Potentiation of cytotoxic chemotherapy by growth hormone-releasing hormone agonists

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The dismal prognosis of malignant brain tumors drives the development of new treatment modalities. In view of the multiple activities of growth hormone-releasing hormone (GHRH), we hypothesized that pretreatment with a GHRH agonist, JI-34, might increase the susceptibility of U-87 MG glioblastoma multiforme (GBM) cells to subsequent treatment with the cytotoxic drug, doxorubicin (DOX). This concept was corroborated by our findings, in vivo, showing that the combination of the GHRH agonist, JI-34, and DOX inhibited the growth of GBM tumors, transplanted into nude mice, more than DOX alone. In vitro, the pretreatment of GBM cells with JI-34 potentiated inhibitory effects of DOX on cell proliferation, diminished cell size and viability, and promoted apoptotic processes, as shown by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide proliferation assay, ApoLive-Glo multiplex assay, and cell volumetric assay. Proteomic studies further revealed that the pretreatment with GHRH agonist evoked differentiation decreasing the expression of the neuroectodermal stem cell antigen, nestin, and up-regulating the glial maturation marker, GFAP. The GHRH agonist also reduced the release of humoral regulators of glial growth, such as FGF basic and TGFβ. Proteomic and gene-expression (RT-PCR) studies confirmed the strong proapoptotic activity (increase in p53, decrease in v-myc and Bcl-2) and anti-invasive potential (decrease in integrin α3) of the combination of GHRH agonist and DOX. These findings indicate that the GHRH agonists can potentiate the anticancer activity of the traditional chemotherapeutic drug, DOX, by multiple mechanisms including the induction of differentiation of cancer cells.

Significance

Glioblastoma multiforme (GBM) is one of the most aggressive human cancers, and the afflicted patients inevitably succumb. The dismal outcome of this malignancy demands great efforts to find improved methods of treatment (1). Many compounds have been synthesized in our laboratory in the past few years that have proven to be effective against diverse malignant tumors (2–14). These are peptide analogs of hypothalamic hormones: luteinizing hormone-releasing hormone (LHRH), growth hormone-releasing hormone (GHRH), somatostatin, and analogs of other neuropeptides such as bombesin and gastrin-releasing peptide. The receptors for these peptides have been found to be widely distributed in the human body, including in many types of cancers (2–14). The regulatory functions of these hypothalamic hormones and other neuropeptides are not confined to the hypothalamo–hypophysal system or, even more broadly, to the central nervous system (CNS). In particular, GHRH can induce the differentiation of ovarian granulosa cells and other cells in the reproductive system and function as a growth factor in various normal tissues, benign tumors, and malignancies (2–4, 6, 11, 14–18). Previously, we also reported that antagonist cytotoxic derivatives of some of these neuropeptides are able to inhibit the growth of several malignant cell lines (2–14).

Our earlier studies showed that treatment with antagonists of LHRH or GHRH rarely effects complete regression of glioblastoma-derived tumors (5, 7, 10, 11). Previous studies also suggested that growth factors such as EGF or agonistic analogs of LHRH serving as carriers for cytotoxic analogs and functioning as growth factors may sensitize cancer cells to cytotoxic treatments (10, 19) through the activation of maturation processes. We therefore hypothesized that pretreatment with one of our GHRH agonists, such as JI-34 (20), which has shown effects on growth and differentiation in other cell lines (17, 18, 21, 22), might decrease the pluripotency and the adaptability of GBM cells and thereby increase their susceptibility to cytotoxic treatment.

In vivo, tumor cells were implanted into athymic nude mice, tumor growth was recorded weekly, and final tumor mass was measured upon autopsy. In vitro, proliferation assays were used for the determination of neoplastic proliferation and cell growth. Changes in stem (nestin) and maturation (GFAP) antigen expression was evaluated with Western blot studies in vivo and with immunocytochemistry in vitro. The production of glial growth factors (FGF basic, TGFβ) was verified by ELISA. Further, using the Human Cancer Pathway Finder real-time quantitative PCR, numerous genes that play a role in the development of cancer were evaluated. We placed particular emphasis on the measurement of apoptosis, using the ApoLive-Glo Multiplex Assay kit and by detection of the expression of the proapoptotic p53 protein. This overall approach permitted the evaluation of the peptide analogs | targeted therapy

Growth hormone-releasing hormone (GHRH) and its agonistic analogs, besides augmenting the release of growth hormone from the pituitary, can exert direct stimulatory effects on various extrapituitary tissues. Present oncolic evaluation of growth of a glioblastoma cell line revealed that the combination of GHRH agonist, JI-34, with doxorubicin (DOX) produced greater inhibition in vivo than either drug alone. In vitro, JI-34 also potentiated the effects of DOX, decreased the expression of the neuroectodermal stem-cell antigen, nestin, and up-regulated the glial maturation marker, GFAP. These findings indicate that GHRH agonists can induce differentiation of cancer cells, increasing the response to DOX. These observations expand the known spectrum of activities of GHRH and its analogs and have potential clinical implications for therapy.


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all four treatment groups. The treatment with GHRH agonist resulted in adherent cultures of neuroectodermal cells with more prominent glial projections. Conversely, DOX treatment elicited characteristic fusiform changes in morphology due to arrested mitoses and the mitotic collapse that precedes apoptosis. The combination exerted a devastative effect, perhaps due to the mitotic synchronizing and sensitizing activity of JI-34.

Effect of GHRH Agonist JI-34 and Its Combination with DOX on the Expression of Genes Related to Cell Proliferation, Apoptosis, Cell Cycle, Angiogenesis, Invasion, and Metastasis. Quantitative real-time PCR array studies revealed a significant effect, of the combination of JI-34 and DOX treatment for 6 wk, on the expression of several markers of tumor growth, invasion, and metastasis formation in U-87 cell-derived tumors (Table 1). The combination of JI-34 and DOX increased the expression of the proapoptotic BCL-2-associated agonist of cell death (BAD) and decreased the expression of the anti-apoptotic B-cell lymphoma 2 (Bcl-2). The increase in BAD seems to be of special importance as DOX itself had an opposite effect on BAD expression. The combination treatment also attenuated the expression of the contact activator integrin α3 subunit (by approximately twofold). In addition, an almost threefold decrease was observed in the metastasis promoting S100 calcium binding protein A4 (S100-A4).

GHRH Agonist, JI-34 Facilitates the Apoptotic Activity and Increases the Intracellular Retention of DOX. GHRH agonist JI-34 alone (1 μM) had no effect on apoptosis, but DOX at the 100-nM concentration and the combination of DOX 100 nM and JI-34 1 μM elicited a significant increase (182% and 263%, respectively) in apoptosis as measured by the ApoLive-Glo Multiplex assay after 24 h of treatment (Fig. 3A; F3,17 = 41.31; Tukey’s P < 0.01 vs. DOX vs. control and combination vs. control and Fisher’s P < 0.05 combination vs. DOX). The combination of the DOX and JI-34 treatment also decreased the viability of the cells by 41% (F3,17 = 7.00, Tukey’s P < 0.01 vs. control and Fisher’s P < 0.05 vs. DOX).

In the multidrug resistance (MDR) assay both cyclosporin-A (manufacturer’s recommended control) and DOX treatment increased calcine retention (17% and 7%, respectively; Fig. 3B; F3,86 = 28.856, P < 0.01; Tukey’s post hoc test: P < 0.01 vs. control). The JI-34 pretreatment significantly augmented calcine retention in the combination group by 23% compared with the control (F3,86 = 28.856, P < 0.01; Tukey’s post hoc test: P < 0.01 vs. control and P < 0.01 vs. DOX). It appears that JI-34 pretreatment

Results

In Vivo Effects of the GHRH Agonist JI-34 Alone or in Combination with DOX on the Growth of Glioblastoma U-87 MG Xenografts in Nude Mice. Nude mice bearing U-87 MG tumors were treated with the GHRH agonist, JI-34 (50 μg/kg/d), DOX (13 μmol/kg/wk), or with their combination for 6 wk. Control animals received daily saline injections. The administration of DOX reduced growth of U-87 MG tumors by 35%. The combination of JI-34 with DOX elicited an even greater (63%) inhibition of tumor growth (Fig. 1A) and repeated measure ANOVA revealed a significant difference (F3,66 = 1.962, P < 0.01, Fisher’s post hoc test: P < 0.05 between J-34 + DOX and control). Similar suppressive effects of these therapies were observed on the basis of the reduction of final tumor weights at sacrifice (Fig. 1B) and increases in tumor doubling times (Fig. 1C), although these results did not prove to be statistically significant.

GHRH Agonist JI-34 Augments the Inhibitory Effects of DOX on Proliferation and Growth of U-87 GM Cells in Vitro. The effect of JI-34, DOX, or their combination on the proliferation rate of U-87 MG cells was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Exposure to GHRH agonist, JI-34, alone at 1 μM concentration or 100 nM DOX alone as separate drugs, had no significant effect on the proliferation of U-87 MG cells in vitro. When cells were pretreated with 1 μM JI-34 for 24 h before exposure to the combination of DOX + JI-34 for 48 h, the inhibitory effect (47% inhibition) of the combination treatment proved to be statistically significant (Fig. 2A) (ANOVA; F2,20 = 11.37, Tukey’s post hoc: P < 0.01 vs. both control and DOX).

In a different experiment, cells were cultured in T-25 flasks, and drugs were applied repeatedly (1 μM JI-34 daily and DOX weekly at 100 nM) for 3 wk (Fig. 2B). Cell number and cell volume were then determined by cell counting and cell volumetric assays, respectively. JI-34 alone had no significant effect, but both DOX and its combination with JI-34 significantly decreased the number of cells (52% and 84% decrease, respectively; F2,26 = 19.51 and Tukey’s post hoc: P < 0.01 for DOX vs. control and combination vs. control, whereas Fisher post hoc: P < 0.05 for combination vs. DOX). DOX and its combination with JI-34 also reduced the volume of the cells (20% and 30% decrease, respectively; F2,26 = 9.87 and Tukey’s post hoc: P < 0.01 for DOX vs. control and combination vs. control) as assessed by intracellular water content. Fig. 2C shows representative microscopic images of

Fig. 1. (A) The effect of treatment with the GHRH agonist JI-34 (50 μg/kg/d or 1 μg/mouse/d) (J) and doxorubicin (DOX; D) (13 μmol/kg/wk) alone and in combination on the growth of U-87 MG, human GBM tumors xenotransplanted to nude mice. Numbers at labels represent the number of successfully implanted tumors. (B) Final weights of necropsied tumor samples compared with the control. (C) Numbers at the end of each curve show the tumor doubling times. *P < 0.05 vs. control.

Fig. 2. The effect of single exposure (A) or repeated exposure (B) to GHRH agonist JI-34 (1 μM) (J) doxorubicin (100 nM DOX; D), and their combination on the proliferation of U-87 MG cells in vitro. *P < 0.05 vs. control, †P < 0.01 vs. DOX. For conditions see Materials and Methods and Results. (C) Phase-contrast images of the U-87 MG cell cultures. Images are shown at 400x magnification with 20-megapixel resolution. Representative sections of the visual field were cropped and fitted. (Scale bars, 100 μm.)
leads to decreased expression of MDR transporter proteins and increased calcine and DOX retention.

Effects of GHRH Agonist JI-34, DOX, and Their Combination on the Levels of Intracellular p53 or Secreted FGFB and TGFβ. The changes in the expression of these regulators of tumor growth and differentiation in vitro were detected by ELISA experiments from either cell culture supernatants or from homogenates of U-87 cells treated with 100 nM DOX, 1 μM JI-34, or their combination (Fig. 3C). Using homogenized U-87 MG cell culture samples, we detected a significant (68%) increase in the level of the tumor suppressor p53 upon exposure of the cells to the treatment with combination of JI-34 + DOX (F3,12 = 3.6; Tukey’s P < 0.05 vs. control). The levels of both the glial growth factor, bFGF (23, 24) (F3,16 = 5.15; Fisher’s P < 0.05 JI-34 vs. control), and the dedifferentiation and tumor promoting factor TGFβ (25) (F3,10 = 4.5; Fisher’s P < 0.05 JI-34 vs. control), were significantly lower (37% and 24%, respectively) in the supernatant of the cell cultures exposed to JI-34 treatment, demonstrating the inhibitory effects of JI-34 on the production of these cytokines.

Effects of GHRH Agonist JI-34 and DOX, Alone and in Combination, on the Expression of GHRH Receptors, Nestin and Glial Fibrillary Acidic Protein. Western blot studies (Fig. 4) verified the expression of pituitary type GHRH receptor (pGHRH-R) and its splice variant, SV1, in samples of U-87 MG xenografts. None of the treatments significantly altered the expression of these receptors. The GHRH agonist decreased and the treatment with DOX increased the expression of the neuroectodermal stem-cell marker, nestin, but the effects on the maturation antigen glial fibrillary acid protein (GFAP) (28) were opposite. In the case of both intermediary filaments, statistically significant differences could be observed according to the integrated density values. The combination treatment produced an 80% decrease in nestin expression compared with the control (F3,8 = 7.991; Tukey’s P < 0.01 for JI-34 + DOX vs. DOX, P < 0.05 for JI-34 + DOX vs. control and Tukey’s P < 0.05 for JI-34 vs. DOX), whereas the JI-34 pretreatment caused a 70% increase in GFAP expression compared with the control (F3,8 = 7.991; Fisher’s P < 0.01 for JI-34 vs. DOX, P < 0.05 for JI-34 vs. control and Fisher’s P < 0.05 for JI-34 + DOX vs. DOX).

When U-87 MG cells were treated with the GHRH agonist, JI-34 (1 μM) in reduced serum-containing growth medium, cells underwent morphological changes, having more/longer astrocytic processes than cells in the control group. Representative phase-contrast images from control and GHRH agonist-treated groups are shown in Fig. 5. To further demonstrate the change in differentiation state following the administration of GHRH agonist, cells were fixed and stained for the detection of the astrocyte marker, GFAP, and the neuroectodermal stem cell marker, nestin. The elevation in the intensity of GFAP-labeling indicates that the GHRH agonist-treated cells attained a higher differentiation level than the untreated cells. The fluorescence staining with nestin antibody, however, did not show a significant change in the level of nestin following the administration of GHRH agonist.

Discussion

Our results successfully demonstrate that the concurrent administration of an agonistic analog of GHRH (20) and a traditional cytotoxic drug, DOX, augments the antineoplastic action of the latter. Our findings show that the treatment with the combination of the GHRH agonist and DOX inhibits the in vivo growth of xenotransplanted U-87 MG tumors as well as decreasing the multiplication and growth of these glioblastoma cells in vitro. This effect may be attributed to the ability of the GHRH agonist to induce changes in maturation state consequently decreasing the pluripotency of the neoplastic cells. Traditional cancer therapies frequently fail because of a phenomenon which may be called “survival of the fittest” (27), based upon the natural selection of cancer cells under evolutionary pressure exerted by the treatment itself, and which is analogous to the development of bacterial antimicrobial resistance under antibiotic therapy. This phenomenon may imply the presence of “cancer stem cells” (26, 27). These cancer stem cells can provide an inexhaustible pool of cellular adaptation upon challenge, when there is no time for dedifferentiation, and the induction of the expression of resistance genes.

Previously, our group reported that EGF, a well-known growth factor in both physiological and pathological states, is able to sensitize cancer cells to cytotoxic treatment (19). Our prior results also suggested that an agonistic LHRH analog could have some sensitizing activity, either as a carrier molecule for a cytotoxic agent or as a cotreatment with a cytotoxic agent (10), but it is more likely that the increased therapeutic efficacy is due to targeting capacity to the receptors. Based on our present findings, we can postulate that this sensitizing activity may be related to a maturation effect on the glioblastoma cells, a process characterized by the down-regulation of levels of nestin (a common neuroectodermal marker), and the up-regulation of levels of GFAP (28). Whereas DOX elicited relative increases in nestin and decrease in expression of GFAP, GHRH agonist JI-34 elicited opposite changes in the expression pattern of intermediary filaments in the present study.

Previously we demonstrated that antagonistic analogs of GHRH potentiate the inhibitory effects of docetaxel on growth of human MX-1 and MDA-MB-231 breast cancers and H460 nonsmall cell lung cancers in vivo (29–31). We believed that the increased efficacy of the response was due to the combined effect of GHRH antagonists and docetaxel or to their synergistic action. However, to our knowledge, no beneficial effects of GHRH agonists on tumor growth have been reported previously.

Abrogation of cancer stem cells would be an initial step, a very special one, leading to the successful treatment of GBM and other malignancies by decreasing the resilience of the tumor and its ability to generate recurrence. Facilitation of maturation has
already been demonstrated to be effective in the treatment of certain malignancies; destruction of the nonsense fusion protein denoted t(11;22) with arsenic-trioxide (AsO3); liberation of RARα; administration of the strong maturation factor, retinoic acid, brings about long-lasting regression in acute promyelocytic leukemia (32). Retinoic acid is required for the differentiation of many epithelial cells (33). In addition, retinoids, as a sole treatment of tumor phenotypes with bioavailable receptors, have proven efficacy in basal cell carcinoma and Verruca vulgaris (34). Retinoids play an important role in the maturation of glial cells (28) and have already been successfully used in the therapy of gliomas (35).

The down-regulation of GHRH receptors by our potent GHRH agonist (20), which could lead to the development of resistance to the treatment, may be excluded as indicated by our Western blot studies. This finding supports our view (2, 3, 10, 11) that GHRH and LHRH analogs may manifest a broad range of activities and their pharmacologic profile cannot be limited to the down-regulation of their receptors, as occurs in prostate tumors and contributes to the well-known therapeutic effect of pharmacological castration (12).

The combination treatment of GHRH agonist and DOX also stimulated apoptosis and decreased the viability of the otherwise immortal U-87 cells in vitro (36, 37). Activation of programmed cell death may be related to several initiating events, such as the synthesis of the most important tumor suppressor p53, a protein whose gene is often mutated in GBMs (38, 39). Furthermore, we observed a decrease in the expressions of the anti-apoptotic Bcl-2 and increase in the proapoptotic BAD genes (39, 40). The impact of the combination on BAD, which triggers apoptosis by inactivating Bcl-2 and Bcl-xL (41), was completely opposite to the effect of DOX alone.

Moreover, in sharp contrast with DOX, JI-34 elicited a down-regulation of two important glial growth factors (42, 43), FGF basic and TGFβ. This may also reflect stem cell maturation and a tendency to increased cellular mortality, because growth-promoting cytokines (42) are able to stimulate the survival cascades (44). The decrease in TGFβ is especially important, because this cytokine not only stimulates malignant transformation and growth (45), but also promotes angiogenesis, epithelial–mesenchymal transition (EMT), invasion, and suppresses peritumoral immune responses (45). Further, deprivation of growth factors (2) may suspend the constitutive stimulatory activity of extracellular signal-regulated kinases/mitogen activated protein kinases (ERK/ MAPKs) over Bcl-2 (37). The combination significantly mitigated the transcription of the contact activator integrin domain, integrin α3 subunit. This molecule, as are other integrins, is frequently overexpressed in GMB tumors and plays an important role in the migration of neuroectodermal cells in both physiological and pathological circumstances (46). Upon binding laminin, fibronectin, or vitronectin (47), the integrin facilitates cell proliferation, EMT, and invasion through the activation of integrin-linked (48) and focal adhesion kinases (FAKs) (49). The down-regulation of the transcription of S100 calcium binding protein A4 (S100-A4) gene, which regulates microtubule polymerization and migration, may also contribute to the inhibition of cancer cell motility and metastatic spread. Moreover, S100-A4, beside activating motility, plays additional roles in proteolysis, EMT, angiogenesis, and cell survival (50).

Our demonstration that a change in the differentiation state of U-87 MG cells triggered by a GHRH agonist highly increases the susceptibility to cytotoxic treatments is particularly important for determining future strategies in the treatment of glioblastoma. GHRH analogs are promising candidates in this respect, as they readily bypass the blood–brain barrier (51). These findings suggest the potential for the development of new therapeutic paradigms in the treatment of brain cancers using agonistic as well as antagonistic analogs of hypothalamic GHRH. These GHRH analogs (2, 4, 6, 11, 20–22) may augment the direct cytotoxic effect of traditional chemotherapeutic drugs, by stimulation and maturation of stem cells and eliciting cellular synchronization of the cell cycle. To further investigate this phenomenon, other studies are necessary to assess the outcome of the combination of GHRH analogs and improved forms of chemotherapeutic agents such as liposomal doxorubicin.

More sophisticated pretreatment or cotreatment with GHRH agonists may further augment the effect of chemotherapeutic agents on malignancies. The impact on cell cycle is especially important, in this regard, because synchronization of dormant cells may make the tumor more susceptible to intercalating agents, such as DOX (52). To clarify these possibilities, further studies are needed.

**Materials and Methods**

**Peptides and Chemicals.** The GHRH agonist, JI-34, was synthesized in our laboratory by solid-phase method and purified by reversed-phase HPLC as described previously (20). The structure of JI-34 is [Dat1, Orn12,21, Abu15, Nle27, Asp28, Agm29]hGH-RH-(1–29) (Abu, a-aminobutyric acid; Agm, agmatine; Dat, desaminotyrosine; Nle, norleucine; Orn, ornithine). For studies in vivo, we used daily s.c. injections of JI-34 dissolved in 0.1% DMSO (Sigma) in 10% (vol/vol) DMSO–FBS-containing growth medium. DOX was prepared freshly from a stock solution in DMSO.

**Phase-contrast images of live cells were taken after 2 d and then cells were fixed and stained for the differentiation markers, GFAP and nestin.**

**Fig. 4.** Western blot images (A) and integrated density values (IDVs) (B) for the expression of GHRH receptors and differentiation antigens in necropsied in vivo samples. GHRH-R, pituitary type growth hormone releasing hormone receptor; SV1, splice variant-1 of GHRH receptor; DOX or D, doxorubicin; J, JI-34; GFAP, glial fibrillary acidic protein. *P < 0.05 vs. control.

**Fig. 5.** GHRH agonist induces the differentiation of U-87 MG cells in reduced serum-containing medium in vitro. Cells were treated with GHRH agonist JI-34 (1 μM) or DMSO in 0.1% FBS-containing growth medium. Phase-contrast images of live cells were taken after 2 d and then cells were fixed and stained for the differentiation markers, GFAP and nestin. The GHRH agonist-treated cells possess a higher tendency toward outgrowth of projections; moreover, high levels of GFAP were detected in these cells, indicative of glial differentiation. The protein level of nestin did not change notably following the treatment with GHRH agonist. Nuclei were stained with DAPI. (Scale bars, 100 μm.)
aqueous propylene glycol (vehicle solution). For in vitro experiments, JI-34 was dissolved in 0.1% DMSO and diluted with incubation medium. DOX (Chenmec Leuna) was administered i.v. and dissolved in 0.01 N acetic acid and diluted with 5% (v/v) mannitol.

Animal Experiments. Six-wk-old nude mice (Ncr nu/nu) were obtained from the National Cancer Institute (Bethesda, MD). The animals were housed in sterile cages in a temperature-controlled room with a 12-h light/12-h dark schedule and were fed with autoclaved chow and water, ad libitum. The Institutional Animal Care and Use Committee, VA Medical Center Miami, fully approved the animal protocols. For the therapy study, 10^5 cells per mouse of the human glioblastoma cell line, U-87 MG (American Type Culture Collection, ATCC), were injected into the flanks of four nude mice (Ncr nu/nu) under isoflurane anesthesia (Baxter). A month later, the resulting tumors were harvested and minced into ∼3-mm^2 pieces and transplanted into flanks of nude mice, under anesthesia, using a minitocarc. When the tumors reached ∼32 mm^3, the mice were randomized into four groups. The animals received the following treatments for 6 wk: (Group 1) control, 16 tumors, vehicle solution; (group 2) agonist JI-34, 1 μg/kg of s.c., daily, 19 tumors; (group 3) DOX, 260 nmol/20 g, i.v., weekly on Thursdays, 19 tumors; and (group 4) JI-34 (1 μg/kg daily) + DOX (260 nmol/20 g), 16 tumors. Tumor volume was measured with microcalipers once a week and calculated using the formula: \[ V = \frac{4}{3} \pi r^3 \] where \( r \) is the tumor radius. Tumor doubling time was calculated using the formula: \[ \text{doubling time} = \frac{\ln 2}{\lambda} \] where \( \lambda \) is the growth rate of the tumor. Necropsy was performed. Samples free of necrotic debris were immediately snap frozen in liquid nitrogen and stored at −80 °C for further analyses.

Cell Maintenance and Cell Counting. U-87 MG cells were cultured in Eagle’s minimum essential medium (ATCC) medium supplemented with 10% FBS (ATCC) and 0.1% penicillin/streptomycin at 37 °C and 5% CO_2 atmosphere. For cell count and cell size determination, 10^3 cells were seeded into T-25 flasks. The medium was changed two to three times a week. The following treatment in vitro groups were set up: (Group 1) control, vehicle solution; (group 2) DOX; (group 3) DMSO; and (group 4) JI-34 (1 μM final concentration), weekly on Thursdays; and (group 4) JI-34, 1 μM and DOX, 100 nM. After 3 wk, cell count and cell size were determined by using 2 Series Coulter Counter (Beckman Coulter).

Cell Volume Determination. Cell volume was estimated by measuring the intracellular water space by the method of Kletzein et al. (53), as modified by Bender and Noreenberg (54). Briefly, 1 mM 3-O-methylglycose (3-MOG) and 0.5 μCi/mL [3H]3-MOG were added to the culture 6 h before the volume assay. At the end of the incubation period, culture medium was aspirated, rinsed with ice-cold buffer containing 460 mM sucrose, 1 mM Tris-buffered saline (TBS), and an aliquot was saved for radioactivity determination. Cells were washed rapidly six times with ice-cold buffer containing 229 mM sucrose, 1 mM Tris, 1 μM calcium nitrate, 0.5 mM calcium nitrate, and 0.1 mM phloretin, pH 7.4. Cells were harvested into 0.5 mL of 1 N sodium hydroxide. Radioactivity in the cell samples was determined, and an aliquot of the cell extract was used for protein estimation with the Bio-Rad bicinchoninic acid kit. Values were normalized to protein level, and cell volume was measured with microcalipers once a week and calculated using the formula: \[ V = \frac{4}{3} \pi r^3 \] where \( r \) is the tumor radius. Necropsy was performed. Samples free of necrotic debris were immediately snap frozen in liquid nitrogen and stored at −80 °C for further analyses.

Total RNA Isolation and Reverse Transcription. Total RNA was isolated from representative, DNase treated, U-87 MG dissected tumor samples using a NucleoSpin kit according to the manufacturer’s instructions (Macherey-Nagel). Four tumor samples from each group were analyzed. The yield and the quality of RNA samples were determined spectrophotometrically using 260 nm, and 260/280- and 260/230-nm ratio. The synthesis of cDNA was performed as described (55). Briefly, 1 μg of RNA from each sample was reverse transcribed into cDNA by a RT First Strand kit (Qiagen). Reverse transcription was done in a Veriti 96-well thermal cycler (Applied Biosystems).

Immunocytochemistry. Cells were seeded onto coverslips in six-well plates (50,000 cells per well) in 10% FBS-containing growth medium. This medium was replaced with serum-free medium the following day for 24 h. Thereafter, GHRH agonist (JI-34, 1 μM) was added in medium supplemented with 0.1% FBS and remained for the consecutive 2 d. Control cells received vehicle (DMSO). At the end of the treatment, phase-contrast images of live cells were collected and cells were fixed in ice-cold acetone for 10 min, washed with PBS three times, and blocked with 2% goat serum in PBS for 30 min. GFAP (Abcam; 1:500 dilution) or nestin (BD Transduction Laboratories; 1:75 dilution) antibodies were added in PBS for 1 h. Anti-rabbit and anti-mouse secondary antibodies (Alexa Fluor 488, Jackson Immunoresearch) were also applied for 1 h. Coverslips were mounted in Vectashield mounting medium containing DAPI for nuclear staining (Vector Laboratories). Images were acquired on a Nikon Eclipse Ti fluorescence microscope (Nikon Instruments).

MDR Assay. The multidrug resistance assays were performed according to the manufacturer’s instructions (Cayman Chemical). U-87 MG cells were seeded, 5 × 10^4 cells per well in 100 μL medium in 100-well black, clear-bottom microtiter plates and grown over night in a humidified incubator at 37 °C. The next day the medium was discarded, and the cells were treated according to the following protocol: Groups 1 (control) and 3, vehicle solution; groups 2 and 4, JI-34 (1 μM final concentration). After another 24 h, the cells received the following treatments: Group 1, vehicle solution; group 2, JI-34, Group 3, DOX (100 nM final concentration); and group 4, JI-34 + DOX. During the second treatment, half of the control wells were treated with cyclosporin-A solution in 1/10,000 dilution as positive control according to the manufacturer’s instructions. Afterward, the cells were incubated for 1 h, then calcein AM/Hoechst dye combined staining solution was added. Fifteen minutes later, both cell density (at excitation and emission wavelengths of 355 nm and 465 nm, respectively) and calcein retention (at excitation and emission wave-lengths of 485 nm and 535 nm, respectively) were detected with the help of a Victor3 multilabel counter. Relative calcein retention values were expressed as a function of cell density.

Cancer Pathway Finder Quantitative PCR Array. The Human Cancer Pathwayfinder quantitative PCR array (PAHS-033A; Qiagen) used in our study contains the unique genes related to cell proliferation, cell-cycle progression, angiogenesis, invasion, and metastasis. All PCR arrays were performed using the iQS Multicolor Real-Time Detection system (Bio-Rad). All genes represented by the array showed a single peak on the melting curve characteristic of the specific products. Experiments were run in triplicate for each study group. Analysis of gene expression data was performed using Excel-based PCR Array Data Analysis software provided by the manufacturer (Qiagen). Fold changes in gene expression were calculated using the ΔΔCT method and five stably expressed housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, and ACTB) were used for normalization of the results.

Statistical Analyses. Statistical analyses were performed using either t test for independent samples, univariate analysis of variance (ANOVA), or repeated measure ANOVA (RMANOVA). ANOVA was followed by Tukey’s post hoc test. Results are expressed as the means ± SEM. Differences, compared with the control, with P < 0.05 considered as statistically significant. Statistical analyses and data reductions were performed by SigmaPlot 11.0 (Systat software) and IBM SPSS Statistics 20.0. Additional information is provided in SI Text.
Supporting Information

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SI Text

ELISA. Glioblastoma cells (10^5 cells per well) were seeded onto six-well plates, cultured overnight, and then exposed to compounds used in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assays: Groups 1 (control) and 3, vehicle solution; groups 2 and 4, GHRH agonists JI-34 (1 μM final concentration). After another 24 h, the cells received the following treatments: Group 1, vehicle solution; group 2, JI-34; group 3, doxorubicin (DOX) (100 nM final concentration); and group 4, JI-34 + DOX. Concentrations of the specific proteins in the medium (in the case of FGF basic or TGFβ-1) or in samples of scraped and homogenized cells (in the case of p53) were determined after 24 h using ELISA kits according to the manufacturer’s instructions. Human TGFβ and FGF basic ELISA kits were obtained from AbCam, whereas p53 was measured with a PathScan Sandwich ELISA kit (Cell Signaling Technology). Readings were normalized to protein concentrations as determined by NanoDrop (NanoDrop Technologies).

Western Blot Analyses. Protein from the tumor tissue was isolated using the NucleoSpin kit (Macherey-Nagel). Protein concentrations were determined by NanoDrop (NanoDrop Technologies). Equal amounts of protein were resuspended in sample loading buffer (0.25 M Trizma Base, 8% SDS, 40% glycerol, 0.004% bromophenol blue, 4% β-mercaptoethanol; pH 6.8), boiled for 3 min and separated by 12% SDS-polyacrylamide gel electrophoresis. Proteins from the gel were then transferred onto nitrocellulose membranes, which were blocked with 50–50% Tris-buffered saline (20 mM Tris-HCl pH 7.5, 150 mM NaCl) and Odyssey blocking buffer for 1 h at room temperature, followed by an overnight incubation at 4 °C with the following primary antibodies: growth hormone-releasing hormone receptor (GHRH-R), (ab28692) nestin (ab92391), glial fibrillary acid protein (GFAP) (ab48050) (all from AbCam), β-actin (A5441; Sigma-Aldrich), or p53 (9282; Cell Signaling Technology). The GHRH-R antibody is targeted against the polypeptide segment found in both pituitary (p)GHRH and splice variant-1 (SV1) receptors. The signals were developed by incubating the nitrocellulose membrane for 1 h at room temperature with the appropriate Infrared IRDye-labeled secondary antibodies (1:10000; LI-COR Biosciences) and were then visualized with the Odyssey Infrared Imaging system (LI-COR Biosciences). The protein bands were quantified using V3.0 software (LI-COR Biosciences); integrated density values of triplicate experiments were plotted.