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

Tania Villanova\textsuperscript{a,1}, Iacopo Gesmundo\textsuperscript{a,1}, Valentina Audrito\textsuperscript{b,c}, Nicoletta Vitale\textsuperscript{d}, Francesca Silvagnino\textsuperscript{a}, Chiara Musuraca\textsuperscript{a}, Luisella Righi\textsuperscript{a}, Roberta Libener\textsuperscript{a}, Chiara Riganti\textsuperscript{a}, Paolo Bironzo\textsuperscript{a}, Silvia Deaglio\textsuperscript{b,c}, Mauro Papotti\textsuperscript{a}, Renzhi Cai\textsuperscript{a,h}, Wei Sha\textsuperscript{h,i}, Ezio Ghigo\textsuperscript{a}, Andrew V. Schally\textsuperscript{h,i,j,k,l,2}, and Riccarda Granata\textsuperscript{a,2}

\textsuperscript{a}Division of Endocrinology, Diabetes and Metabolism, Department of Medical Sciences, University of Turin, 10126 Turin, Italy; \textsuperscript{b}Department of Medical Sciences, University of Turin, 10126 Turin, Italy; \textsuperscript{c}Italian Institute for Genomic Medicine, 10126 Turin, Italy; \textsuperscript{d}Department of Molecular Biotechnology and Health Sciences, University of Turin, 10126 Turin, Italy; \textsuperscript{e}Department of Oncology, San Luigi Gonzaga Hospital, University of Turin, 10043 Orbassano, Italy; \textsuperscript{f}Pathology Unit, SS. Antonio e Biagio General Hospital, 15121 Alessandria, Italy; \textsuperscript{g}Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, Miller School of Medicine, University of Miami, Miami, FL 33136; \textsuperscript{h}Endocrine, Polyepitope and Cancer Institute, Veterans Affairs Medical Center, Miami, FL 33125; \textsuperscript{i}Sylvester Comprehensive Cancer Center, Department of Medicine, Miller School of Medicine, University of Miami, Miami, FL 33136; \textsuperscript{j}Division of Hematology/Oncology, Department of Medicine, Miller School of Medicine, University of Miami, Miami, FL 33136; and \textsuperscript{k}Department of Pathology, Miller School of Medicine, University of Miami, Miami, FL 33136

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Malignant pleural mesothelioma (MPM) is an aggressive malignancy associated with exposure to asbestos, with poor prognosis and no effective therapies. The strong inhibitory activities of growth hormone-releasing hormone (GHRH) antagonists have been demonstrated in different experimental human cancers, including lung cancer; however, their role in MPM remains unknown. We assessed the effects of the GHRH antagonists MIA-602 and MIA-690 in vitro in MPM cell lines and in primary MPM cells, and in vivo in MPM xenografts. GHRH, GHRH receptor, and its main splice variant SV1 were found in all the MPM cell types examined. In vitro, MIA-602 and MIA-690 reduced survival and proliferation in both MPM cell lines and primary cells and showed synergistic inhibitory activity with the chemotherapy drug pemetrexed. In MPM cells, GHRH antagonists also regulated activity of apoptotic molecules, inhibited cell migration, and reduced the expression of matrix metalloproteinases. These effects were accompanied by impairment of mitochondrial activity and increased production of reactive oxygen species. In vivo, s.c. administration of MIA-602 and MIA-690 at the dose of 5 μg/d for 4 wk strongly inhibited the growth of MPM xenografts in mice, along with reduction of tumor growth factors. Overall, these results suggest that treatment with GHRH antagonists, alone or in association with chemotherapy, may offer an approach for the treatment of MPM.

**Significance**

Malignant pleural mesothelioma (MPM) is an aggressive cancer with poor prognosis and limited treatment options. MPM remains a serious public health problem, and novel therapeutic strategies are urgently needed. The antitumor properties of growth hormone-releasing hormone (GHRH) antagonists have been demonstrated in different cancers; however, their influence in MPM remains unexplored. Our work shows that GHRH antagonists MIA-602 and MIA-690 reduce survival, proliferation, and migration of human MPM cell lines and primary MPM cells in vitro by modulating apoptotic and oncogenic pathways. In vivo, GHRH antagonists inhibited the growth of MPM xenografts and blunted the production of growth factors in tumors. Overall, the inhibitory activities described in this study suggest that GHRH antagonists may be considered for development of therapies for MPM.
mechanisms mediated by GHRH-R and its splice variant type 1 (SV1) (5, 6, 14). The stimulatory loop, formed by tumor-derived GHRH and its receptors, can be blocked by GHRH antagonists, resulting in inhibition of tumor growth in experimental models. In fact, GHRH antagonists suppress in vitro and in vivo the proliferation of many experimental human cancers, including lung cancer (14–17).

Recently, new GHRH antagonists of the Miami (MIA) series have been synthetized, such as MIA-602 and MIA-690, with greatly increased anticancer activity, higher binding affinity, and low endocrine effect on the GH/insulin-like growth factor I (IGF-I) axis. These analogs display potent inhibitory actions in thyroid, lung, gastric, renal, prostate, and endometrial cancer, as well as in glioblastoma, colorectal adenocarcinoma, lymphoma, and retinoblastoma (16–25). To date, however, the role of GHRH antagonists in MPM remains to be determined.

In this study, we evaluated the potential inhibitory activities of MIA-602 and MIA-690 on the survival and proliferation of human MPM cell lines and primary MPM cells. The effects on apoptosis and cell migration, along with mitochondria metabolism, were also analyzed in MPM cell lines. Finally, the antitumor activities of GHRH antagonists were assessed in vivo, in mice xenografted with human MPM cells.

**Results**

**Expression of pGHRH-R, SV1, and GHRH in MPM Cells.** Results from Western blot analysis showed that both pGHRH-R and SV1 as well as GHRH proteins are expressed in pleural mesothelial cells (MeT-5A) and in pleural biphasic and epithelioid MPM cell lines (MSTO-211H and REN, respectively) (Fig. 1).

**GHRH Antagonists Inhibit Cell Survival and Proliferation, Promoting Apoptosis in MPM Cell Lines.** The inhibitory efficacy of MIA-602 and MIA-690 was initially determined in human MPM cell lines treated with the antagonists for 24 h at concentrations of 1 to 2,000 nM. We found a similar dose-dependent reduction in survival and proliferation of MSTO-211H and REN cells with both MIA-602 (SI Appendix, Fig. S1 A–D) and MIA-690 (SI Appendix, Fig. S1 E–H), particularly from 50 to 2,000 nM, compared with controls (IC50 for MIA-602: 3,925 nM in MSTO-211H and 2,330 nM in REN; IC50 for MIA-690: 2,779 nM in MSTO-211H and 2,431 nM in REN). Likewise, in cells treated for 48 h, the effect was observed from 50 nM for survival (SI Appendix, Fig. S2 A–D) and from 100 nM for proliferation (SI Appendix, Fig. S2 E–H) in both cell lines. Interestingly, in MeT-5A pleural mesothelial cells, both antagonists reduced survival only at the highest concentrations (SI Appendix, Fig. S3 A and B), while having no effect on proliferation (SI Appendix, Fig. S3 C and D). Because of the striking decrease with both antagonists in cell survival at 1,000 nM (1 μM) (MIA-602: 23% and MIA-690: 27.4% for MSTO-211H; MIA-602: 31.54% and MIA-690: 32.2% for REN) and proliferation (MIA-602: 23.63% and MIA-690: 24.93% for MSTO-211H; MIA-602: 25.59% and MIA-690: 27.34% for REN), 1 μM was chosen as concentration for subsequent studies.

We next examined the possible combinatorial cytotoxic activity produced by MIA-602 or MIA-690 with the anticancer drug PEM. MPM cells were treated for 72 h with each antagonist at 1 μM and with PEM at 10 or 50 nM in MSTO-211H cells or at 50 or 100 nM in REN cells, which are less sensitive to the cytotoxic effect of the drug. MIA-602 (Fig. 2A–D) and MIA-690 (SI Appendix, Fig. S1 I–L) were synergistic in inhibiting cell survival in both MPM cell lines, with Combination Index (CI) values <1 at all of the concentrations of PEM tested. The proapoptotic effect of the antagonists was assessed at 12, 24, and 48 h in the MPM cell lines. MIA-602 and MIA-690 increased the activity of proapoptotic caspase-3 (Fig. 2 E and G) at 24 h in both MPM cell lines. Moreover, expression of the antia apoptotic protein Bcl-2 was reduced by MIA-602 in both MPM cell lines at 24 and 48 h (Fig. 2 F and H) and by MIA-690 at 48 h in MSTO-211H cells (SI Appendix, Fig. S1 M) and at 12, 24, and 48 h in REN cells (SI Appendix, Fig. S1 P). Accordingly, in both MPM cell lines, the tumor suppressor protein p53 was up-regulated by the antagonists at 48 h (Fig. 2 J and K and SI Appendix, Fig. S1 N and O), whereas oncogenic c-Myc was reduced at 24 and 48 h (Fig. 2 L and SI Appendix, Fig. S1 O and R).

**GHRH Antagonists Inhibit Growth and Migration of MPM Cell Lines.** To further assess whether GHRH antagonists suppress the growth of MPM cells, we performed colony formation assay. Our data indicated a similar reduction in the ability to form colonies after 10 d in both MSTO-211H and REN cells treated with MIA-602 (Fig. 3 A and B) and MIA-690 (SI Appendix, Fig. S4 A and B), compared with untreated cells. The ability of MPM cells to migrate and invade the pleura is a major characteristic of tumor growth (4); thus, we next assessed cell migration by wound-healing assay. MIA-602 and MIA-690 strongly inhibited the wound closure in both MPM cell lines to a similar extent. In REN cells, the effect was detected at 1 μM and was even increased at 2 μM for both antagonists (Fig. 3 C and SI Appendix, Fig. S4 C). In MSTO-211H cells, only 1 μM was effective for both antagonists (SI Appendix, Fig. S4 D). Moreover, in both cell lines, MIA-602 and MIA-690 equally reduced the mRNA levels of molecules implicated in cell migration and tumor growth [i.e., metalloproteinase (MMP)-2 and MMP-9] (Fig. 3 D and E and SI Appendix, Fig. S4 E and F).

**MIA-602 and MIA-690 Induce Mitochondrial Damage.** Since mitochondria are central players in apoptosis (26), and we show here that GHRH antagonists promote apoptosis in MPM cells, we evaluated the role of mitochondria in the antitumor effects of MIA-602 and MIA-690. Mitochondrial membrane potential (∆Ψm), an indicator of mitochondrial activity, was dramatically reduced in MSTO-211H and REN cells treated with the antagonists for 48 h, as measured by flow cytometry analysis of the mitochondrial-sensitive dye JC-1 (SI Appendix, Fig. S5 A and D). This result was accompanied by elevation of reactive oxygen species (ROS) (SI Appendix, Fig. S5 B and E), which causes impairment of ∆Ψm, release of mitochondrial proteins, and activation of caspase cascades (27). Moreover, MIA-690, but not MIA-602, blunted the expression of superoxide dismutase 2 (SOD-2), a key mitochondrial antioxidant enzyme (SI Appendix, Fig. S5 C and F).

**GHRH Antagonists Inhibit Cell Survival and Proliferation in Human Primary MPM Cells.** To next determine the effects of MIA-602 and MIA-690 in MPM cells obtained from pleural biopsy tissues of patients with MPM, we first analyzed the presence of GHRH-R and GHRH in biphasic, epithelioid, and sarcomatoid cells. Western blot and RT-PCR analysis showed expression of both...
GHRH-R and GHRH protein (Fig. 4 A and B) and mRNA (SI Appendix, Fig. S6 A and B) in all MPM types that were also positive for SV1 mRNA (SI Appendix, Fig. S64). MIA-602 and MIA-690 displayed similar dose-dependent inhibitory activity on survival and proliferation in biphasic (Fig. 4 C and D and SI Appendix, Fig. S6 C and D), epithelioid (Fig. 4 E and F and SI Appendix, Fig. S6 E and F), and sarcomatoid cells (Fig. 4 G and H and SI Appendix, Fig. S6 G and H). MIA-602 was slightly more effective than MIA-690, being significant from 1 nM on survival, and from 50 nM on proliferation of epithelioid (Fig. 4 E and F) and sarcomatoid cells (Fig. 4 G and H). MIA-690, in turn, was more effective than MIA-602 in biphasic cells, starting from 1 nM for survival (SI Appendix, Fig. S6C) and from 100 nM for proliferation (SI Appendix, Fig. S6D).

**GHRH Antagonists Inhibit the Growth of MPM in Vivo.** The therapeutic potential of MIA-602 and MIA-690 was evaluated in NOD/SCID/gamma chain−/− mice xenografted with MSTO-211H cells. When the tumors became palpable, mice were randomly assigned to receive a daily s.c. injection of MIA-602 or MIA-690 (5 μg/d) or vehicle for 4 wk. Both antagonists showed a remarkable inhibitory effect on tumor growth, as demonstrated by the reduction in tumor volume and weight (Fig. 5 A and B, P < 0.001). Moreover, in xenografts, the antagonists reduced the production of IGF-I protein (Fig. 5 C) and blunted mRNA levels of VEGF (Fig. 5D). Histopathological examination of the tumors showed a uniform growth of neoplastic cells, morphologically consistent with biphasic MPM. More- or less-extensive areas of necrosis were observed in all cases, with a slight increase in treated animals (mean values 35% and 36% for treatments with MIA-602 and MIA-690, respectively, compared with 29% of untreated xenografts) (SI Appendix, Fig. S7).

**Discussion**

MPM is an aggressive tumor with poor prognosis due to the unavailability of effective therapies. Even though MPM is a rare cancer, its incidence is expected to increase in the next two decades because of the worldwide exposure to asbestos over the past years (1).

MIA-602 and MIA-690 are part of the latest MIA series of GHRH antagonists with potent antitumor activity in different cancers, including lung cancer (16–25); however, their inhibitory effects in MPM remain to be investigated. This study shows that MIA-602 and MIA-690 can potently—and to a similar extent—inhibit the growth of human MPM cell lines and primary MPM cells in vitro and display antitumor effects in vivo in MPM xenografts.

GHRH-R and its splice variant SV1 have been implicated in the antitumor effects of GHRH antagonists (6, 14, 15). Importantly, the expression of nonhypothalamic GHRH, pGHRH-R, and SV1 has been demonstrated in different tumors and cancer cell lines, suggesting that locally produced GHRH might function as an autocrine/paracrine growth factor in various cancers. Interestingly, cancer cells transfected with SV1 exhibited increased cell proliferation, suggesting that blockade of ligand-independent activity of SV1 would lead to the development of...
and 1.15 F 0.01, and ***SEM. Inhibitory effects of MIA-602 in primary MPM cells. Representative ***< and 3. ns, not significant.

50 50 50 ***

Biph ± SEM. **< (Sarc Epit REN 3. Real-time PCR for μSarc 0.001 vs. c; 0.01 vs. c; < Biph < 3. (0.01 and ***Epit ± <

Fig. 3. Inhibitory effects of GHRH antagonists on cell growth and migration. Representative colony formation in MTO-211H (A) and REN (B) cells untreated (c, control) or treated for 10 d with 1 μM MIA-602. 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-602 at the indicated concentrations. (Scale bar: 20 μM.) Histogram (Right) shows the wound closure efficiency. Results, expressed as percent of control, are mean ± SEM. **P < 0.01 and ***P < 0.001 vs. c; n = 4. Real-time PCR for MMP-2 (D) and MMP-9 (E) mRNA expression normalized to 18S rRNA in REN cells treated for 24 h with 1 μM MIA-602 or MIA-690. Results, expressed as fold change of control, are mean ± SEM. *P < 0.05 and **P < 0.01 vs. c; n = 3.

Fig. 4. Inhibitory effects of MIA-602 in primary MPM cells. Representative Western blot of GHRH-R (A) and GHRH protein (B) in biphasic (Biph), epithelioid (Epit), and sarcomatoid (Sarc) MPM cells. LNCaP prostate cancer cells and MCF-7 breast cancer cells were used as positive control (+) for GHRH-R and GHRH, respectively. 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 (c, control) for 12 h, and then treated for 24 h with MIA-602 at the concentrations indicated. Results, expressed as percent of control, are mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. c; n = 3. ns, not significant.

In addition to the inhibitory effects per se, GHRH antagonists acted synergistically with PEM to reduce MPM cell survival, suggesting the ability to sensitize the cells to chemotherapy-induced toxicity. Several factors may contribute to this synergism, including induction of proapoptotic signaling and inhibition of oncogenic and antiapoptotic molecules by GHRH antagonists. Moreover, different survival pathways are involved in resistance to chemotherapy in MPM, including PI3K/Akt, a downstream component of IGF-I receptor and a major antiapoptotic pathway and frequently overexpressed in cancer cells (29). Herein, MIA-602 and MIA-690 down-regulated IGF-I in MPM xenografts, providing, at least in part, a mechanistic explanation for the inhibitory effect on tumor growth. Thus, it will also be important to explore the role of Akt and its downstream effector proteins in the antitumor activities of GHRH antagonists in MPM. In fact, the mechanisms of action of GHRH antagonists in various cancers have been the subject of intense investigations (16, 19, 23, 30). Along with the endocrine effects on the GH/IGF-I axis, direct mechanisms include blockade of the autocrine/paracrine activity of GHRH and IGF-I/II production in tumors (14, 15). Suppression of tumor growth factors, like VEGF, has also been reported (24, 31, 32). Other emerging mechanisms comprise the up-regulation of molecules involved in cell cycle arrest and apoptosis, such as p53 and caspases, and the inhibition of oncogenic and antiapoptotic pathways like c-Myc and Bcl-2 (20, 25, 31, 33, 34). Accordingly, in MPM cells, MIA-602 and
Inhibitory effects of GHRH antagonists on the growth of MPM in vivo.

MIA-690 elevated p53 and reduced c-Myc levels, strongly increased caspase-3 activity, and inhibited Bcl-2 expression. Notably, p53 is mutated in ~15% of MPMs, with hyperactivation of survival and antiapoptotic pathways and resistance to chemotherapy. Expression and activity of p53, in turn, can be dramatically increased by DNA damage, leading to cell cycle arrest, apoptosis, and inhibition of VEGF, which is highly expressed in MPM and implicated in tumor growth and angiogenesis (4, 31, 35, 36). It has been also shown that inhibition of c-Myc promotes apoptosis and sensitizes MPM cells to cytotoxicity by kinase inhibitors (37). Moreover, in line with recent findings (20, 25), MIA-602 and MIA-690 in the present study strongly inhibited cell motility and invasion of MPM cells and blunted the expression of MMP-2 and MMP-9, key regulators of tumor growth, metastasis, and angiogenesis (38).

It has been demonstrated that GHRH and its agonistic analogs, like MR-409 and MR-356, display extraparitary activities, including cardioprotection, an increase in survival of pancreatic islets after transplantation, and acceleration of wound healing, suggesting their potential clinical use in the fields of cardiology, diabetes, and others (5–11, 13). GHRH, however, also functions as a stimulatory growth factor in cancer cells, thus raising serious concerns for its therapeutic use (14, 25). Importantly, alterations on the synergistic effect of GHRH antagonists and PEM have been reported in MPM, suggesting implication in suppression of tumor growth. Moreover, recent studies demonstrated that in the prostate, GHRH antagonists suppress IGF-I signaling, which can be ligand-independently activated by tumor-derived GHRH (25). Importantly, alterations in IGF-system components have been reported in MPM, suggesting implication in tumorigenesis (44). That VEGF expression is reduced in xenografts further strengthens the antitumor and antiangiogenic role of MIA-602 and MIA-690 in MPM, in agreement with studies in other cancer cell models, including non-small cell lung cancer (24, 32).

In conclusion, our results demonstrate in vitro and in vivo, the antitumor activities of MIA-602 and MIA-690 in MPM, suggesting that GHRH antagonists could be considered for use in future therapeutic strategies, alone or in combination with standard therapies.

Methods

Please see SI Appendix, Materials and Methods for more information.

Cell Lines and Reagents. The MSTD-211H (human biphasic MPM) cell line and MeT-SA (human mesothelioma) cells were obtained from the American Type Culture Collection and cultured following the manufacturer’s instructions. The REN (human epithelioid MPM) cell line was kindly provided by Giorgio Scaglotti, Department of Oncology, University of Turin, Orbiassano, Italy and cultured as described previously (45). GHRH-R antagonists MIA-602 ([PhAc-AdA]-Tyr₁, ß-Arg₂, Fpa₅, Al₈, Har₆, Tyr(Me)₁₄, His₁₉, Orn₂₁, Abu₂₃, His₂₅, Orn₂₆, Ne²₂, ß-Arg₂₈, Har₂₉GHR-RH(1-29)NH₂) and MIA-690 ([PhAc-AdA]-Tyr₁, ß-Arg₂, Fpa₅, Al₈, Har₆, Tyr(Me)₁₄, His₁₉, Orn₂₁, Abu₂₃, His₂₅, Orn₂₆, Ne²₂, ß-Arg₂₈, Har₂₉GHR-RH(1-29)NH₂) were synthesized and purified in the A.V.S. laboratory as described previously (18).

Human Primary MPM Cells. Human primary biphasic, epithelioid, and sarcomatoid MPM cells were isolated after diagnostic thoracoscopies of MPM patients and cultured as described previously (46). The study was approved by the ethical committees of the Biological Bank of Mesothelioma, the S.S. Antonio e Biagio General Hospital (Alessandria, Italy), and the San Luigi Gonzaga Hospital (Orbiassano, Italy) (studies: #9/11/2011; #126/2016). MPM patients gave their informed consent for the use of primary cells obtained from thoracoscopies.

Cell Survival and Proliferation. Cell survival and proliferation were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide (MTT) assay
Drug synergistic effect was performed according to the Chou-Talalay method of synergy quantitation using Compusyn software (47). Using a cell survival assay and computerized software data, CI values were generated. A CI of 1 indicates an additive effect between two drugs; a CI greater than 1 indicates antagonism; and a CI less than 1 indicates a synergistic effect.

**Caspase-3 Activity**. Caspase-3 activity was assessed by Caspase-3 Colorimetric Assay Kit (Biovision) in cell lysates, according to the manufacturer’s instruction.

**Western Blot Analysis**. Western blotting was performed as described previously (9).

**RT-PCR and Real-Time PCR**. RT-PCR and real-time PCR analyses were performed as described previously (9, 12).

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**Tumor Xenographs**. MSTO-211H cells (2 × 10^6) were resuspended in PBS 1/4 matrigel (Matrigel, Corning) solution (ratio 1:1) and s.c. injected in the right flank of 6- to 8-wk-old NOD/SCID/γδT−/− mice. Male mice. When tumors became palpable, mice were injected with MIA-602 or MIA-690 (5 μg/d) or vehicle, for 4 wk. 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 approved by the Italian Ministry of Health (protocol #52/2018-PR).

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