GHRH antagonists can suppress tumor growth indirectly by blocking GH release from the pituitary and consequently the hepatic production of IGF-I (6,12), which is an established mitogen for various cancers (13), is likewise inhibited. Nevertheless, GHRH antagonists, exert their main antitumor effects through direct mechanisms (12,14), by the inhibition of tumoral GHRH and IGF-I and/or IGF-II (6,14-16), which serve as autocrine/paracrine growth factors. Pituitary-type GHRH receptors (pGHRH-R) and their splice variants (SVs) have been detected in different human cancers and various cancer cell lines (17-21).

Earlier reports have shown that GHRH antagonists decrease cell viability of breast cancers *in vitro* (9-11) and inhibit the growth and metastases of orthotopic breast cancer models *in vivo* (8,16). Previously, we demonstrated that GHRH antagonists induce cell death through the major apoptotic pathways and by a membrane destabilizing effect on the mitochondrial membrane potential in two glioblastoma cell lines (22). In the present study, our goal was to screen a number of breast cancer cell lines, differing in their expression of estrogen/progesterone and HER-2 receptors, for their response to treatment with a GHRH antagonist. This is the first report comparing the effects of a novel, potent GHRH antagonist, MIA-602, on

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*Correspondence to:* Dr Szabolcs Belyei, Veterans Affairs Medical Center and South Florida Veterans Affairs Foundation for Research and Education, 1201 Northwest 16th street, Research (151), Miami, FL 33125, USA  
E-mail: belyeisz@gmail.com

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proliferation of breast cancer cells as well as on the key cell signaling pathways.

## Materials and methods

**Peptides and chemicals.** GHRH antagonist, MIA-602, was synthesized in our laboratory by solid-phase methods and purified by reversed-phase HPLC as described previously (18). The chemical structure of MIA-602 is [(PhAc-Ada)<sup>0</sup>-Tyr<sup>1</sup>, D-Arg<sup>2</sup>, Fpa<sup>56</sup>, Ala<sup>8</sup>, Har<sup>9</sup>, Tyr(Me)<sup>10</sup>, His<sup>11</sup>, Orn<sup>12</sup>, Abu<sup>15</sup>, His<sup>20</sup>, Orn<sup>21</sup>, Nle<sup>27</sup>, D-Arg<sup>28</sup>, Har<sup>29</sup>]hGH-RH(1-29)NH<sub>2</sub>. GHRH(1-29)NH<sub>2</sub> was also synthesized in our laboratory. For the experiments, GHRH(1-29)NH<sub>2</sub> and MIA-602 were dissolved in 0.1% DMSO and diluted with incubation media.

**Cell lines.** The cell lines (HCC1806, MDAMB468, MDAMB435S, MCF7, T47D, HCC1937, BT474, MX-1) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured at 37°C in a humidified 95% air/5% CO<sub>2</sub> atmosphere. The culture media were purchased from Gibco (Carlsbad, CA).

**Polymerase chain reaction (PCR).** Total RNA was extracted using the Nucleospin kit (Macherey-Nagel, Germany). The yield and quality of total RNA was determined spectrophotometrically using the absorbance at 260 and 260/280 nm ratio, respectively. RNA (2 µg) with a final volume of 40 µl was reverse transcribed into cDNA with the QuantiTect<sup>®</sup> reverse transcription kit (Qiagen, Valencia, CA) using Veriti<sup>™</sup> 96-well thermal cycler (Applied Biosystems, Foster City, CA). We evaluated the expression of mRNA for human pGHRH-R, splice variant 1 of GHRH-R (SV1) and β-actin. Probes and primers for pGHRH-R, and β-actin and sense- and antisense-specific primers for SV1 were described previously (23). All real-time PCR reactions were performed in the iCycler iQ<sup>™</sup> real-time PCR detection system (Bio-Rad, Hercules, CA). All thermal cycling conditions were described in former studies (23). All samples were run as described earlier (24). Briefly, samples were run in triplicate and each well of PCR reaction contained 25 µl as final volume including 2 µl of cDNA, 200 nM of gene specific primers and 400 nM of probes. iQ<sup>™</sup> Supermix (Bio-Rad) was used in the PCR reactions for pGHRH-R, and β-actin and iQ<sup>™</sup> SYBR-Green Supermix (Bio-Rad) for SV1. The efficiencies of all primers (Invitrogen Life Technologies, Carlsbad, CA) and probes (Integrated DNA Technologies, Coralville, IA) were tested prior to the experiments and found to be efficient in the range of 95-105%. Normal human pituitary was used as positive control and human β-actin as a housekeeping gene. Negative samples were run in each reaction consisting of no-RNA in reverse transcriptase reaction and no-cDNA in PCR reaction.

**Proliferation assay.** Cells were seeded onto 96-well-plates at a starting density of 2500 cells/well, cultured overnight, starved for 24 h with medium containing no FBS and then treated with GHRH(1-29)NH<sub>2</sub> or GHRH antagonist, MIA-602 for 48 h. After the treatment, the relative number of viable cells was measured in comparison with the untreated control and the solvent control, using Cell Titer 96 AQueous Assay (Promega) at 490 nm in a Victor3 multilabel counter (Perkin-

Table I. GHRH, SV-1 and ER receptor status of the nine breast cancer cell lines.

Breast cancer cell lines	GHRH receptor	SV-1 receptor	ER receptor
HCC1806	+	+	+
MDAMB468	+	+	-
HCC1937	+	+	-
MCF7	+	+	+
BT474	+	+	+
MX1	+	+	-
T47D	-	+	+
MDMB435S	-	+	-
MDAMB231	-	+	-

Elmer, Waltham, MD, USA) according to the manufacturer's instructions. All experiments were done at least in quadruplicate and repeated three times. The percentage of cell survival was determined by comparing the absorbance value of the vehicle control.

**Isolation of subcellular fractions.** Cells were harvested and centrifuged at low-speed, then the pellet was dispersed by vortexing in lysis buffer [50 mM Tris-HCl (pH 8.0), 1% Triton X-100, 10% glycerol, 1 mM EDTA, 250 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 100 mM sodium fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin and 10 mg/ml pepstatin] for 10 min at 4°C. Isolation of cytosol, nuclear and mitochondrial fractions was carried out by standard protocols as described previously (25).

**Western blot.** Cells were washed with PBS, and directly lysed in lysis buffer. Cell lysates were adjusted to equal protein concentrations (NanoDrop Technologies, Inc., Wilmington, DE), resuspended in 2X sample loading buffer containing 4% SDS, 20% glycerol, 120 mM Tris and bromophenol blue, and boiled for 5 min. Protein samples were then subjected to SDS-polyacrylamide gel electrophoresis. Proteins on the gel were transferred onto nitrocellulose membranes that were blocked with 50-50% Odyssey buffer and phosphate-buffered saline (PBS) for 1 h at room temperature. Subsequently, the membranes were incubated with the indicated primary antibodies overnight at 4°C. p-AKT, p-ERK1/2, pGSK3β and phospho-p38 primary antibodies were purchased from Cell Signaling. α-tubulin primary antibody was obtained from Calbiochem. After washing with PBS containing 0.1% Tween-20, the membranes were incubated with the appropriate secondary antibody. The immunoreactive bands were visualized with the Odyssey Infrared Imaging System and V. 3.0 software was used (LI-COR Biosciences, Lincoln, Nebraska).

**Statistical analysis.** Results are expressed as means ± SE. Results were compared using Student's t-test, p<0.05 being accepted as statistically significant.

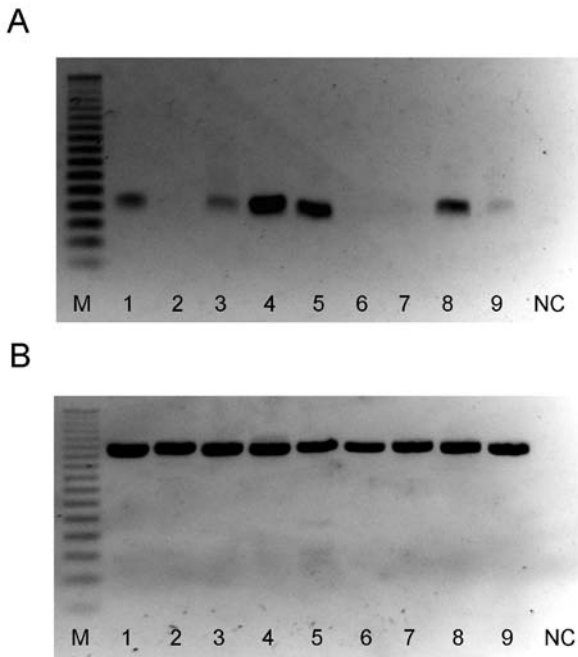


Figure 1. Reverse-transcription PCR gel electrophoresis of receptors of GHRH (A) and SV1 (B) from 9 different breast cancer cell lines. M, 50-bp DNA size marker; 1, MDAMB468; 2, MDAMB435S; 3, BT447; 4, MX1; 5, HCC1806; 6, T47D; 7, MDAMB231; 8, HCC1937; 9, MCF7; NC, negative control.

## Results

*The presence of pGHRH-R and SV1 on nine different breast cancer cell lines.* Reverse transcribed mRNA from nine different breast cancer cell lines (HCC1806, MDAMB468, MDAMB435S, MCF7, T47D, HCC1937, BT474, MX-1) was subjected to PCR to determine the expression of the pituitary type of GHRH receptor (Fig. 1A) and its main truncated splice variant, SV1 (Fig. 1B). The 523 bp amplicon for SV1 beginning within the intron between exon 3 and 4 of the GHRH-R gene and ending in the exon 7 was identified in all the nine cell lines. Specific primers for the pGHRH-R spanning from exon 3 to exon 8 were used and the amplification of a 145 bp fragment was detected in 6 (HCC1806, MDAMB468, MCF7, HCC1937, BT474, MX-1) of the nine cell lines (Fig. 1A and B, and Table I). Our results are in line with previous reports on the presence of pGHRH-R and SV1 on breast cancer cell lines (11,26).

*The inhibitory effect of GHRH antagonist MIA-602 on the cell viability of breast cancer cell lines.* Nine breast cancer cell lines were treated with GHRH antagonist MIA-602 at 0.1, 1, 5 and 10  $\mu$ M concentrations for 48 h. The untreated cells served as negative controls. The exposure to MIA-602 at all concentrations resulted in a reduced cell proliferation.

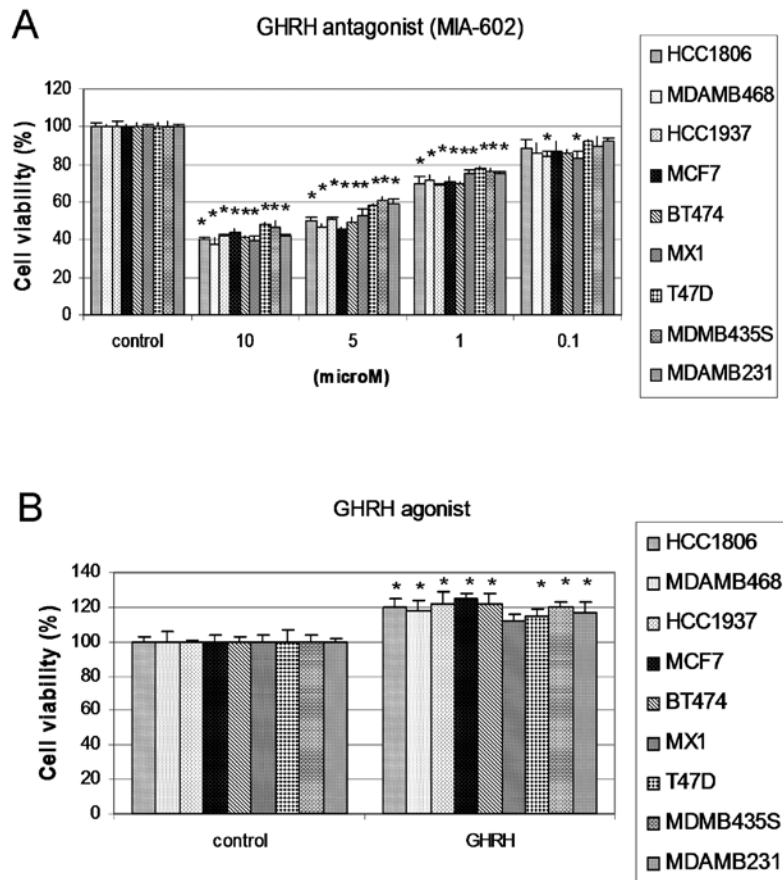


Figure 2. Inhibitory effect of GHRH antagonist, MIA-602 (A), at different concentrations, on 9 different breast cancer cell lines. The effect of GHRH(1-29)NH<sub>2</sub> (B) at 1  $\mu$ M concentration on the different breast cancer cell lines cell lines. Cell viabilities were measured by an MTT (methylthiazolydiphenyl-tetrazolium bromide) assay and were expressed as percentage of untreated cells. The results are the mean  $\pm$  SEM of three independent experiments performed at least in quadruplicate. \*p<0.05.

We determined that the concentration at which cellular growth was inhibited by 30% ( $IC_{30}$ ) was 1  $\mu$ M and hereafter used this concentration in subsequent experiments. After treatment with 1  $\mu$ M MIA-602, the cell viability decreased significantly in all cancer cell lines with a slightly greater reduction in the 6 GHRH-R-positive cell lines (Fig. 2A, Table I). Treatment with GHRH(1-29) $NH_2$ , on the other hand, augmented the proliferation of the cell lines (Fig. 2B). Used at the same concentration, MIA-602 inhibited cell proliferation and abolished the effect of exogenous GHRH(1-29) $NH_2$  (data not shown).

*The effect of MIA-602 on key cell signaling pathways in breast cancer cell lines.* Previously, we reported that the exposure of glioblastoma cells to MIA-602 changed the levels of the anti-apoptotic proteins, phospho-Akt, phospho-GSK3 $\beta$  and phospho-ERK1/2 (38). In this study we treated nine breast cancer cell lines, differing in their receptor positivity, with 1  $\mu$ M MIA-602, and using Western blotting determined the levels of the anti-apoptotic proteins at 0, 5, 10 and 30 min following exposure. In accord with earlier findings, treatment with MIA-602 caused a significant decrease in the expression of p-Akt and pGSK3 $\beta$  at 5 min, a greater reduction at 10 min, then an increase to their original, 0 min, level at 30 min (Fig. 3A and B). This pattern of reduction and elevation was also apparent in the p-ERK1/2 levels (Fig. 3C). It is interesting to note, that the degree of change in the levels of antiapoptotic proteins correlated with the GHRH-R positivity of the cell lines: the cell lines with both GHRH and SV1 receptors displayed a more striking reduction in the levels of the anti-apoptotic proteins, than those with only SV1 positivity (Fig. 3A, B and C). When the cells were treated with GHRH(1-29) $NH_2$  the levels of p-Akt, p-GSK3 $\beta$  and pERK1/2 rose continuously at 5 and 10 min then gradually fell to their initial levels at 30 min (Fig. 3A, B and C). The amount of phospho-p38 expression was examined at 0 time point and 24 h following treatment. The former time point served as a control. Exposure to MIA-602 produced a clear activation of p38 MAP kinase phosphorylation (Fig. 3D). In contrast, the level of p38 activation in GHRH(1-29) $NH_2$ -treated cells was only slight (data not shown). We did not detect a difference between the intensities of phosphorylation of p38 in the nine breast cancer cell lines after MIA-602 treatment.

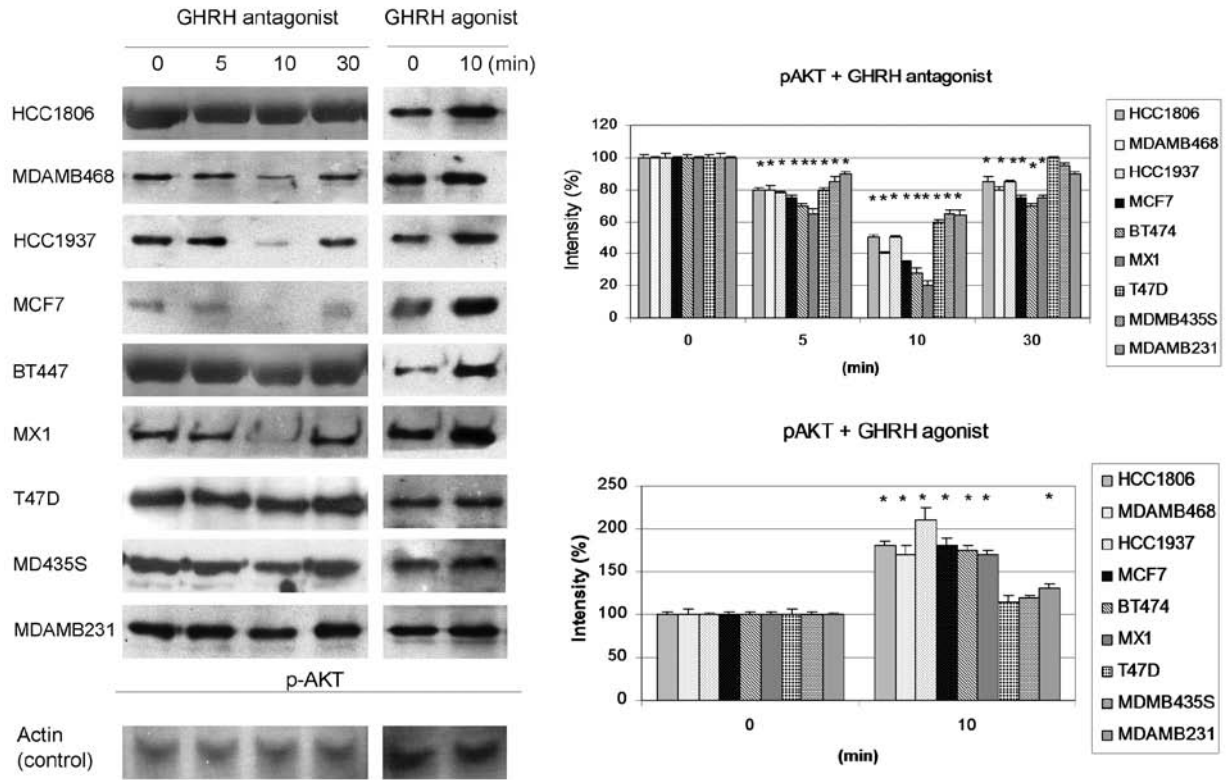
## Discussion

GHRH is known to be expressed in various tumors, including prostatic, ovarian, endometrial and breast cancers (27-32). Extra hypothalamic production of GHRH has been linked to increased mitogenic activity in lymphocytes and testicular germ cells (33). Furthermore, a recent study demonstrated that GHRH stimulates the proliferation of MDA-MB-231 breast cancer cells in a dose- and receptor-dependent manner, through the Ras/Raf/MAPK pathway (34). During the past decade, the efficacy of GHRH antagonists on tumor growth inhibition has been reported in multiple publications (6). The evidence indicates that GHRH antagonists act primarily by blocking the binding of autocrine GHRH to cancer cells (8,35). The receptors which mediate the effects of GHRH and GHRH antagonists have been identified and four truncated splice variants (SVs) of the pGHRH-R have been found (26). In our study, in accordance with earlier

investigations (9,26) we could detect the presence of SV1 in all breast cancer cell lines examined, while pGHRH-R was present in only 6 cell lines. The occurrence of either type of GHRH receptors was not related to the presence of estrogen/progesterone or HER-2 receptors. SV1 of GHRH-R is thought to be the main SV that transmits the effects of GHRH and its antagonists in cancers (26). Accordingly, the MTT assay showed that GHRH antagonist, MIA-602, was effective in significantly decreasing cell viability in all nine cell lines. The proliferation of the two cell lines, MDAMB435 and T47D, expressing only SV1 receptors, was inhibited to a lesser degree than that in cells positive for both the pGHRH-R plus SV-1 cells, however the difference was not significant. These results support the previous findings that GHRH antagonists can exert their antiproliferative effects on tumors and cell lines, even when the pGHRH-R cannot be detected and only the SV-type receptors are present (11,36).

The role of GHRH in the promotion of proliferation and prevention of apoptosis in cancer cells is well known (34,37). Earlier reports have shown that GHRH stimulates ERK and Elk-1 activation (38), as well as the activation of the transcription factors CREB and CHOP (39,40). Recently, we studied the signal transduction in glioblastoma cell lines following treatment with a GHRH antagonist and found that it affects cell death through the key pro-apoptotic pathways: the reduction of phosphorylated Akt, GSK3 $\beta$  and p44/42 MAPK, the cleavage of PARP and caspase-3 as well as through the intracellular translocation of proteins AIF, EndoG and cyt c (22). Our group also demonstrated that antagonism of GHRH abolishes the integrity of the mitochondrial membrane, thus promoting apoptosis (22,41). In the present study we investigated the level of phosphorylation of Akt, GSK3 $\beta$  and p44/42 MAPK following exposure to a novel GHRH antagonist, MIA-602, in nine different breast cancer cell lines. As expected, there was a reduction in the levels of phosphorylation of all three anti-apoptotic proteins in all the cell lines following treatment with MIA-602 and an increase in the intensities of phospho-p38 compared to GHRH(1-29) $NH_2$ -treated cells. Nevertheless, it was not previously shown, that the levels of phosphorylated Akt, GSK3 $\beta$  and ERK1/2 would be significantly lower at 5 and 10 min in SV1 plus pGHRH-R-positive cells than in cells expressing only SV-1. Similarly, differences in the reduction of cell viability of such cells have not been observed in the MTT assay. This finding may be explained by the assumption that, signal transduction is more rapid where the signal is mediated through both types of receptors and thus results in a diminished phosphorylation in a shorter time. The difference, nevertheless, becomes smaller by 48 h, by the time the MTT assay is completed. It is clear from our study, that the anti-apoptotic effect of GHRH antagonists is transmitted through the main truncated splice variant of the GHRH-R, SV1, almost as well as when both types of receptors are present on the cells. Whether the presence of both pGHRH-R and SV1 provides a long-term disadvantage for breast cancer cells compared to cells expressing only SV1 is not established and further studies are required. Altogether, our results indicate that, it might be useful to test breast cancer specimens not only for the presence of estrogen/progesterone and HER-2 receptors, but also for pGHRH-R and SV1 positivity in the future, if a therapy with GHRH antagonists is contemplated.

A



B

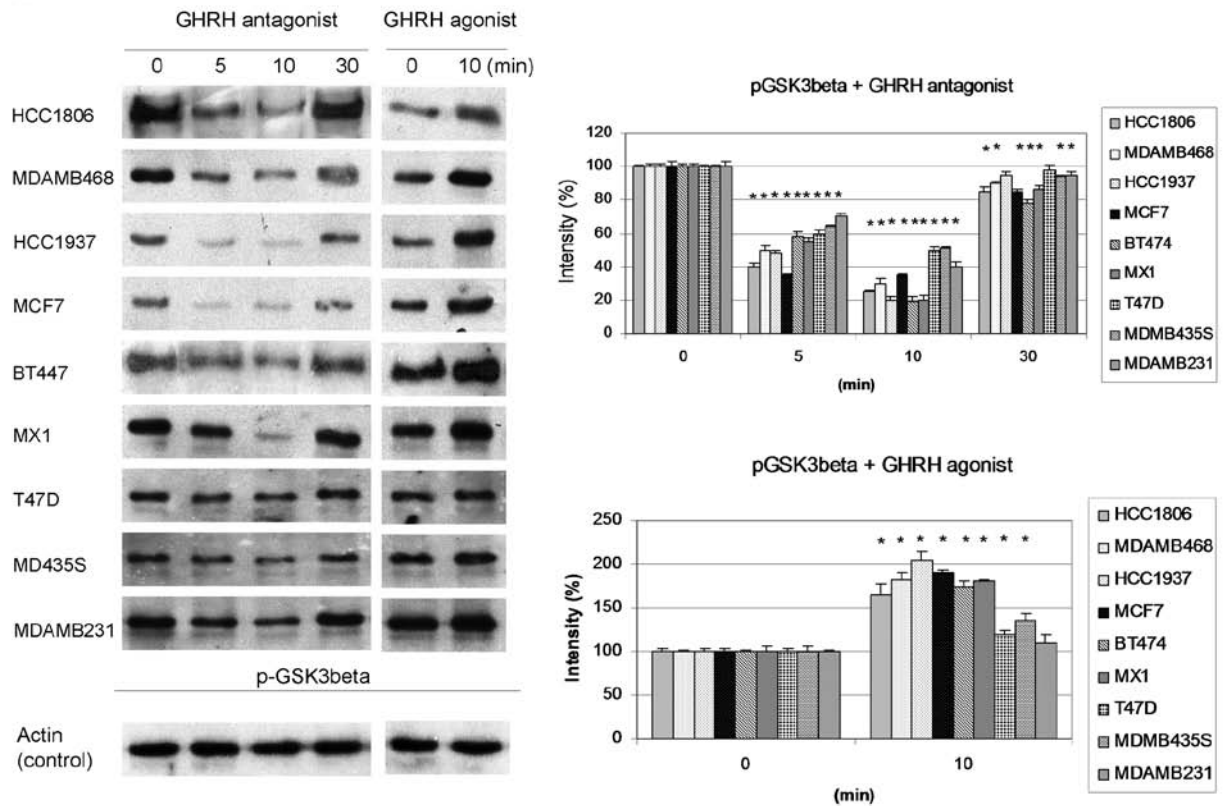
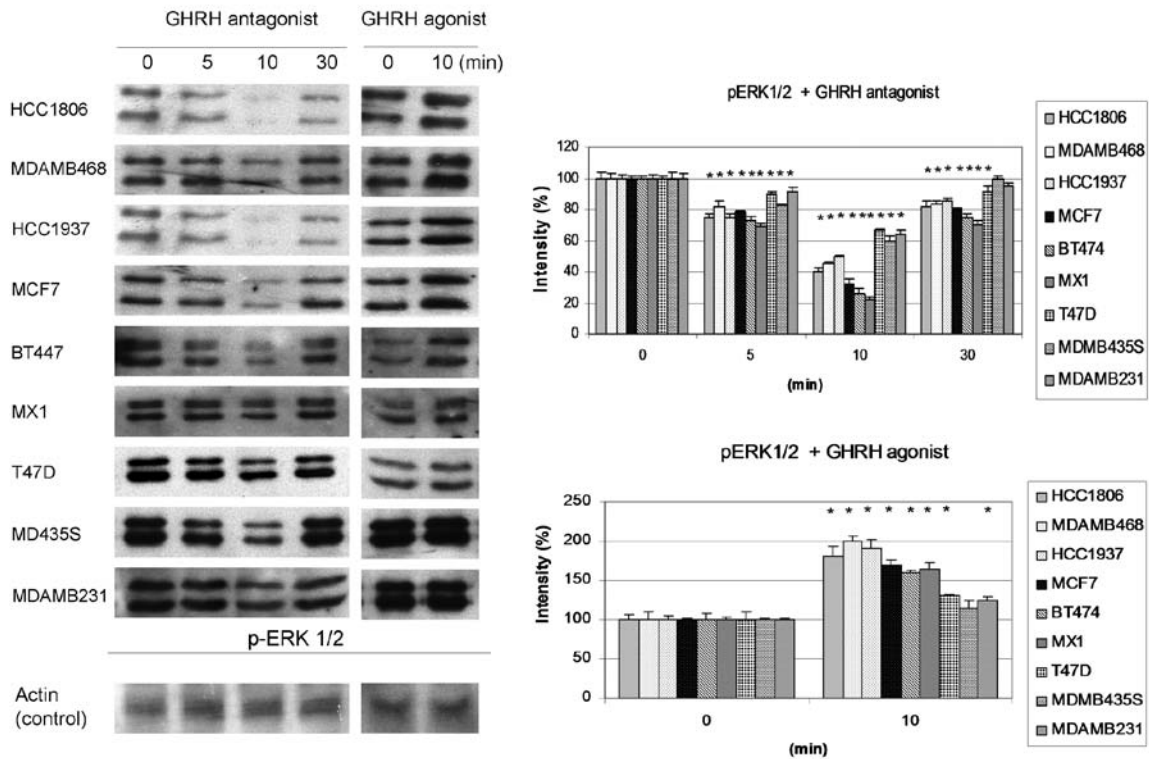


Figure 3 (A and B). The effect of GHRH antagonist, MIA-602 and GHRH(1-29)NH<sub>2</sub> on the activation of (A) Akt (B) GSK3β.

In conclusion, this is the first report on screening nine different breast cancer cell lines and comparing their responses

to GHRH antagonist treatment. The responses to GHRH antagonist treatment have not been investigated previously in

C



D

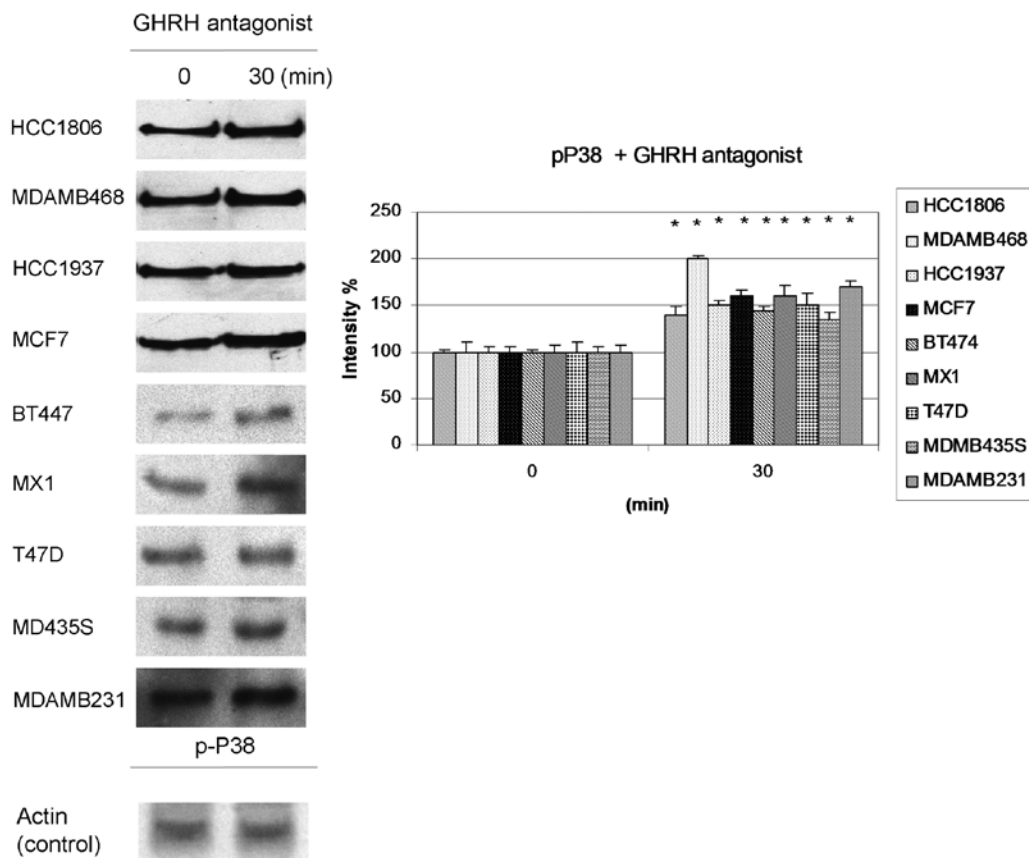


Figure 3 (C and D). The effect of GHRH antagonist, MIA-602 and GHRH(1-29)NH<sub>2</sub> on the activation of (C) ERK and (D) p38 kinases in samples from nine different breast cancer cell lines. The detection of pAkt, pGSK3 $\beta$  and pERK was carried out at 0, 5, 10 and 30 min and detection of phospho-p38 at 0, 30 min, and all were demonstrated by immuno-blotting utilizing phosphorylation specific primary antibodies against the given kinase.  $\alpha$ -tubulin immunoreactivity was used to show even loading. Representative blots of three independent experiments are presented. The results are mean  $\pm$  SEM of three independent experiments performed at least in quadruplicate. \* $p$ <0.05.

such a variety of breast cancer cell lines. We found, that the effect of MIA-602 is mediated almost as well in cells expressing only the SV1 receptor as in cell lines in which both SV1 and pGHRH-R are present, although a difference can be observed at the level of cell signaling. The current study clearly shows, that the new GHRH antagonist, MIA-602, is very efficacious against a wide spectrum of breast cancer cells, independently of their status for estrogen/progesterone and HER-2 receptors. Thus, the use of GHRH antagonists such as MIA-602, after further experimental studies and therapeutic trials, could provide an approach to the treatment of triple negative breast cancer, where endocrine therapy or other targeted therapies are not an option.

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