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Supplementary Information for

Antagonists of growth hormone-releasing hormone (GHRH) inhibit the growth of human malignant pleural mesothelioma

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Supplementary Information Text

SI Materials and Methods

Reagents.
The GHRH-R antagonists MIA-602 and MIA-690 were synthesized and purified in the laboratory of Dr. Andrew V. Schally at the Veterans Affairs Medical Center, University of Miami, Miami, FL as described previously (1). For *in vitro* experiments, MIA-602 and MIA-690 were dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and diluted with appropriate incubation medium. The concentration of DMSO never exceed 0.1% (vol/vol). Pemetrexed (PEM), 2,5-diphenyl tetrazolium bromide (MTT), RPMI-1640 medium, Ham's F12 medium, fetal bovine serum (FBS), bovine serum albumin (BSA), penicillin, amphotericin B, L-glutamine, primers and cell culture reagents were from Sigma-Aldrich. GHRH (sc-10281), GHRH-R (sc-54201), Bcl-2 (sc-783), c-Myc (sc-40), p53 (sc-1313) and actin (sc-376421) antibodies were from Santa Cruz Biotechnology (DBA). The antibody for GHRH-R, which also recognizes SV1, was from Abcam (ab28692). RT-PCR and Real-Time PCR reagents were from Life Technologies.

Cell Lines.
The human biphasic MPM cell line MSTO-211H and the human mesothelial cell line MeT-5A were purchased from American Type Culture Collection. The human epithelioid MPM cell line REN was kindly provided by Dr. Giorgio Scagliotti (Department of Oncology, University of Turin, Orbassano, Turin, Italy). Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere in RPMI-1640 with 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml) and 250 ng/mL amphotericin B.

Isolation and Culture of Human Primary MPM cells.
Human Primary MPM cells (3 epithelioid MPM, 3 biphasic MPM, 3 sarcomatoid MPM) were isolated from diagnostic thoracoscopies of MPM patients, as previously described (2). Briefly, tissues were digested in medium containing 1 mg/ml collagenase and 0.2 mg/ml hyaluronidase for 1 h at 37 °C. Cells were seeded in culture and used within passage 6. The study was approved by the Ethical Committees of the Biological Bank of Mesothelioma, SS. Antonio and Biagio General Hospital, Alessandria, Italy, and San Luigi Gonzaga Hospital, Orbassano, Turin, Italy (#9/11/2011; #126/2016). Primary MPM cells were grown in Ham's F12 medium with 10% of FBS (normal medium, NM). All culture mediums were supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 250 ng/mL amphotericin B. All the cell lines were cultured at 37 °C in a 5% CO_2 humidified atmosphere.

Cell Survival and Proliferation.
Cells were seeded in 96-wells plates at the concentration of 2 x 10^3 cells/well. After 48 h, cells were serum-starved for 12 h and incubated with the different stimuli for further 24 h or 72 h. Cell survival was assessed by MTT assay. Cells were incubated with 1 mg/ml of MTT for approximately 2 h, then the medium was removed, and formazan products solubilized with 100 μl DMSO. Cell viability was assessed by spectrophotometry at 570 nm absorbance using the LT-4000 microplate reader (Euroclone). Cell proliferation was assessed using the 5-bromo-2-deoxyuridine (BrdU) incorporation ELISA kit (Roche Diagnostic). Briefly, the cells were incubated with BrdU labeling solution for 2 h at 37° C. After removal of the labeling solution, cells were fixed, denatured, and incubated for 90 min with anti-BrdU antibody conjugate, which was subsequently removed by rinsing three times. Finally, cells were incubated in substrate solution at room temperature and proliferation assessed by colorimetric detection at 450 nm absorbance using the LT-4000 microplate reader (Euroclone).

IC50 Calculation.
The 50% inhibitory concentration (IC50) values were defined as the drug concentrations required to reduce cell survival (evaluated by MTT assay) to 50% of the untreated control well. They were calculated by weighted nonlinear regression analysis using Prism GraphPad software 6.0 for statistics.

Colony Formation.
To examine the influence of GHRH antagonists on colony formation, MSTO-211H and REN cells were seeded into 60 mm tissue culture plates, at a concentration of 1 x 10^3 cells, and maintained in RPMI-1640 with 10% FBS (normal medium, NM) for 10 days. Then cells were fixed with methanol, colonies were stained with crystal violet (0.05%) and plates were photographed using a digital camera (ChemiDoc XRS). Colonies were counted with ImageJ software (http://rsbweb.nih.gov/ij/).

Caspase-3 Activity.
Cells were seeded into a 6-wells plate at a concentration of 3 x 10^4 cells/well. Caspase-3 activity was assessed by Caspase-3 Colorimetric Assay Kit (BioVision) in cell lysates, according to the manufacturer's instruction. Briefly, cells were resuspended in Cell Lysis Buffer, incubated 10 min at 4° C, centrifuged and cytosolic extract was used for protein quantification. Next, samples were incubated for 2 h with DEVD-pNA substrate. Caspase-3 activity was assessed by colorimetric detection at 405 nm absorbance with LT-4000 microplate reader (Euroclone).

Western blot Analysis.
Protein extraction and Western blot analysis were performed as described previously (3). Proteins (60 or 70 µg) were separated by SDS-PAGE (13% for GHRH, 12% for SV1 and Bcl-2, and 10% for GHRH-R, c-Myc and p53), transferred to a nitrocellulose membrane and incubated overnight at 4 °C with the specific antibodies (dilution 1:2000 for SV1, and 1:500 for GHRH-R, GHRH, Bcl-2, c-Myc and p53). Blots were reprobed with actin (dilution 1:500) for protein normalization. Immunoreactive proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit or mouse anti-goat (1:4000) secondary antibodies by enhanced chemiluminescence substrate (ECL) using ChemiDoc XRS (Bio-Rad), densitometric analysis was performed with Quantity One software (Bio-Rad).

Wound-healing Assay.
MSTO-211H and REN cells were seeded into a 24-wells plate at a concentration of 1.5 x 10^4 cells/well and grown to monolayer confluency. Confluent cells were subsequently wounded with a pipette tip and, after washing twice with PBS to remove floating cells, incubated in medium with 2.5% FBS for 24 h. Photographs were taken with inverted microscope using a Leica DFC340FX camera and the distance between the edges was measured with ImageJ software (http://rsbweb.nih.gov/ij/) 3 times for each condition.

RT-PCR.
Total RNA isolation and reverse transcription to cDNA (3 µg RNA) from MeT-5A, MSTO-211H, REN and primary MPM cells were performed as described previously (4). Briefly, nine microliters of cDNA were amplified in a 50 µL volume using AmpliTaq Gold Polymerase in a GeneAmp PCR System (Perkin-Elmer). For GHRH a second PCR was permorfed on the primary PCR products. Amplifications were assessed in the following conditions: 95 ºC for 30 sec, annealing for 30 sec (60 ºC for *GHRH-R*; 60 ºC and 62 ºC for the first and second amplification of *GHRH*, respectively; 62 ºC for the *SV1*) and 72 ºC for 60 sec, 72 ºC for 7 min for the elongation step. The final PCR products (144 bp for *GHRH-R*, 150 bp for *GHRH*, 523 bp for *SV1* and 120 bp for 18S rRNA) were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The following primer were used: *GHRH-R* forward 5'-ATGGGCTGCTGTGCTGGCCAAC-3', reverse 5'-TAAGGTGGAAAGGGCTCAGACC-) (NM_000823.3) (5); *GHRH* forward 5'-ATTTGAGCAGTGCCTCGGAG-3', reverse 5'-TTTGTTCTGCCCACATGCTG-3'(XM_011528788.2) for the first PCR and forward 5'-ATGCAGATGCCATCTTCACCAA-3', reverse 5'-TGCTGTCTACCTGACGACCAA-3' for the second PCR (XM_011528788.2) (6); *SV1* forward 5'-TGGGGAGAGGGAAGGAGTTGT-3', reverse 5'-GCGAGAACCAGCCACCAGAA-3'(AF282259) (7); *18S rRNA* forward 5'-GTGGAGCGATTTGTCTGGTT-3', reverse 5'-CGCTGAGCCAGTTCAGTGTA-3' (NR_146119.1) (designed with the Primer 3 Software, http://www.primer3.org/). The LNCaP (human prostate cancer) or MCF-7 (human breast cancer) cell lines (American Type Culture Collection) were used as positive control. *18S rRNA* served as internal control; the negative control consisted of no RNA.

Real-Time PCR.
Total RNA isolation and reverse transcription to cDNA (1 µg RNA) from mice xenograft tumors, treated with TRIzol reagent (Invitrogen), MSTO-211H and REN cells, were performed as described previously (4). For real-time PCR, cDNAs were treated with DNA-free DNase (Life Technologies) and reaction performed with 50 ng cDNA, 100nmol/L of each primer and IQ-SYBR-green Mastermix (Bio-Rad) using the ABI-Prism 7300 (Applied Biosystems). The following primer pairs were used: *MMP-9*, forward 5'-TTGACAGCGACAAGAAGTG-3', reverse 5'-GCCATTCACGTCGTCCTTAT-3' (NM_004994.2) (8); *MMP-2*, forward 5'-ACCTGGATGCCGTCGTGGAC-3', reverse 5'-TGTGGCAGCACCAGGGCAGC-3' (NM_001302510.1) (8); *VEGF*, forward 5'-ATCTTCAAGCCATCCTGTGTGC-3', reverse 5'-CAAGGCCCACAGGGATTTTC-3'(NM_001287044.1) (9); *18S* rRNA, forward 5'-

CCCATTCGAACGTCTGCCCTATC-3', reverse 5'-TGCTGCCTTCCTTGGATGTGGTA-3'(NR_146144.1), (designed with the Primer 3 Software, http://www.primer3.org/). *18S* rRNA was used as endogenous control. Real-time PCR for *SOD-2* was performed using the 7900 HT Fast Real Time PCR system (SDS2.3 software) using commercially available primers (TaqMan Gene Expression Assays; Thermo Fisher Scientific): Hs00167309_m1 (SOD2). Actin beta was used as housekeeping gene: Hs99999903_m1 (ACTB). Relative quantification was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Mitochondrial Membrane Potential (ΔΨm).
The mitochondrial membrane potential (ΔΨm) was assessed by cytofluorimetric analysis using JC-1 (Thermo Fisher Scientific), a cationic dye that indicates mitochondrial polarization by shifting its fluorescence emission from green (530 nm) to red (590 nm) (10). Briefly, after 48 h of treatment with MIA-602 or MIA-690 cells were harvested by trypsinization, washed with PBS and incubated with JC-1 dye (2 μg/ml) at 37 °C for 30 min. The amount of JC-1 retained by 1 x 10^4 cells per sample was measured at 530 nm (FL-1 green fluorescence) and 590 nm (FL-2 red fluorescence) using a flow cytometer and analyzed using Cell Quest Alias software. ΔΨm was determined as FL2/FL1 ratio.

Measurement of Intracellular ROS Production.
Cells were harvested and incubated for 15 min with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich). As previously described (11), the ROS-dependent DCF fluorescence was determined at an excitation wavelength of 504 nm and an emission wavelength of 529 nm, using a Packard EL340 microplate reader (Bio-Tek Instruments). Fluorescence values were normalized to the protein concentration and expressed as percent of control.

In vivo Tumor Growth.
All procedures were performed according to institutional guidelines in compliance with national (D.L. N.26, 04/03/2014) and international law and policies (new directive 2010/63/EU). All mice were bred at the Animal Facility of the Molecular Biotechnology Center (Turin, Italy) recognized and was approved by the Italian Ministry of Health (protocol n°52/2018-PR). 6/8-weeks-old NOD/SCID/gamma chain$^{-/-}$(NSG) male mice were subcutaneously injected in the right flank with 2 x 10^6 MSTO-211H cells resuspended in 100 μL PBS 1X/matrigel (Matrigel®, Corning) solution (ratio 1:1). When tumors became palpable, the mice were randomly divided into three groups: control, MIA-602 and MIA-690 (n=15). MIA-602 and MIA-690, dissolved in 0.1% DMSO and 10% aqueous propylene glycol solution (vehicle solution), were subcutaneously daily administered at a dose of 5 μg/day for 4 weeks. Mice of control group were treated in the same way administering a vehicle solution without drugs. Tumor volumes were measured once a week until the end of the experiment. Animals were sacrificed at the end of the treatments. Tumors were resected, and volume was calculated with the formula $v = (a \times b^2)/2$, where a is the long axis and b is the short axis. Next, tumors were weighed and divided for RNA and proteins isolation or fixed in 10% buffered formalin. Paraffin sections were conventionally stained with hematoxylin/eosin (H/E) for immunohistochemistry analysis. Images acquisition and automated evaluation of necrosis areas were performed with Aperio Scanscope XT (Leica Biosystem).

IGF-I Analysis.
Tumor xenograft samples were homogenized in RIPA buffer (Sigma-Aldrich), sonicated and centrifuged at 14,000 rpm (4 °C for 15 min). Total protein lysates were quantified with Bicinchoninic Acid kit (BCA) from Sigma-Aldrich. IGF-I levels were measured following the manufacturer's protocol using mouse IGF-I ELISA Kit (Abcam). Briefly, loading buffer was used to dilute proteins, followed by overnight incubation at 4 °C. After washing three times with washing buffer, 1X biotinylated IGF-I antibody was added to each well and incubated for 1 h at room temperature with gentle shaking. Next, the wells were washed three times with washing buffer and 100 μl of 1×HRP-Streptavidin solution was added. The samples were incubated for 30 min at RT with 100 μl of TMB solution and results assessed by colorimetric detection at 450 nm absorbance using LT-4000 microplate reader (Euroclone).

Statistical Analysis.
Results are presented as mean ± SEM. Significance was calculated by two-tailed Student's *t* test or 2-way ANOVA followed by Bonferroni's multiple comparison test for post hoc analysis. Analysis was performed using GraphPad Prism

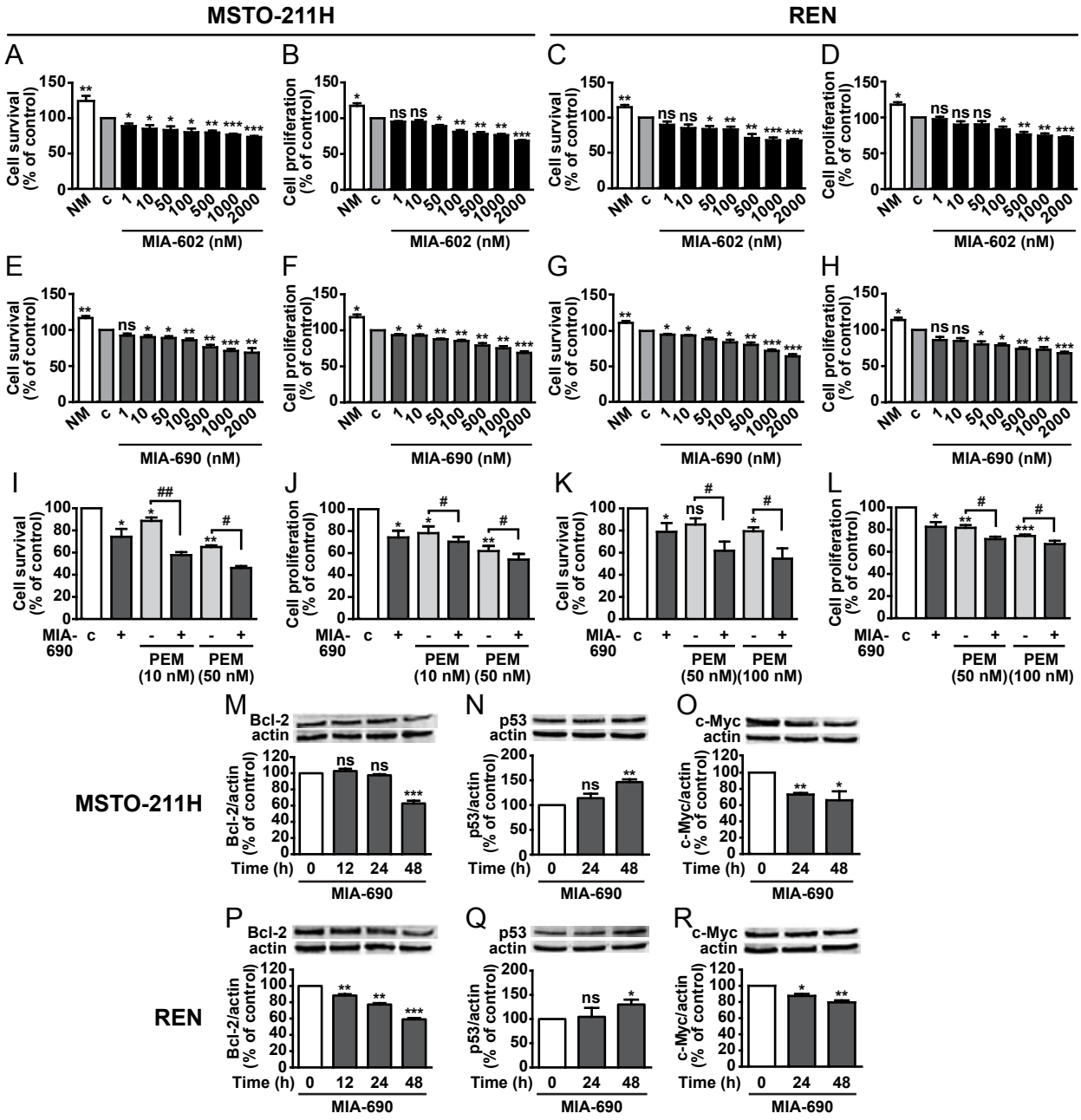

Fig. S1. Effect of GHRH antagonists on survival, proliferation and apoptosis of MSTO-211H and REN cells. Cell survival and proliferation assessed by MTT and BrdU, respectively, in cells serum-starved for 12 h, then cultured for 24 h with MIA-602 (*A-D*) or MIA-690 (*E-H*), at the concentrations indicated. Results, expressed as percent of control (c) are mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. c; ns, not significant; *n*=4. Cell survival (*I* and *K*) and proliferation (*J* and *L*) in cells treated for 72 h in medium with 2.5% serum (c, control medium) and with 1 μM MIA-690, alone or with pemetrexed (PEM), at the concentrations indicated. Results, expressed as percent of control, are mean ± SEM. **P* < 0.05, ***P* < 0.01 vs. c; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01; *n*=4. Representative Western blot for Bcl-2, p53 and c-Myc in MSTO-211H (*M-O*) or in REN cells (*P-R*) treated with 1 μM MIA-690 for the indicated times. Results, normalized to actin and expressed as percent of control (c) are mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. Time 0; ns, not significant; *n*=3.

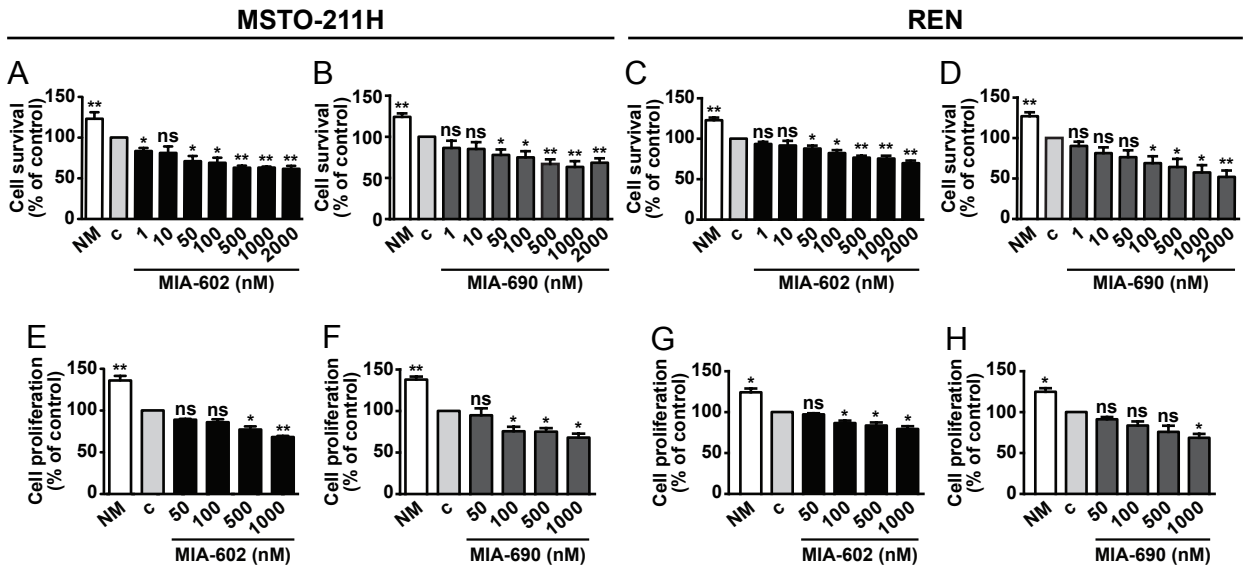

Fig. S2. Cell survival and proliferation in MPM cells treated with MIA-602 and MIA-690 for 48 h. MSTO-211H and REN cells were cultured in either normal medium (NM) or in serum-deprived medium (c, control) for 48 h with MIA-602 or MIA-690 at the concentrations indicated. Cell survival *(A-D)* and proliferation *(E-H)* were assessed by MTT and BrdU, respectively. Results, expressed as percent of control are mean ± SEM. **P* < 0.05, ***P* < 0.01 vs. c; ns, not significant; *n*=4.

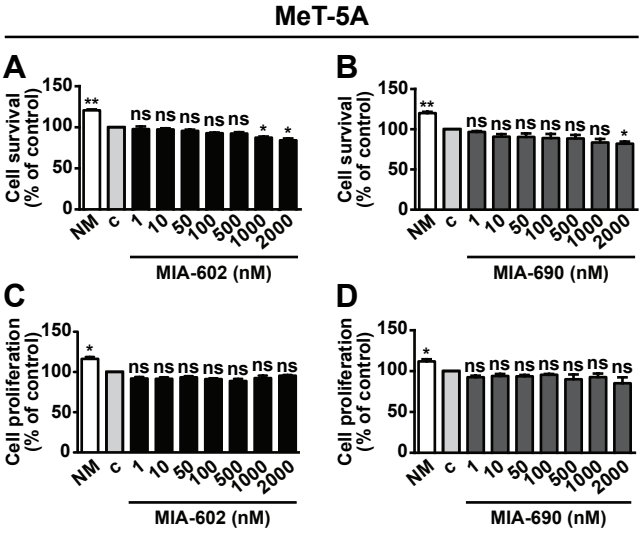

Fig. S3. Cell survival and proliferation in pleural mesothelial cells treated with MIA-602 and MIA-690. MeT-5A cells were cultured in normal medium (NM) or in serum-deprived medium (c, control) for 12 h and then for further 24 h with MIA-602 *(A and C)* and MIA-690 *(B and D)*, at the concentrations indicated. Cell survival and proliferation were assessed by MTT and BrdU, respectively. Results, expressed as percent of control are mean ± SEM. **P* < 0.05 vs. c; ns, not significant; *n*=3.

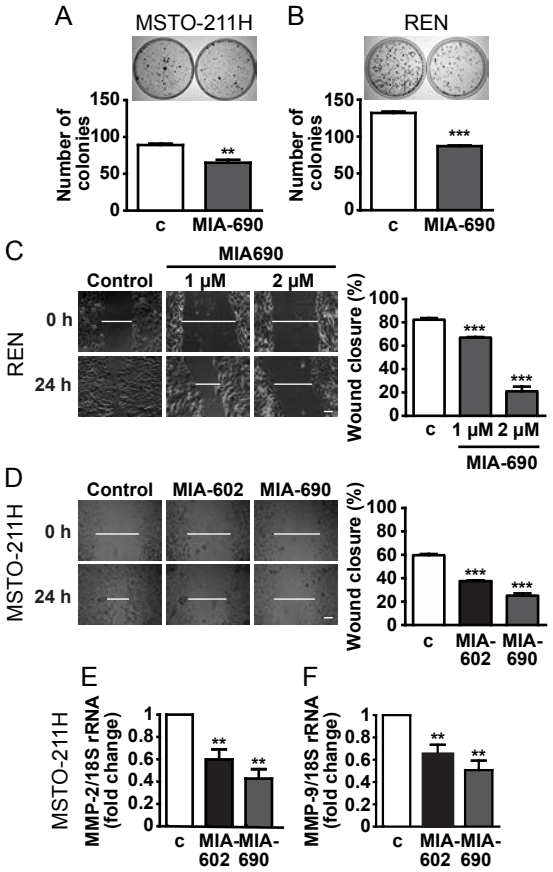

Fig. S4. Cell growth and migration in MPM cells treated with GHRH antagonists. Representative colony formation in MSTO-211H (A) and REN (B) cells, untreated (c, control) or treated for 10 days with 1 μM MIA-690. Results are mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. c; n=3. (C) Representative images of wound-healing assay in REN cells cultured in medium with 2.5% serum and treated for 24 h with MIA-690, at the indicated concentrations, and in MSTO-211H cells treated with 1 μM MIA-602 and MIA-690 (*D*) (scale bars: 20 μm). Histograms on the right shows the wound closure efficiency. Results, expressed as percent of control, are mean ± SEM. $^{***}P < 0.001$vs. c; n=3. Real-time PCR for MMP-2 (*E*) and MMP-9 (*F*) mRNA normalized to 18S rRNA in MSTO-211H cells treated for 24 h with 1 μM MIA-602 and MIA-690. Results, expressed as fold change of control (c), are mean ± SEM. $^{**}P<0.01$ vs. c; n=3.

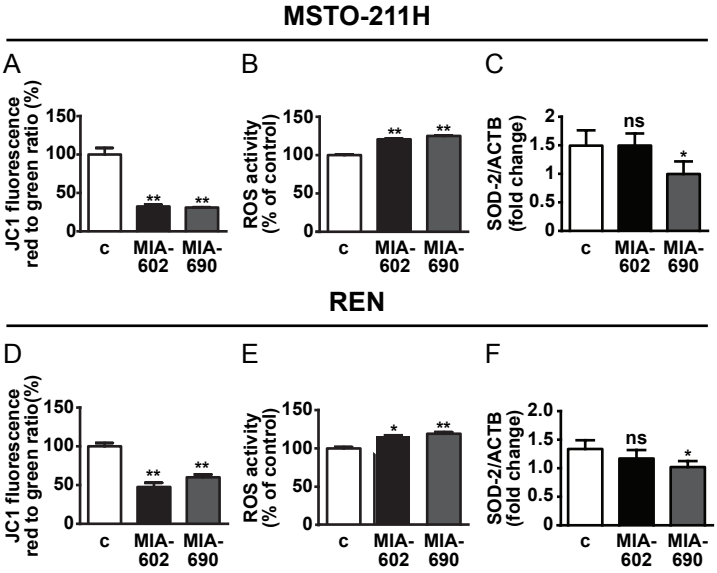

Fig. S5. Effect of MIA-602 and MIA-690 on mitochondrial metabolism and production of ROS. MSTO-211H and REN cells were incubated for 48 h in control medium with 1% serum (c, control) with or without 1 μM MIA-602 or MIA-690. (*A* and *D*) Mitochondrial membrane potential (ΔΨm) examined by flow cytometry analysis of JC-1. For each experimental condition the FL-2/FL-1 ratio was calculated and expressed as percent of the value obtained for untreated cells. Mean ± SEM. **P* < 0.01 vs. c; *n*=3. (*B* and *E*) Intracellular ROS activity evaluated by fluorescence assay. Results, expressed as percent of control are mean ± SEM. **P* < 0.05, ***P* < 0.01 vs. c; *n*=3. (*C* and *F*) Real-time PCR analysis for superoxide dismutase 2 (SOD-2) mRNA normalized to actin beta (ACTB). Results, expressed as fold change of control (c), are mean ± SEM. **P* < 0.05 vs. c; *n*=3.

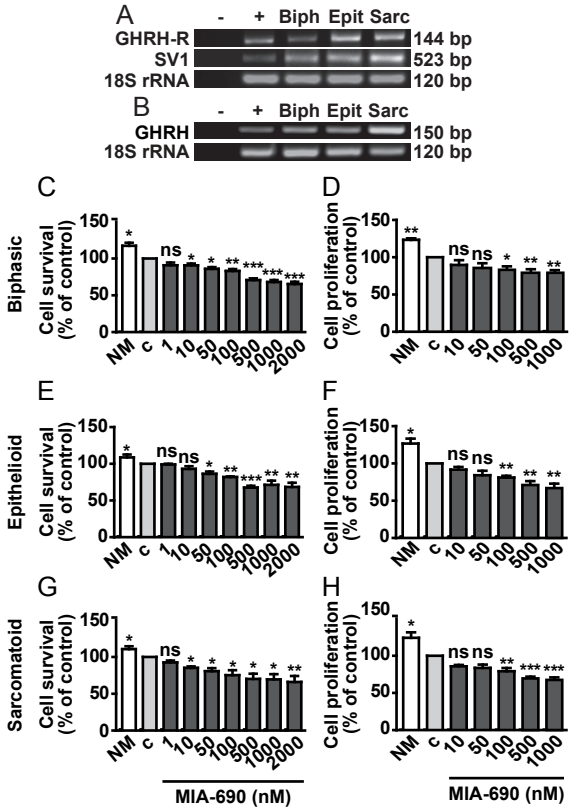

Fig. S6. Inhibitory effects of MIA-690 in primary MPM cells. Representative RT-PCR of GHRH-R, SV1 (*A*), and GHRH mRNA (*B*) in biphasic (Biph), epithelioid (Epit) and sarcomatoid (Sarc) MPM cells. LNCaP prostate cancer cells were used as positive control (+) for GHRH-R and SV1; MCF-7 breast cancer cells were used as positive control (+) for GHRH. Buffer alone was used as negative control (-). Cell survival (MTT assay) and proliferation (BrdU assay) in biphasic (*C* and *D*), epithelioid (*E* and *F*) and sarcomatoid (*G* and *H*) cells cultured in either normal medium (NM) or serum-deprived medium for 12 h (c, control), then for 24 h with MIA-690, at the concentrations indicated. Results, expressed as percent of control are mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. c; ns, not significant; *n*=3.

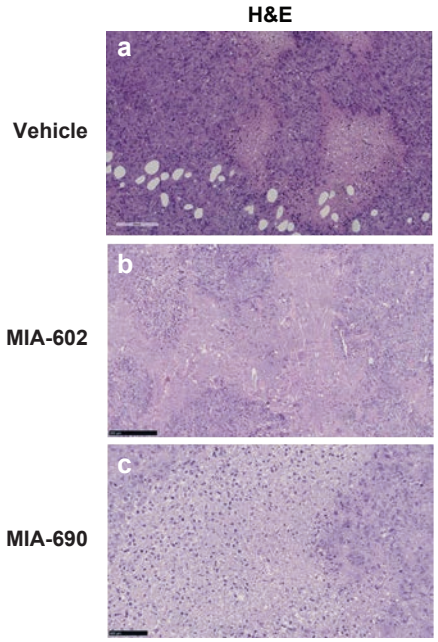

Fig. S7. Morphology of mesothelioma xenografts. Untreated mice (a, vehicle) and mice treated with MIA-602 (*b*) or MIA-690 (*c*). Necrosis is more extensive in treated cases. Scale bar, 100 μm.

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