Gonadotropin-Releasing Hormone Receptor Levels and Cell Context Affect Tumor Cell Responses to Agonist In vitro and In vivo

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Abstract

Activation of gonadotropin-releasing hormone (GnRH) receptors inhibits proliferation of transformed cells derived from reproductive tissues and in transfected cell lines. Hence, GnRH receptors represent a therapeutic target for direct action of GnRH analogues on certain proliferating cells. However, more cell biological data are required to develop this particular application of GnRH analogues. Therefore, we compared the effects of GnRH receptor activation in transfected HEK293 cells (HEK293[SCL60]) with transfected human ovarian cancer cell lines SKOV3 and EFO21, human hepatoblastoma HepG2 cells, and rat neuroblastoma B35 cells. Marked differences in receptor levels, magnitude of inositol phosphate generation, and dynamics of inositol phosphate turnover occurred in the different cells. Activation of GnRH receptors, expressed at high or moderate levels, inhibited the growth of HEK293[SCL60] and B35 cells, respectively. Western blotting detected markers of apoptosis [cleaved poly(ADP-ribose) polymerase, caspase-9] in HEK293[SCL60] and B35 following treatment with 100 nmol/L D-Trp6-GnRH-I. Cell growth inhibition was partially or completely rescued with inhibitor Q-VD-OPh or Ro32-0432. Low levels of GnRH receptor expression in transfected SKOV3, EFO21, or HepG2 activated intracellular signaling but did not induce apoptosis or significantly affect cell proliferation. Tumor xenografts prepared from HEK293[SCL60] regressed during treatment with D-Trp6-GnRH-I and growth of xenografts derived from transfected B35 was slowed. SKOV3 xenografts were not growth inhibited. Therefore, differences in levels of GnRH receptor and signaling differentially affect the apoptotic machinery within cell lines and contribute to the cell type–specific effects of GnRH on growth. Further studies should exploit the growth-inhibitory potential of GnRH receptor activation in abnormal cells in diseased human tissues. [Cancer Res 2008;68(15):6331–40]

Introduction

Gonadotropin-releasing hormone (GnRH) analogue therapy is potentially useful in the treatment of certain malignancies, particularly those that are sex steroid hormone dependent, due to the sex steroid–lowering effects of GnRH administration. Application of long-acting analogues such as D-Trp6-GnRH-I superagonist (1, 2) in second-line therapy for ovarian cancer (3), uterine cancer (4), and premenopausal breast cancer (5, 6) has been studied in small (3, 4) or moderately sized clinical trials (2) since the late 1980s. Larger data sets indicate that a relatively poor, widely variable, yet favorable additive effect on response rates can occur when GnRH is used in combination therapy (5, 6). Most studies have focused on patients with advanced cancer, with fewer analyzing the effects of early-stage treatment. A recent meta-analysis encompassing a large sample size (11,900 cases) reported small benefits (12% additional decrease in disease recurrence rate) associated with GnRH analogues in the treatment of steroid receptor–positive breast cancer when used in combination with tamoxifen (5). On the other hand, GnRH agonists are widely and effectively used clinically to suppress androgen levels and extend survival in men with advanced prostate cancer (7).

In certain tumors, a direct antiproliferative effect of GnRH receptor activation on malignant cells represents an added, but currently unexploited, benefit of GnRH analogue therapy (8–16). However, the extent to which this direct action may be applicable to cancer therapy remains uncertain. The incidence of GnRH receptor–positive tumors is undefined and many phenotypic variables probably influence the effect of GnRH receptor activation on cell fate, including oncogenic mechanisms likely to mediate resistance to GnRH action.

One concern is that cell types in the mammalian body express GnRH receptors in different contexts. For instance, high levels of receptor occur in pituitary gonadotropes and in certain neuronal cells within the central nervous system whereas much lower levels of receptor expression occur in peripheral reproductive tissues (17), including pathological specimens (8, 18). More studies are required to address the practical significance of variations in receptor expression levels and other cell-specific differences to enable improved application of GnRH analogues in the treatment of proliferative disease.

Thus far, diverse in vitro studies using cells that endogenously express low levels of GnRH receptor have confounded the evaluation of the applicability of GnRH-mediated cell growth inhibition. For instance, low levels of receptor expression have been described in a number of cell lines derived from human reproductive tissue malignancies [including breast (MCF-7 and MDA-MB-231), ovary (OV-1063, OVCA-3, SKOV3, EFO21, EFO27, and Caov-3), uterine epithelium (Hec1A, Ishikawa, and RL95-2), and prostate (AT-1, DU145, and LNCaP); refs. 8–16]. Very significant cell growth–inhibitory effects of GnRH analogues on all these cell lines have been reported (often >30% inhibition within 5–9 days). Effects on transformed cells from nonreproductive tissues, including liver, pancreas, and melanoma, have also been described (19, 20). Growth of some of these cells as tumor xenografts in nude mice can be inhibited with GnRH analogues.

However, contradictory data exist in that the absence of response to GnRH analogues has been described for some cell

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In vitro (10) or in xenograft studies (21). One possibility is that fluctuations in levels of receptor expression occur during cell passage in vitro or as a consequence of differences in culture conditions. Certain cells (e.g., LNCaP) show either a proliferative or an antiproliferative response to GnRH analogue depending on analogue concentration (15). Hormones such as estrogen (22), insulin-like growth factors, or members of the epidermal growth factor family may counteract the growth-inhibitory effects of GnRH (17).

The relevance of comparisons between effects of GnRH on different cell lines in vitro or in xenograft experiments to the clinical situation is currently difficult to interpret. Nevertheless, in vitro studies have implicated various intracellular signaling pathways in GnRH analogue–mediated cell growth inhibition (23–31), with cell cycle arrest and apoptosis clearly established as outcomes in target cells. However, whether different cell types share a common mechanism for GnRH-mediated growth inhibition is not clear. One way to rigorously investigate this question, and other uncertainties, is to examine different cell types engineered in parallel to express the GnRH receptor by genetic manipulation. In transfected cells, activation of the mammalian type 1 GnRH receptor, including the human isoform (32), reproducibly inhibits growth in vitro, largely irrespective of passage number under standard culture conditions and without the need to manipulate steroid hormone content or other culture components. However, recently studied cell lines, either transfected with GnRH receptor cDNA (e.g., HEK293 and MCF-7) or isolated from transgenic mice (e.g., L/ST2 or αT3-1 embryonic gonadotropes), express the GnRH receptor at relatively high levels, unlike the situation in cells derived from tumors. Therefore, levels of GnRH receptor expression at the cell surface have been carefully titrated in MCF-7 and PC3 cells using an adenosiral vector. Cell-surface receptor level was shown to be a major influence on the extent of GnRH-mediated growth inhibition in vitro (33, 34).

The behavior of cell lines stably transfected with GnRH receptor has not previously been studied simultaneously in vitro and in vivo, where pharmacokinetic and systemic factors may influence cell proliferation. Therefore, we compared the effects of GnRH receptor activation on the growth of five different transfected cell lines stably expressing rat GnRH receptor over a range of levels. The cells were studied both in vitro and in vivo with the aim of identifying factors important in determining the effects of d-Trp<sup>6</sup>-GnRH-I on growth. We confirmed that the level of GnRH receptor expression correlates with the extent of growth inhibition both in vitro and in vivo, and we established that in vitro growth inhibition can be prevented in a cell type–specific fashion using exogenous chemical inhibitors of signaling. The results suggest strategies to develop more precisely targeted applications of GnRH analogues on appropriate GnRH receptor–positive cells.

Materials and Methods

**Reagents.** Most chemicals were purchased from Sigma: n-Trp<sup>6</sup>-GnRH-I, sulforhodamine B, and 8-bromo-cyclic AMP (8-BrcAMP). d-Trp<sup>6</sup>-GnRH-I was dissolved in 20% propylene glycol (Sigma). Certain reagents were from Calbiochem: Ro32-0432, Q-VDP-OPh, PD98059, SB203580, rotllerin, fumonisin B1, 2-aminoethoxydiphenylborate (2-APB), and m-3msFBS. Radioactive reagents, Na<sup>125</sup>I and myo[<sup>3</sup>H]inositol, were from Amersham.

**Cell culture.** Cells were grown in DMEM containing 10% FCS, glutamine, and antibiotics. Nontransfected cell lines HEK293 (35), B35 (36), SKOV3 (37), and HepG2 (38) were obtained from the American Type Culture Collection. Nontransfected EFO21 cells (39) were a gift from Prof. Günter Emmons (Georg-August-University, Göttingen, Germany). HEK293 cells expressing the rat GnRH receptor HEK293[5C4Lc6] cells were generated by our colleagues in our laboratory (40).

**Isolation of cell clones stably expressing rat GnRH receptor.** Stably transfected cell lines were prepared following electroporation or Fugene 6 transfection (Roche) of rat GnRH receptor cDNA in pCDNA3.1 (Invitrogen) into cells followed by G418 antibiotic selection and clone isolation.

**GnRH binding assay.** <sup>125</sup>I-radiolabeled (His<sup>5</sup>-α-Tyr<sup>9</sup>)-GnRH-I analog bound to cells in 12-well plates was displaced with different concentrations of unlabeled native GnRH-I, each in triplicate. The number of cells per well was determined using a hemocytometer following trypsination of wells from a plate prepared in parallel. Binding data were analyzed using Prism software (GraphPad).

**Inositol phosphate assay.** Cells in 12-well plates were assayed for generation of <sup>3</sup>H-radiolabeled inositol phosphate following treatment with GnRH or phospholipase C activator m-3msFBS at various concentrations (each in triplicate) using a method adapted for batch chromatography with AG 1-X8 resin (Bio-Rad) in 12 × 75 mm plastic tubes. The number of cells per well was determined as described above.

**In vitro growth assay.** Cells growing in 12-well plates (200,000 per well with 2-mL medium) were treated with GnRH in triplicate and growth was monitored using the sulforhodamine B staining assay (41). Growth rates for untreated cells in 12-well plates were also measured by daily sampling in duplicate over a period of 120 h by manual counting with a hemocytometer following trypsination. The data were analyzed using Prism software.

**Western blotting.** Cells were cultured in six-well plates for short time-course experiments (i.e., for treatments up to 6–8 h) or in 6-cm dishes for time courses up to 5 d. Cells were lysed in ice-cold NP40 lysis buffer. Cellular fractions were collected following 10-min centrifugation at 14,000 rpm and mixed with SDS-PAGE loading buffer. For quantitative analysis, treatments were done in triplicate. Western blotting was done with SDS-PAGE mini-gels (Invitrogen), prestained broad-range molecular weight markers (Bio-Rad), and polyvinylidene difluoride membrane (Perkin-Elmer). Rabbit polyclonal antibodies were purchased from Cell Signaling Technology: anti–phospho-extracellular signal–regulated kinase (ERK)-1/2, anti–total ERK1/2, anti–phospho-p38, anti–cleaved poly(ADP-ribose) polymerase (PARP; human or rat specific), anti–cleaved caspase-9, and anti–phospho-Fas-associated death domain (FADD); β-actin was from Abcam. Specific antibody binding was detected by enhanced chemiluminescence with alkaline phosphatase–conjugated secondary antibody (Sigma) and enhanced chemiluminescence substrate (GE Healthcare) on a Typhoon phosphoimager (Amersham Biosciences). Bands were measured using ImageQuant software (Amersham Biosciences).

**Tumor xenograft experiments.** Cloned cell lines were expanded in vitro in the presence of 500 μg/mL G418 before implantation into nude mice. Cells (5–10 million) were implanted s.c. (bilaterally) into the flanks of groups of adult female nude mice. At least five animals per group were studied. Pharmacologic treatment was initiated when tumors were 50 to 100 mm<sup>3</sup> in size. Tumor volumes were measured in two diameters using calipers and volumes were calculated (V = π × D × d<sup>2</sup> / 6, where D and d represent the larger and smaller diameters, respectively). Mean tumor volumes were measured and expressed as a ratio of the value on day 0 of treatment.

**Statistical analyses.** Statistical analysis of data was done using online software (Simple Interactive Statistical Analysis<sup>3</sup> and the SD calculator<sup>4</sup>).
and expanded. Cell clones were screened for GnRH receptor expression using a $^{125}$I-radioligand binding assay and compared with HEK293[SCL60] and αT3-1 cells. Stability of receptor expression was verified over the course of subsequent cell passages. Transfected cell clones exhibited differences in relative levels of receptor expression (Fig. 1). Nontransfected cell lines did not possess detectable endogenous specific binding (displaceable radioligand binding never exceeded 1% that observed in transfected cells, see dashed line in Fig. 1). HEK293[SCL60] cells expressed ~8.6-fold more GnRH receptor at the cell surface than transfected SKOV3-clone 4 cells (SKOV3-4), which expressed the lowest level of receptor in the range of cell clones studied here. Rat neuronal cell clones B35-2b, B35-20, and B35-2 expressed 5.1-, 2.3-, and 1.9-fold higher levels of receptor per cell than SKOV3-4. Clones EFO21-1 and HepG2-3 expressed only slightly more receptor per cell than SKOV3-4 (30% of receptor per cell). Clones EFO21-1 and HepG2-3 expressed 5.1-, 2.3-, and 1.9-fold higher levels of receptor compared with receptor binding in mouse αT3-1 gonadotrope cells. Very low levels of displaceable radioligand binding were detected in nontransfected cells (Fig. 2). Typically, HEK293[SCL60] cell growth was inhibited by 70% relative to vehicle-treated cells within 96 hours (i.e., sulfrohormadine B staining was 30% that of controls). Growth of B35-2b cells was inhibited by 85% relative to vehicle-treated cells after 96 hours. B35-20 and B35-2 cells were growth inhibited by 22% to 23% after 96 hours. Dose-response analysis indicated that 5 nmol/L D-Trp$^5$-GnRH-I was sufficient to induce growth inhibition as effectively as 25 or 100 nmol/L D-Trp$^5$-GnRH-I. For HEK293[SCL60] and B35-2b, relative growth inhibition was detectable within 24 hours and was cumulative during the 96-hour time course. A brief exposure to D-Trp$^5$-GnRH-I (10 min) was sufficient to elicit growth inhibition but continuous exposure was marginally more effective. Vehicle-treated cell cultures became confluent within 96 hours but cell viability was not compromised following further incubation for at least 24 hours without the need to change culture medium.

GnRH-stimulated production of inositol phosphate. Clones exhibiting specific GnRH binding were characterized by $[^3]$H][inositol phosphate assay. The amount of inositol phosphate production reflected the level of receptor expression determined by binding assay (Fig. 3). HEK293[SCL60] cells generated 3- to 8-fold more inositol phosphate per cell than transfected B35 cells and 11- to 12-fold more inositol phosphate than transfected SKOV3-4 or EFO21-1 cells. The HepG2-3 clone exhibited weakest inositol phosphate production. Maximum relative levels of inositol phosphate production per cell after 90 minutes of stimulation were HEK293[SCL60], 11.4; B35-2b, 3.8; B35-2, 2.4; B35-20, 1.6; EFO21-1, 1.0; SKOV3-4, 0.6; and HepG2-3, 0.3. The maximum levels of $[^3]$H][inositol phosphate were at least 2-fold above basal in cells expressing low levels of GnRH receptor (i.e., EFO21-1, SKOV3-4, and HepG2-3) and were much higher than basal in HEK293[SCL60] cells (at least 50- to 70-fold) and B35 clones (3- to 6-fold).

The dynamics for inositol phosphate generation were similar between cell clones, reaching a peak at ~90 minutes after receptor activation. On replacement of culture medium to remove GnRH agonist and to release inositol phosphate inhibition (GnRH and LiCl washed out with DMEM), inositol phosphate levels returned to basal in B35-20 cells within 2 to 2.5 hours, but did not return to basal levels in HEK293[SCL60], B35-2b, or B35-2 cells. In these clones, inositol phosphate remained 9.9-, 2.0-, and 1.5-fold above basal, respectively, for at least another hour (i.e., 3–3.5 hours in total after removal of GnRH and LiCl).

D-Trp$^5$-GnRH-I was more potent than native GnRH-I in stimulation of inositol phosphate production. In HEK293[SCL60] cells, 5 nmol/L native GnRH-I elicited 18% of maximal inositol phosphate production whereas 5 nmol/L D-Trp$^5$-GnRH-I elicited 90% of maximum inositol phosphate production. In B35-2 cells, 5 nmol/L native GnRH-I elicited 47% of maximal inositol phosphate production before calculation relative to SKOV3-4. The highest relative level expression using a 125I-radioligand binding assay and compared with HEK293[SCL60] and αT3-1 cells. Stability of receptor expression was verified over the course of subsequent cell passages. Transfected cell clones exhibited differences in relative levels of receptor expression (Fig. 1). Nontransfected cell lines did not possess detectable endogenous specific binding (displaceable radioligand binding never exceeded 1% that observed in transfected cells, see dashed line in Fig. 1). HEK293[SCL60] cells expressed ~8.6-fold more GnRH receptor at the cell surface than transfected SKOV3-clone 4 cells (SKOV3-4), which expressed the lowest level of receptor in the range of cell clones studied here. Rat neuronal cell clones B35-2b, B35-20, and B35-2 expressed 5.1-, 2.3-, and 1.9-fold higher levels of receptor per cell than SKOV3-4. Clones EFO21-1 and HepG2-3 expressed only slightly more receptor per cell than SKOV3-4 (30% of receptor per cell). Clones EFO21-1 and HepG2-3 expressed 5.1-, 2.3-, and 1.9-fold higher levels of receptor compared with receptor binding in mouse αT3-1 gonadotrope cells. Very low levels of displaceable radioligand binding were detected in nontransfected cells (Fig. 2). Typically, HEK293[SCL60] cell growth was inhibited by 70% relative to vehicle-treated cells within 96 hours (i.e., sulfrohormadine B staining was 30% that of controls). Growth of B35-2b cells was inhibited by 85% relative to vehicle-treated cells after 96 hours. B35-20 and B35-2 cells were growth inhibited by 22% to 23% after 96 hours. Dose-response analysis indicated that 5 nmol/L D-Trp$^5$-GnRH-I was sufficient to induce growth inhibition as effectively as 25 or 100 nmol/L D-Trp$^5$-GnRH-I. For HEK293[SCL60] and B35-2b, relative growth inhibition was detectable within 24 hours and was cumulative during the 96-hour time course. A brief exposure to D-Trp$^5$-GnRH-I (10 min) was sufficient to elicit growth inhibition but continuous exposure was marginally more effective. Vehicle-treated cell cultures became confluent within 96 hours but cell viability was not compromised following further incubation for at least 24 hours without the need to change culture medium.

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phosphate production, whereas 5 nmol/L d-Trp⁶-GnRH-I elicited 89% of maximum inositol phosphate production (data not shown). A phospholipase C activator, m-3m3FBS, elicited a small additive effect on production of inositol phosphate in HEK293[SCL60] cells treated with d-Trp⁶-GnRH-I over the first 5 to 30 minutes of stimulation (30% elevation), followed by marked inhibition of further inositol phosphate production (62% less inositol phosphate relative to cells treated with d-Trp⁶-GnRH-I alone for 90 minutes). An additive effect of m-3m3FBS plus d-Trp⁶-GnRH-I on production of inositol phosphate was also detectable in SKOV3-4 cells (2.7-fold relative increase in inositol phosphate at 90-minute stimulation) but there was no feedback inhibition of phospholipase C in these cells relative to cells treated with d-Trp⁶-GnRH-I alone (data not shown).

GnRH receptor–activated protein kinase signaling in different cell lines. Treatment with GnRH elicited rapid transient phosphorylation of ERK1/2 in transfected HEK293[SCL60], EFO21-1, SKOV3-4, and HepG2-3 cells. In contrast, transfected B35 cells exhibited transient dephosphorylation of ERK1/2 (Fig. 4). Rapid initial transient activation of the stress-activated protein kinase p38 was readily detectable in HEK293[SCL60] cells but not in the other transfected cell lines. Elevated levels of phosphorylated p38 were detectable in both HEK293[SCL60] and B35-2b cells at later stages of exposure to d-Trp⁶-GnRH-I (48–96 hours; Fig. 4C).

Time course for detection of markers of apoptosis following GnRH treatment. Elevated levels of cleaved PARP were readily detectable in HEK293[SCL60] cells following 24 to 48 hours of treatment with d-Trp⁶-GnRH-I. Small elevations in the levels of cleaved caspase-9 and phosphorylated FADD were also detectable in these cells (Fig. 4C). Much lower levels of cleaved PARP were detectable in B35-2b cells compared with HEK293[SCL60]. Elevations in these markers attributable to d-Trp⁶-GnRH-I treatment were not detected in EFO201-1 or SKOV3-4 cells. However, elevation in the level of cleaved PARP was detected in SKOV3-4 cells following treatment with m-3m3FBS (data not shown).

Manipulation of GnRH effects in vitro. In some experiments, cells were treated with a combination of a signal-modulatory compound plus d-Trp⁶-GnRH-I (Table 1). Inhibition of protein kinase C (PKC) using Ro32-0432 rescued transfected HEK293[SCL60] cells from GnRH-mediated growth inhibition (Table 1; Fig. 5). Dose-response analysis indicated that 100 nmol/L Ro32-0432 enabled complete protection (100% rescue) and attenuated the appearance of cleaved PARP (Fig. 5B). The general caspase inhibitor Q-VD-OPh (20 μmol/L) enabled partial rescue of these cells in the presence of 100 nmol/L d-Trp⁶-GnRH-I (40% more cell growth after 96 hours compared with cells treated with GnRH agonist alone). However, transfected B35 cell clones were partially protected from the effects of d-Trp⁶-GnRH-I by Q-VD-OPh (also 40% more cell growth for each clone at 96 hours) but were not protected by Ro32-0432.

Certain agents exerted an additive growth-inhibitory effect when combined with d-Trp⁶-GnRH-I whereas others had no effect (see Table 1). Growth of SKOV3-4 cells was inhibited by treatment with the phospholipase C activator m-3m3FBS (60 μmol/L), and cotreatment with Trp6-GnRH-I elicited a small poorly reproducible additive effect (up to 10% increase in growth inhibition after 96 hours; data not shown). The mitogen-activated protein (MAP) kinase inhibitors PD98059 and SB203580 did not rescue cells from the growth-inhibitory effects of 100 nmol/L d-Trp⁶-GnRH-I (Table 1; Fig. 5C and D).

Effects of GnRH on tumor growth in vivo. Cells were implanted s.c. into the flanks of female athymic nude mice and tumors were derived. Tumors were further propagated by xenografting. Growth of transfected HEK293[SCL60] xenografts could be inhibited with daily i.p. doses of 10-μg d-Trp⁶-GnRH-I, resulting in markedly reduced tumor growth rate within 7 days and persisting for at least 25 days (Fig. 6). Growth of xenografts prepared from untransfected HEK293 cells was not affected by d-Trp⁶-GnRH-I (data not shown). A much less dramatic but significant inhibition of tumor growth was observed with B35-2b.
and B35-2 cell xenografts. Transfected B35-20 and SKOV3-4 cell xenografts did not exhibit significant growth inhibition. Attempts to generate tumors using transfected EFO21-1 cells failed.

Discussion

The aim of this study was to engineer new sublines of cells expressing different levels of rat GnRH receptor at the cell surface and to use them to assess factors influencing GnRH-mediated cell growth inhibition in vitro and in vivo. Our data confirm and extend previous in vitro data obtained with transfected HEK293 cells, MCF-7, and PC3 cells (26, 28, 32–34) and contribute some novel observations.

Three independent clones of rat B35 neuroblastoma cells (B35-2b, B35-2, and B35-20) and a derivative of the human ovarian cancer cell line SKOV3 (SKOV3-4) were informative in in vitro and in vivo studies done in parallel with HEK293[SCL60] cells (26, 28).

HEK293[SCL60] cells exhibited higher GnRH binding than αT3-1 gonadotrope cells (Fig. 1). In contrast, the B35 and SKOV3 cell clones possessed moderate or low levels of cell-surface GnRH receptor (Fig. 1). The functional effects of setting different levels of GnRH receptor expression using recombinant DNA have hitherto been described only in in vitro studies using MCF-7 and PC3 cells (33, 34).

Contrary to previous reports, we could not verify endogenous GnRH receptor expression in nontransfected cell lines (EFO21, SKOV3, and HepG2) using binding assay (Fig. 1), inositol phosphate production, MAP kinase activation, or in vitro growth inhibition assays. Differences in passage number, culture conditions, or trafficking (42) of the human GnRH receptor to the cell surface may account for loss of low-level endogenous GnRH receptor expression in these cell lines. Hence, stably transfected cells in which the level of receptor expression can be verified represent a good tool for functional studies. The rat GnRH receptor is particularly amenable to such studies because cell clones possessing different levels of surface receptor can be readily isolated and the receptor possesses pharmacologic properties similar to the human receptor.

In our cell clones, levels of rat GnRH receptor expression and receptor-stimulated inositol phosphate production both correlated with the extent of cell growth inhibition in vitro (Figs. 2 and 3). Relative growth inhibition ranged from 12% to 80% following 96 hours of treatment with triptorelin (d-Trp⁶-GnRH-I), depending on the particular clone, and these differences were reflected by the magnitude of inositol phosphate production elicited by GnRH in each clone.

GnRH receptor–activated signaling varied between cell types. B35 cells exhibited a different pattern of GnRH-stimulated ERK phosphorylation compared with the other cell types (a variable and transient dephosphorylation rather than clear-cut elevated phosphorylation; Fig. 4). However, the role of ERK phosphorylation in determining the cellular growth response remains uncertain (30, 43). Nevertheless, cell growth inhibition characteristically involved elevation in levels of cleaved PARP in both HEK293[SCL60] and B35 cells (Fig. 4).

Elevated levels of cleaved caspase-3 and cleaved PARP have previously been described in HEK293[SCL60] cells following treatment with GnRH (28). The levels of cleaved PARP in HEK293[SCL60] and B35-2b cells reflected the level of GnRH receptor expression and

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Figure 3. A, time courses for accumulation of ³H-radiolabeled total inositol phosphates (inositol 1-phosphate, inositol 1,4-phosphate, and inositol 1,4,5-phosphate) following stimulation of cells with 1 µmol/L GnRH-I in the presence of 10 mmol/L lithium chloride. Representative data of assays done in triplicate. B, time courses for turnover of ³H-radiolabeled total inositol phosphates. Cells were stimulated with 1 µmol/L GnRH-I for 90 min in the presence of 10 mmol/L LiCl. Then the medium was replaced with GnRH-I-and LiCl-free medium (dotted line) before assay. Representative data for assays done in triplicate.
Figure 4. A, qualitative Western blotting showing effects of GnRH receptor activation on phosphorylation of ERK1/2 in cell lines stably transfected with rat GnRH receptor cDNA. Cells were treated with 100 nmol/L D-Trp6-GnRH-I for the stated periods of time. Note that B35 clones such as B35-2b show variable decreases in the level of phosphorylated ERK1/2 (pERK1/2) following 10-min stimulation whereas the other cell clones exhibit transient increases in ERK1/2 phosphorylation. \( \beta \)-Actin serves as a sample loading control. B, quantitative Western blotting analysis of effects of GnRH receptor activation (via 100 nmol/L D-Trp6-GnRH-I) on phosphorylation of ERK1/2 or p38. Data from three independent analyses were quantified and are expressed as fold increase in band intensity standardized according to the level of total ERK1/2 protein. C, Western blot analysis examining changes in the abundance of selected proteins during treatment of cells with 100 nmol/L D-Trp6-GnRH-I. In HEK293[\[SCL60\]] cells (top), there is transient activation of ERK1/2 and p38 and accumulation of phosphorylated FADD, cleaved caspase-9 (c-caspase-9), and cleaved PARP (c-PARP; 4-fold elevation at 72–96 h of treatment in this instance). There is transient dephosphorylation of ERK1/2 but no evidence for rapid activation of p38 in B35-2b cells (bottom). Accumulation of cleaved PARP is less marked in these cells. \( \beta \)-Actin serves as a loading control.
growth inhibition at the doses used in this study (Fig. 5).

Inhibitors PD98059 and SB203580 were unable to rescue cells from reasons for this cell-specific effect. In contrast, the MAP kinase (44) and more work is required to elucidate the extent of growth inhibition, being more marked in HEK293[SCL60] cells (Fig. 4C). Only small elevations in the levels of cleaved caspase-9 (upstream to cleaved caspase-3 and cleaved PARP, and a marker of the intrinsic apoptosis pathway) and phosphorylated FADD (a marker of death-receptor signaling via the extrinsic apoptosis pathway) were detectable in HEK293[SCL60] cells. Cleaved caspase-9 was detectable within 24 hours of treatment.

Although the level of GnRH receptor expressed in other transfected cells was low (SKOV3-4, EFO21-1, and HepG2-3), it was possible to detect low levels of inositol phosphate elevation and transient activation of ERK in these cells following treatment with GnRH. However, the signaling response was weaker (see Fig. 4 for example) and was insufficient to elicit growth inhibition (Fig. 2). In SKOV-3 cells, inositol phosphate production was significantly stimulated by phospholipase C activator m-3mFBS (60 μmol/L) but could be only slightly enhanced by treatment with triptorelin (data not shown). Hence, m-3mFBS treatment was also accompanied by cell growth inhibition.

The in vitro growth-inhibitory effects of triptorelin could be partially prevented by the caspase inhibitor Q-VD-OPh (20 μmol/L; Fig. 5). Furthermore, a marked cell type-specific rescue was possible with the protein kinase C inhibitor Ro32-0432 (in HEK293[SCL60] cells but not B35 clones; Fig. 5). This is a novel finding, but it is important to note that Ro32-0432 may inhibit other classes of protein kinases in addition to PKCα (44) and more work is required to elucidate the reasons for this cell-specific effect. In contrast, the MAP kinase inhibitors PD98059 and SB203580 were unable to rescue cells from growth inhibition at the doses used in this study (Fig. 5). Inhibition of cellular Ca²⁺ elevation using 2-APB had no effect on GnRH-mediated cellular growth inhibition in the cells studied here (Table 1). However, phospholipase Cβ activity also influences a range of proteins, including Rac1, a small GTPase involved in regulation of apoptosis (45). In HEK293[SCL60] cells, Rac mediates GnRH-stimulated cytoskeletal reorganization (46), which may potentially disrupt the highly coordinated cytoskeletal rearrangements required for cell division. In this respect, it is interesting that a proportion of HEK293[SCL60] cells become blocked at the G2 phase of the cell cycle after treatment with GnRH (28). Arrest at the G2-M checkpoint can trigger apoptosis. Elevation in the level of phosphorylated p38 is a recognized marker of G2-M checkpoint stress. We observed activation of p38 in HEK293[SCL60] and B35-2b cells 48 to 96 hours after treatment with triptorelin (Fig. 4).

Among numerous target proteins, PKCα can activate PKCδ and phospholipase D1. PKCδ can activate apoptosis via stimulation of sphingomyelinase-mediated ceramide production and alteration of mitochondrial membrane lipid composition (47). However, we found that rottlerin, an inhibitor of PKCδ, did not affect GnRH-mediated growth inhibition (Table 1); neither did fumonisin B1, an inhibitor of sphingosine N-acyltransferase and de novo ceramide biosynthesis. More investigations are required to determine the mechanism of PKC signaling during GnRH treatment.

Exogenous 8-Br-cAMP acted in an additive fashion with triptorelin to inhibit in vitro cell growth (Table 1). This suggests that GnRH receptor signaling through Gαs and adenylate cyclase may be contextually different from the effects of exogenous 8-Br-cAMP.

### Table 1. Effects of exogenous agents on in vitro cell growth inhibition elicited by 100 nmol/L d-Trp⁶-GnRH-I

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>HEK293[SCL60] Cells</th>
<th>B35-2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺ chelator</td>
<td>5 μmol/L</td>
<td>Additive*</td>
<td>Additive*</td>
</tr>
<tr>
<td>IP3 Ca²⁺ channel blocker</td>
<td>75 μmol/L</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>MEK inhibitor</td>
<td>18 μmol/L</td>
<td>Partial rescue (8%)</td>
<td>No effect</td>
</tr>
<tr>
<td>P38 inhibitor</td>
<td>10 μmol/L</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>PKC inhibitor</td>
<td>100 μmol/L</td>
<td>Complete rescue (100%)*</td>
<td>Additive*</td>
</tr>
<tr>
<td>PKC inhibitor</td>
<td>0.5 μmol/L</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>cAMP</td>
<td>20 μmol/L</td>
<td>Partial rescue (40%)*</td>
<td>Additive*</td>
</tr>
<tr>
<td>Caspase inhibitor</td>
<td>20 μmol/L</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Ceramide synthesis block</td>
<td>0.3 μmol/L</td>
<td>No effect</td>
<td>No effect</td>
</tr>
<tr>
<td>Fas ligand antagonist</td>
<td>300 μmol/L</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with agents and growth was monitored at daily intervals over a period of 4 d. Where percentage figures are quoted, these refer to the effect on growth at day 4.

Abbreviations: MEK, MAP kinase/ERK kinase; IP3, inositol 1,4,5-trisphosphate.

*Represents statistically significant effects where P < 0.002; all analyses were done in triplicate.
Figure 5. A, results of in vitro cell growth assay indicating that the general caspase inhibitor Q-VD-OPh (20 μmol/L) elicits partial rescue of HEK293[SC60] or B35-2b cells from growth inhibition by 100 nmol/L D-Trp6-GnRH-I. B35-20 or B35-2 cells were not significantly rescued by Q-VD-OPh. Cell growth was measured following 4 d of treatment. Representative data for assays done in triplicate. B, results of in vitro cell growth assay indicating that the protein kinase C inhibitor Ro32-0432 (100 nmol/L) rescues HEK293[SC60] cells from growth inhibition by 100 nmol/L D-Trp6-GnRH-I (top). Cell growth was measured on days 0, 2, 3, and 4 following treatment. Representative data for assays done in triplicate. Western blotting showed that increased levels of cleaved PARP following treatment with 100 nmol/L D-Trp6-GnRH-I are inhibited by 100 nmol/L Ro32-0432 (bottom). β-Actin serves as a loading control. C, results of in vitro cell growth assay indicating that 18 μmol/L PD98059 or 20 μmol/L SB203580 does not rescue HEK293[SC60] cells from growth inhibition by 100 nmol/L D-Trp6-GnRH-I (top), although these agents do prevent rapid activation of p38 or ERK1/2 respectively (bottom). Ro32-0432 at 100 nmol/L prevents ERK1/2 activation, but not p38 activation. β-Actin serves as a loading control. D, results of in vitro cell growth assay indicating that different doses of Ro32-0432 (90 nmol/L or 180 nmol/L) do not rescue B35 cell clones from growth inhibition by 100 nmol/L D-Trp6-GnRH-I. Representative data for assays done in triplicate.
Previous data obtained using pertussis toxin to inhibit Goi may be misleading in view of the recent discovery that the toxin is also an inhibitor of acid sphingomyelinase and ceramide production (48).

Differences in the level of GnRH receptor expression also correlated with the extent of tumor xenograft growth inhibition in vivo (Fig. 6). Growth of HEK293[SCL60] cell tumors expressing high levels of receptor was completely inhibited (Fig. 6). This is a remarkable effect.

B35-2b cell tumors expressing a moderate level of GnRH receptor were more marginally growth inhibited by daily administration of triptorelin than B35-2 tumors, which express lower receptor levels (Figs. 1 and 6). The smaller effect on tumor growth inhibition obtained with transfected B35-2 cell xenografts suggests that 20% in vitro growth inhibition (Fig. 2) translates into poor tumor growth retardation in vivo (Fig. 6).

SKOV3-4 cells expressing low levels of GnRH receptor were not growth inhibited in vitro or in vivo by triptorelin. Because low levels of GnRH receptor expression do elicit intracellular signaling in SKOV3-4 (and EFO21-1) cells (Fig. 4), attempts to manipulate this signaling toward stimulation of cell growth inhibition may be one way to improve the effectiveness of GnRH treatment. How this may be achieved is the focus of ongoing investigation.

The tumor xenograft experiments complemented our in vitro analyses, enabling confirmation of in vitro data. However, in many circumstances the in vivo effects of GnRH are likely to be mediated by a combination of direct and indirect actions on proliferating cells. Blood sex steroid concentrations are decreased following systemic administration of GnRH analogue. Growth of steroid-dependent cells is therefore indirectly inhibited. It would be informative to study cells proven to be steroid hormone dependent using our approach to determine whether steroid hormone receptor function is modified (e.g., by posttranslational modification) by GnRH signaling. Likewise influences of GnRH receptor signaling on tumor growth via effects on angiogenesis or activity of the innate immune system need to be addressed in further investigations. Nevertheless, HEK293[SCL60] and transfected B35 cell lines represent useful benchmarks to make further comparisons with cells from human tumors that endogenously express GnRH receptor.

**Disclosure of Potential Conflicts of Interest**

The authors have no conflicts of interest to declare concerning the contents of this research article.

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Figure 6. *In vivo* effects of triptorelin (o-Trp6-GnRH-I) on tumor xenograft growth. HEK293[SCL60] cell tumor xenografts regressed following treatment for 10 d whereas B35-2b and B35-2 tumor xenografts were growth retarded. Growth of SKOV3-4 xenografts was not affected by treatment with triptorelin. Effects on tumor growth correlate with levels of GnRH receptor expression shown in Fig. 1.
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Gonadotropin-Releasing Hormone Receptor Levels and Cell Context Affect Tumor Cell Responses to Agonist *In vitro* and *In vivo*

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