Inhibition of Ovarian Cancer Growth by a Tumor-Targeting Peptide That Binds Eukaryotic Translation Initiation Factor 4E

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Abstract

Purpose: A critical step of protein synthesis involves the liberation of the mRNA cap-binding translation initiation factor elf4E from 4EBP inhibitory binding proteins, and its engagement to the scaffolding protein elf4F4G. elf4E is a candidate target for cancer therapy because it is overexpressed or activated in many types of tumors and has tumorigenic properties. Our aim was to design and evaluate 4EBP-based peptides for their antitumor activity in ovarian cancer.

Experimental Design: The ability of peptides to bind and inhibit elf4E was determined by immunoprecipitation and by assaying cap-dependent reporter synthesis. To target ovarian tumors, the lead candidate 4EBP peptide was fused to an analog of gonadotropin-releasing hormone (GnRH). Cellular uptake of peptide, and effects on cell viability and cell death were determined. The antitumor activity of fusion peptide was evaluated in female nude mice bearing i.p. ovarian tumor xenografts.

Results: 4EBP-based peptides bound elf4E, prevented elf4E from binding elf4G, and inhibited cap-dependent translation. GnRH agonist-4EBP fusion peptide was taken up by, and inhibited the growth of, GnRH receptor-expressing tumor cells, but not receptor-negative cells. Intrapertoneal tumor burden was significantly smaller in mice treated with fusion peptide than in mice treated with saline (P < 0.001). Ascites was also reduced in peptide-treated mice. Significant cytotoxic effects to host tissues were not observed. On the other hand, treatment with GnRH agonist alone did not inhibit tumor growth or ascites.

Conclusion: Because ovarian cancer is rarely cured by conventional chemotherapies, GnRH-4EBP fusion peptide may be of therapeutic potential for treatment of this disease.

The process of protein synthesis, on which cell growth depends, is tightly regulated at the level of translation initiation. The eukaryotic translation initiation factor 4E (elf4E) binds the 5′ cap structure of mRNA, and is sequestered by three related inhibitory proteins (4EBPs). Phosphorylation of the 4EBPs by mammalian target of rapamycin (mTOR), which is activated by the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, releases 4EBPs from elf4E (1, 2). Once liberated, elf4E binds the scaffolding protein elf4F4G. This interaction is essential for delivering mRNA to the 43S preinitiation complex that scans the 5′-untranslated region (UTR) to reveal the initiation codon and triggers ribosome engagement (1, 2).

Levels of free elf4E are commonly elevated in a wide variety of tumors resulting either from overexpression of elf4E or activation of the PI3K/AKT signaling pathway (1, 3). Moreover, elevated levels of elf4E have been shown to promote tumorigenesis (4, 5). Elevated levels of free elf4E are thought to promote tumorigenesis by selectively enabling translation of mRNAs that contain long, highly structured 5′-UTRs – a common feature of mRNAs encoding growth and survival factors (1, 3). This preferential enhancement has been shown for mRNAs encoding many tumor-promoting factors, including vascular endothelial growth factor, ornithine decarboxylase, and survivin (6–8). elf4E also enhances cyclin D1 protein levels by stimulating nuclear export of cyclin D1 mRNA (7). As a focal point of multiple pathways that drive tumor growth, elf4E is an ideal molecular target. However, because elf4E is ubiquitously expressed, the principal challenge is to develop elf4E-inhibitory agents that specifically target tumor cells.

The need for new-generation targeted therapeutics is essential for epithelial ovarian cancer, as 70% of patients present with advanced-stage disease that is rarely cured by conventional taxane-platinum therapies (9). The PI3K/AKT signaling...
Translational Relevance

The process of protein synthesis, on which cell growth depends, is tightly regulated by the translation initiation factor eIF4E that binds the 5′ cap structure of mRNA. eIF4E is a candidate cancer therapeutic target because it is overexpressed or activated in many types of tumors. In this study, we designed and evaluated peptides for their ability to bind eIF4E and inhibit cap-dependent translation. The lead candidate peptide was fused to an analog of gonadotropin-releasing hormone to target its receptor that is widely overexpressed in ovarian and other endocrine cancers. This fusion peptide inhibited the growth of i.p. ovarian tumors and ascites in mice without significant cytotoxic effects to host tissues. Because ovarian cancer is rarely cured by conventional taxane-platinum combination therapy, the fusion peptide may be of therapeutic potential for treatment of this disease.

Materials and Methods

Peptides and other reagents. Peptides were synthesized to >98% purity according to our design by AnaSpec Inc., and dissolved in water. The following antibodies were used: unconjugated eIF4E antibody (BD Biosciences), actin antibody (Sigma), eIF4G antibody (Cell Signaling Technology), anti-GnRH antibody. Immunoprecipitation was likewise carried out using lysates block eIF4E from binding eIF4G, immunoprecipitation was done using agarose-conjugated eIF4E antibody, and Western blot done using eIF4G antibody. Immunoprecipitation was likewise carried out using lysates of cells that had been cultured with addition of peptides at a final concentration of 10 μmol/L for 24 h.

Immunofluorescence staining. For detecting peptide uptake, cells were plated in 2-well chamber slides and incubated with biotinylated peptides at a final concentration of 3 μmol/L for 6 h at 4°C. To determine binding of peptides to eIF4E, peptides were pulled down using streptavidin-agarose, and Western blot done using eIF4E antibody. To determine the ability of peptides to block eIF4E from binding eIF4G, immunoprecipitation was done using agarose-conjugated eIF4E antibody, and Western blot done using eIF4G antibody. Immunoprecipitation was likewise carried out using lysates of cells that had been cultured with addition of peptides at a final concentration of 10 μmol/L for 24 h.

Cell-based translation assays. Five thousand ES-2-DualLuc cells were plated per well in 96-well plates in McCoy's 5A medium (Invitrogen) containing 10% fetal bovine serum (FBS). Following attachment, cells were cultured in FBS-free medium overnight, then cultured in medium containing 10% FBS for 6 h with addition of peptide at final concentrations of 0, 0.3, 1, 3, 10, and 30 μmol/L. Cells were also incubated with rapamycin for 24 h and with cycloheximide for 3 h at concentrations indicated in the text. F-Luc and R-Luc activities in cells were assayed using the Dual-reporter assay kit (Promega). Triplicate wells were set up for each assay, and assays were independently done two times.

Cell viability and TUNEL assays. For cell viability assays, 2,000 cells were plated per well in 96-well plates in medium containing 10% FBS. Following attachment, cells were cultured in FBS-free medium overnight, and thereafter cultured in medium containing 10% FBS for 3 d with addition of peptide at final concentrations of 0, 0.3, 1, 3, 10, and 30 μmol/L. Cells were stained by crystal violet solution, and absorbance measured at 570 nm. Triplicate wells were set up for each assay, and assays were independently done two times. For terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assays, 2 × 10^5 cells were plated in 60-mm dishes, and incubated with or without peptide for 3 d as described above. Cells were fixed in 70% ethanol and the extent of apoptosis was determined by flow cytometric analysis of TUNEL-staining using the APO-BRDU kit (Phoenix Flow Systems).

Animal studies. Animal studies were approved by the Institutional Animal Care and Use Committee. The ES-2 cell line stably expressing the pRES-DualLuc construct. The human GnRH-R1 cDNA clone (purchased from OriGene Technology) was subcloned into the pcDNA3.1+ vector (Invitrogen) to generate the pcDNA3.1+ GnRH-R1 construct.

Cell lines. The sources of cell lines were as follows: OVCAR-3 and SKOV3 (American Type Culture Collection), ES-2 (Patrice Morin, National Institute on Aging, Baltimore MD), and MDA-MB-231, B, and HUVEC (Gordon Mills, Shiyu-Yih Lin, and Lee Ellis, M. D. Anderson Cancer Center, Houston TX). The ES-2-DualLuc line was generated by transfecting ES-2 cells with pRES-DualLuc plasmid using FuGENE6 reagent (Roche) and selecting stably transfected clones using G418 (400 μg/mL) (Invitrogen). The SKOV3+GnRH-R1 line was likewise generated by stably transfecting SKOV3 cells with pcDNA3.1+GnRH-R1 plasmid.

Immunoprecipitation and Western blotting. Biotinylated peptides were incubated with 250 μg of cell lysate at a final concentration of 3 μmol/L for 6 h at 4°C. To determine binding of peptides to eIF4E, peptides were pulled down using streptavidin-agarose, and Western blot done using eIF4E antibody. To determine the ability of peptides to block eIF4E from binding eIF4G, immunoprecipitation was done using agarose-conjugated eIF4E antibody, and Western blot done using eIF4G antibody. Immunoprecipitation was likewise carried out using lysates of cells that had been cultured with addition of peptides at a final concentration of 10 μmol/L for 24 h.

Plasmids. The bicistronic construct containing Renilla luciferase (R-Luc) and firefly luciferase (F-Luc) genes separated by the hepatitis C virus type 2b internal ribosomal entry site (IRES; ref. 18) was provided by Richard Elliott (University of St. Andrews, U.K.). The bicistronic cassette was subcloned into the pCMVScript vector (Stratagene) to generate the pRES-DualLuc construct. The human GnRH-R1 cDNA clone (purchased from OriGene Technology) was subcloned into the pcDNA3.1+ vector (Invitrogen) to generate the pcDNA3.1+GnRH-R1 construct.

Pathway is activated in many epithelial ovarian cancers, and 4EBP phosphorylation is strongly associated with poor prognosis (10). Eighty percent of epithelial ovarian cancers express GnRH-R1, the receptor for gonadotropin-releasing hormone (GnRH; ref. 11). This hypothalamus-derived decapeptide stimulates the pituitary gland to produce gonadotropins that control gonadal steroidogenesis (12). GnRH-R1 is also widely expressed in breast, endometrial, and prostate cancers (13). GnRH agonists are used to treat endometriosis, uterine fibroids, infertility, precocious puberty, and prostate cancer (12, 13). Although the response rates of epithelial ovarian cancer patients to GnRH agonists have been only modest (14), the use of agonists as tumor-targeting moieties has shown considerable promise (15–17). In this study, we generated 4EBP-based eIF4E-binding peptides that prevent eIF4E from binding eIF4G, block cap-dependent translation, and inhibit cell growth. Moreover, 4EBP peptide fused to the GnRH agonist [DLys6]GnRH(R1-exerted potent antitumor effects in a mouse xenograft model of epithelial ovarian cancer without eliciting significant toxicity. [DLys6]GnRH-4EBP peptide may therefore be of therapeutic significance for epithelial ovarian cancer and other endocrine tumors.
Results

Design of 4EBP peptides that bind eIF4E and inhibit binding of eIF4E to eIF4G. Of the highly related 4EBPs, 4EBP1 is the most characterized. Structural studies have identified the region spanning residues 49 to 68 of 4EBP1 to bind eIF4E (20–22). It has also been reported that 4EBPs can bind eIF4E without folded structure (22). We synthesized a peptide comprising residues 49 to 68 of 4EBP1 (4EBP1-WT) and a peptide containing the corresponding region of 4EBP2 (4EBP2-WT; Fig. 1A). Both peptides bound to eIF4E in cell extracts of the epithelial ovarian cancer line OVCAR-3, with 4EBP1-WT showing stronger binding (Fig. 1B). These peptides inhibited eIF4E from binding eIF4G, with 4EBP1-WT showing stronger inhibitory activity (Fig. 1B).

Fusion of TAT to 4EBP peptides enables cellular uptake and does not interfere with eIF4E-binding. To render 4EBP peptides cell-permeable, 4EBP residues were fused to cell-penetrating residues of HIV TAT protein (Fig. 1A). These fusion peptides were water-soluble. TAT-4EBP fusion peptides were taken up by OVCAR-3 cells, whereas 4EBP peptides that lacked TAT were not internalized (Fig. 1C). Fusion of TAT to 4EBP peptides did not interfere with their ability to bind eIF4E (Fig. 1D), and to inhibit eIF4E from binding eIF4G (Fig. 1D). The conserved residues Tyr-54, Arg-56, and Leu-59 of 4EBP proteins are important for binding to eIF4E through hydrophobic and electrostatic interactions, and hydrogen bonds (20, 21). We generated mutant TAT-fusion 4EBP1 and 4EBP2 peptides in which these three residues were substituted by Gly (Fig. 1A). These mutations substantially impaired the ability of TAT-4EBP peptides to bind eIF4E and to inhibit eIF4E from binding eIF4G (Fig. 1D).

Inhibition of cap-dependent translation and cell growth by TAT-4EBP fusion peptides. To determine the efficacy of our peptides, we developed a cell-based system to assay changes in cap-dependent translation. This system is based on a bicistronic cassette in which one reporter, R-Luc, is synthesized in a cap-dependent manner, whereas the other, F-Luc, is synthesized under IRES-mediated control (18). Similar cassettes have been used for assaying translation in vitro (23, 24). We generated a reporter line derived from the epithelial ovarian cancer cell line ES-2 that stably expresses the bicistronic cassette (ES-2-DualLuc; Fig. 2A). We determined the robustness of our model system using rapamycin, which inhibits cap-dependent translation by inhibiting mTOR from phosphorylating 4EBPs (25). Rapamycin inhibited cap-dependent translation (R-Luc readout) in a dose-dependent manner, but did not inhibit cap-independent translation (F-Luc readout; Fig. 2A). In contrast, the elongation inhibitor cycloheximide inhibited both cap-dependent and cap-independent translation (Fig. 2A).

In ES-2-DualLuc cells, TAT-4EBP1-WT peptide inhibited cap-dependent translation but not cap-independent translation at concentrations ranging from 0.3 to 10 μmol/L (Fig. 2B). TAT-4EBP2-WT also inhibited cap-dependent translation, but was slightly less effective than TAT-4EBP1-WT. Under these conditions, peptide treatment did not alter the level of reporter transcript (Supplementary Fig. S1). Cap-dependent translation was not inhibited by mutant peptides or by TAT alone at the same range of concentrations (Fig. 2B). At the highest concentration used (30 μmol/L), some inhibition of cap-independent translation by wild-type and mutant peptides was observed (Fig. 2B).

Treatment of OVCAR-3 cells with TAT-4EBP1-WT and TAT-4EBP2-WT peptides decreased cell viability in a dose-dependent manner, with TAT-4EBP1-WT showing a stronger effect (Fig. 2C). In contrast, cell viability was not affected by mutant peptides or by TAT at concentrations ranging from 0.3 to 10 μmol/L. TUNEL-staining of tissue sections was done using the in situ cell death detection kit (Roche).

Statistical analysis. Data were analyzed using STATISTICA6 software (StatSoft Inc.). Statistical significance of differences in cell viability and luciferase activity was calculated by Student’s t-test. For animal experiments, 11 mice were assigned in each treatment group. This sample size gave >80% power to detect a 50% reduction in tumor burden at a 5% level of statistical significance. Because sample size was small and outcome variables could have a skewed distribution, the nonparametric Mann-Whitney U-test was used. All statistical tests were two-sided. P < 0.05 was considered to be statistically significant.
[DLys6]GnRH-4EBP1-WT prevented eIF4E from binding eIF4G, whereas this binding was not inhibited by [DLys6]GnRH-4EBP1-MT or by the agonist alone (Fig. 3C). Because ES-2 cells express GnRH-RI (Fig. 3A), we used our ES-2-DualLuc reporter line to determine the effect of [DLys6]GnRH-4EBP1-WT inhibited cap-dependent translation, and was almost as effective as 4EBP1 peptide fused to TAT (Fig. 3D). In contrast, cap-dependent translation was not inhibited by mutant peptide or by [DLys6]GnRH (Fig. 3D). Although [DLys6]GnRH-4EBP1-WT inhibited cap-dependent translation in cells, it was important to determine that the peptide binds and inactivates its target within cells that express GnRH-RI. We generated an SKOV3 cell line that stably expresses GnRH-RI (Fig. 3A). Cells of this line (SKOV3+GnRH-RI) were cultured with peptides, and immunoprecipitation experiments were done using lysates of treated cells. Binding of [DLys6]GnRH-4EBP1-WT to eIF4E was detected, whereas neither mutant peptide nor the agonist alone bound eIF4E in cells treated with these peptides (Fig. 4B). Binding of eIF4E to eIF4G was substantially
reduced in SKOV3+GnRH-RI cells treated with [DLys6]GnRH-4EBP1-WT (Fig. 4C). The reduced binding of eIF4E to eIF4G was not due to any decrease in expression of these factors (Fig. 4C). On the other hand, binding of eIF4E to eIF4G was not inhibited in cells that had been treated with mutant peptide or agonist alone (Fig. 4C). The same findings were observed using the GnRH-RI–positive line ES-2 (Fig. 4C). In contrast, binding of eIF4E to eIF4G was not inhibited in GnRH-RI–negative parental SKOV3 cells following treatment with wild-type peptide (Fig. 4C).


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Fig. 2. Inhibition of cap-dependent translation and cell growth by TAT-4EBP fusion peptides. A, top, representation of the pIRES-DualLuc bicistronic construct containing R-Luc and F-Luc genes. Bottom, R-Luc and F-Luc activities were assayed in ES-2-DualLuc cells following treatment for 24 h with rapamycin and for 3 h with cycloheximide with the indicated concentrations of these drugs, and expressed relative to the respective Luc activities in cells incubated without drug. Cell viability was not affected by rapamycin and cycloheximide under these conditions. B, R-Luc and F-Luc activities were assayed in ES-2-DualLuc cells following treatment for 6 h with the indicated concentrations of peptides, and expressed relative to the respective Luc activities in cells incubated without peptide. Shown are results of triplicate assays done independently two times, and statistical significance of differences in relative R-Luc and F-Luc activities in cells treated with peptide at a concentration of 10 μmol/L. C, viability of OVCAR-3 cells at 3 d after addition of the indicated concentrations of peptides, expressed relative to viability of cells incubated without peptide. Shown are results of triplicate assays done in two independent experiments, and statistical significance of differences in viability of cells treated with TAT-4EBP1-WT versus TAT-4EBP1-MT, and of cells treated with TAT-4EBP2-WT versus TAT-4EBP2-MT at a concentration of 10 μmol/L. D, left, OVCAR-3 cells at 3 d after peptide addition (10 μmol/L) viewed under phase-contrast microscopy; right, flow cytometric analysis of TUNEL-staining of OVCAR-3 cells at 3 d after peptide addition (10 μmol/L). Cells incubated for 3 d in the absence of peptide in medium with and without serum are included as controls.
Cell blebbing and detachment were induced by wild-type peptide but not by mutant peptide, even at the highest concentration tested (30 μmol/L; Fig. 5B). Annexin V-binding was detected in GnRH-RI-positive cells following treatment with wild-type peptide, but not with mutant peptide (Supplementary Fig. S4A). In contrast, [DLys6]GnRH-4EBP1-WT failed to inhibit growth of fibroblasts and endothelial cells that do not express GnRH-RI (Fig. 5A). Parental SKOV3 cells were likewise resistant to [DLys6]GnRH-4EBP1-WT, whereas this peptide inhibited growth and induced cell death, as indicated by TUNEL-staining, in SKOV3+GnRH-RI cells (Fig. 5A, B, and C). On the other hand, [DLys6]GnRH-4EBP1-MT or [DLys6]GnRH (Fig. 5A). Cell blebbing and detachment were induced by wild-type peptide but not by mutant peptide, even at the highest concentration tested (30 μmol/L; Fig. 5B). Annexin V-binding was detected in GnRH-RI-positive cells following treatment with wild-type peptide, but not with mutant peptide (Supplementary Fig. S4A). In contrast, [DLys6]GnRH-4EBP1-WT failed to inhibit growth of fibroblasts and endothelial cells that do not express GnRH-RI (Fig. 5A). Parental SKOV3 cells were likewise resistant to [DLys6]GnRH-4EBP1-WT, whereas this peptide inhibited growth and induced cell death, as indicated by TUNEL-staining, in SKOV3+GnRH-RI cells (Fig. 5A, B, and C). On the other
hand, GnRH-RI–positive and -negative lines were equally sensitive to 4EBP1-WT peptide fused to TAT (Fig. 5A and C; Supplementary Fig. S4B). These observations indicate that wild-type 4EBP1 peptide fused to [DLys6]GnRH inhibits growth and induces cell death only in GnRH-RI–positive cells. The morphologic changes, Annexin V-binding, and TUNEL-staining detected in these cells indicate cell death occurs, at least in part, by apoptosis.

To confirm specificity of [DLys6]GnRH as a targeting moiety, we generated a peptide comprising 4EBP1-WT residues fused to a mutant agonist in which His-2 was substituted by Gln (Supplementary Fig. S4B). These observations indicate that 4EBP1-WT fused to mutant agonist was not internalized in GnRH-RI–positive cells and did not inhibit cell growth (Supplementary Fig. S4D and E).

GnRH agonists generally exert only modest growth-inhibitory effects in epithelial ovarian cancer cells (14, 27). Indeed, [DLys6]GnRH alone did not significantly inhibit cell growth (Fig. 5A). GnRH-RI is a G protein-coupled receptor that activates mitogen-activated protein kinase cascades (12, 13). No significant difference was observed in activation of ERK1/2, p38, and JNK in ES-2 cells treated with [DLys6]GnRH-4EBP1-WT as compared with cells treated with agonist alone (Supplementary Fig. S5A). This observation eliminates the possibility that the fusion peptide inhibits growth at least in part by altering agonist-mediated signaling in tumor cells. Furthermore, the fusion peptide did not induce AKT activation (Supplementary Fig. S5B).

Our findings that [DLys6]GnRH-4EBP1-WT binds eIF4E, prevents eIF4E from binding eIF4G, and inhibits cap-dependent translation in GnRH-RI–expressing cells strongly imply that an appreciable proportion of peptide enters the cytoplasm. Immunofluorescent staining revealed that peptides are mostly excluded from the nucleus (Fig. 3B). GnRH agonists are internalized in cells within 1 hour at 37°C (28). At 1 hour after treatment of ES-2 cells with 5-carboxyfluorescein (FAM)-labeled [DLys6]GnRH-4EBP1-WT at 37°C, some peptide was detected in early endosomes (visualized by staining of EEA1; Supplementary Fig. S3A). A small proportion of peptide was detected in late endosomes and lysosomes (visualized by staining of LAMP1 and LAMP2) at 1 hour (Supplementary Fig. S3A), but this proportion did not increase with extended treatment (6 hours). Similar observations were made in OVCAR-3 cells (Supplementary Fig. S3B).

Antitumor activity of [DLys6]GnRH-4EBP1-WT peptide in an epithelial ovarian cancer xenograft model. To determine the efficacy of [DLys6]GnRH-4EBP1-WT peptide in vivo, we used a mouse i.p. xenograft model that we established from an ES-2 cell line that stably expresses GFP (19). This model mimics the typical behavior of epithelial ovarian cancer, including i.p. dissemination and ascites formation. Our study comprised three treatment groups of mice: (a) saline, (b) [DLys6]GnRH agonist, and (c) [DLys6]GnRH-4EBP1-WT, where sample size of each group was n = 11 (Supplementary Table S1). The first dose of peptide or saline was administered i.p. to mice on day 9 after tumor cell inoculation. On day 9, no ascites had yet formed but tumors had established on the omentum, broad ligament, and mesentery which are common sites of epithelial ovarian cancer attachment (Fig. 6A and B). A schedule in which peptide was administered every 2 days was chosen, based on our studies of peptide stability in serum (Supplementary Fig. S2). Based on studies of other agonist-conjugates in xenograft models (15–17), a peptide dose of 3.0 nmol/g body weight was used. Duration of the regimen was 11 days (i.e. 6 doses in total), and mice were sacrificed on day 20. On day 20, saline-treated mice had developed ascites and extensive tumors throughout the abdominal cavity (Fig. 6A, B, and C). Of the original 11 animals in each treatment group, 8 animals in each group completed the regimen and were evaluated (Supplementary Tables S1 and S2). Intrapерitoneal tumor burden, defined

**Fig. 4.** Effect of [DLys6]GnRH-4EBP1 peptide in SKOV3 cells stably expressing GnRH-RI. A, Western blot analysis of GnRH-RI in parental SKOV3 cells and SKOV3 cells transfected with GnRH-RI. B, SKOV3+GnRH-RI cells were treated with biotinylated peptides at a final concentration of 10 μmol/L or incubated without peptide. At 24 h after treatment, lysates were prepared from cells. Peptide was pulled down using streptavidin-agarose. Precipitates and supernatants were analyzed by Western blot using eIF4E antibody. C, parental SKOV3, SKOV3+GnRH-RI, and ES-2 cells were treated with the indicated peptides (10 μmol/L) or incubated without peptide. At 24 h after treatment, lysates were prepared from cells. Immunoprecipitation was done using eIF4E antibody, and Western blot of precipitates was carried out using eIF4G antibody. Western blot of whole cell lysates of treated cells was done using antibodies to eIF4G, eIF4E, and actin.

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as percent of the abdominal cavity, was similar in mice treated with [DLYs6]GnRH agonist alone [mean, 56.4%; 95% confidence interval (95% CI), 41.5-71.3%] and in mice treated with saline (mean, 52.8%; 95% CI, 38.9-66.7%; P = 0.34). In contrast, i.p. tumor burden was substantially smaller in mice treated with [DLYs6]GnRH-4EBP1-WT peptide (mean, 13.7%; 95% CI, 8.4-19.1%; P = 0.0008; Fig. 6C). As compared with ascites volumes in saline-treated mice (mean, 3.44 mL; 95% CI, 1.95-4.93 mL), ascites volumes in mice treated with fusion peptide were also reduced (mean, 1.44 mL; 95% CI, 0.43-2.46 mL; P = 0.020; Fig. 6C).

During the course of treatment, mice in the control and peptide-treated groups were observed to maintain appetite. Neither alterations in urine and stools nor dermal changes were observed.
noted. Impairment of corneal and pedal reflexes was not observed, but mobility was reduced in mice that developed extensive ascites. No gross histologic changes were seen in normal tissues of peptide-treated mice. No TUNEL-staining was detected in breast and pituitary tissues that express GnRH-RI, but some staining was observed in ovaries of mice treated with [DLys6]GnRH-4EBP1-WT (Supplementary Fig. S6). TUNEL-positive cells were not detected in other normal tissues of these
mice, such as brain, heart, lung, and bone marrow. Cells of the liver and pancreas were also TUNEL-negative, whereas TUNEL-positive cells were detected in adjacent tumors (Supplementary Fig. S6). To further assess for pituitary damage, we assayed levels of luteinizing hormone (LH) in serum collected at the end of the regimen. Serum LH levels of mice treated with [DLys6]GnRH-4EBP1-WT were not significantly different from LH levels of saline-treated mice ($P = 0.43$), whereas elevated LH levels were detected in 2 of 8 mice treated with the agonist alone (Supplementary Fig. S7A). We also determined the immunogenicity of [DLys6]GnRH-4EBP1-WT by assaying mouse serum antibodies for reactivity to the peptide. As a positive control, we assayed antibody reactivity to cellular proteins of ES-2 cells. Whereas antibody reactivity to tumor cell proteins was detected in all groups of mice, antibody reactivity to [DLys6]GnRH-4EBP1-WT that was at least two SD above the mean background level in saline-treated mice was detected in 2 of 8 peptide-treated mice (Supplementary Fig. S7B and C).

**Discussion**

Inhibiting translation initiation is a candidate approach for cancer therapy, but presents significant challenges. Rapamycin inhibits 4EBP phosphorylation (25), but its growth-inhibitory effect is attenuated in part by its ability to induce feedback activation of AKT signaling (29). Increasing attention has focused on other agents that inhibit eIF4E and other components of the translation initiation complex. Tumstatin, a fragment of type IV collagen, interacts with αVβ3 integrin and prevents dissociation of eIF4E from eIF4B (30). Other agents include 4EGI-1, a small molecule inhibitor that prevents eIF4E from binding to eIF4G (24); RNA aptamers that bind eIF4G (31); and the marine natural products pateamine and hippuristanol that target the RNA helicase eIF4A (23, 32). However, the specificity of these agents for tumor cells and lack of cytotoxicity in normal cells have not been shown in animal models. One promising agent is ribavirin, a guanosine ribonucleoside analog. Ribavirin inhibits eIF4E-mediated oncogenic transformation by competing with the 7-methyl guanosine mRNA cap for binding to eIF4E (33). It has been reported that eIF4E antisense oligonucleotides inhibit tumor xenograft growth, but also substantially inhibit eIF4E levels in the liver (34).

Based on X-ray crystal structures of 4EBP-eIF4E interactions (20, 21), we designed water-soluble 4EBP peptides that bind eIF4E and prevent eIF4E from binding to eIF4G. These peptides were fused to TAT in initial proof-of-concept studies to facilitate cellular uptake. Cap-dependent translation and cell growth were inhibited by wild-type TAT-4EBP peptides, but not by fusion peptides containing mutations that impaired their ability to bind eIF4E and to prevent eIF4E from binding eIF4G. Our findings differ from a study in which eIF4E-binding peptides fused to the non–cell-specific transporter penetratin were evaluated in MRC5 lung cells in vitro (35). In the earlier study, the peptides only induced cell death under serum-starved conditions, and their ability to inhibit the translational function of eIF4E was not determined. One significant difference is that our peptides comprise 4EBP residues 49 to 68, whereas peptides in the earlier study contained residues 51 to 62 (35). Trp-73 of eIF4E is important for eIF4B binding (20, 21), and interacts with Arg-63 of eIF4B and eIF2B that is present in our peptides. The additional 4EBP residues in our peptides might strengthen their ability to bind eIF4E and prevent eIF4G from binding eIF4E. In our cell-based assay system, inhibition of cap-dependent translation was detected as early as 6 hours when no significant changes in cell viability had yet occurred. This implies that our peptides induce cell death by inhibiting the translational function of eIF4E. Our observations indicate that cell death induced by the peptides occurs at least in part by apoptosis. It should be noted that cap-independent translation was inhibited by TAT-4EBP peptides at the highest concentration used (30 μmol/L). We cannot therefore totally exclude the possibility that 4EBP peptides might induce cell death in part by inhibiting a pathway independent of eIF4E's role in translation, or by modes other than apoptosis.

It is intriguing to note that the small molecule inhibitor 4EGI-1 not only blocks eIF4E from binding eIF4G but also enhances binding of eIF4B to eIF4E (24). Displacement of eIF4G from eIF4E by 4EGI-1 was speculated to free the binding site for eIF4B by removing steric obstruction (24). Because our peptides contain 4EBP residues, it is not surprising that these peptides do not promote binding of 4EBP1 to eIF4E (unpublished data). It is also interesting to note a recent report that elevated levels of 4EBP1 and eIF4E promote a hypoxia-activated switch from cap-dependent to IRES-dependent synthesis of vascular endothelial growth factor and hypoxia-inducible factor 1α (HIF1α; ref. 36). This raises the possibility that a 4EBP1-mimicking peptide might promote tumor growth. However, this was not found to be the case for our 4EBP1 peptide fused to [DLys6]GnRH. Mice that completed treatment with [DLys6]GnRH-4EBP-WT fusion peptide showed a 50% reduction in tumor burden and ascites as compared with mice treated with saline or the agonist alone. Of the 11 mice treated with [DLys6]GnRH-4EBP-WT, one died and two were terminated prior to completing the regimen. However, the death and drop-out rates of the peptide-treated group were not higher than rates in control groups. Furthermore, the tumor burden of peptide-treated drop-out mice was not higher than that of drop-outs of control groups (Supplementary Tables S1 and S2). Moreover, HIF1α expression in tumors was not higher in peptide-treated drop-out mice, as compared with drop-outs of control groups (unpublished data).

An important aspect of this work is the fusion of 4EBP peptide to a GnRH agonist as a targeting moiety. Cell-penetrating peptides such as penetratin and TAT do not facilitate peptide uptake in a cell type–specific manner. Indeed, we observed that 4EBP peptide fused to TAT significantly inhibited growth of nontumorigenic fibroblasts and endothelial cells. Likewise, eIF4E-binding peptides fused to penetratin have been reported to substantially induce cell death in nontumorigenic fibroblasts (35). The rationale for using a GnRH agonist as a targeting moiety was based on several considerations. One significant consideration is the high prevalence of GnRH-R1 overexpression in epithelial ovarian cancers and other endocrine cancers, and its relatively restricted tissue distribution (11, 13). GnRH-R1 is expressed in the ovary, uterus, breast, prostate, and pituitary (13). An important concern with using a GnRH agonist is the potential for pituitary toxicity. In this study, cell death was not detected in pituitary tissues of mice treated with [DLys6]GnRH-4EBP-WT peptide. In addition, LH levels in these mice were not significantly altered, indicating that pituitary function was not impaired. Alterations in reproductive capability could not be assayed due to rapid growth of tumors. However,
increased TUNEL-staining was observed in ovaries of mice treated with [DLys6]GnRH-4EBP1-WT peptide. Another study that evaluated a GnRH-conjugated membrane-disrupting peptide in a breast cancer xenograft model likewise noted some death of ovarian granulosa cells (37).

A second factor in choosing a GnRH agonist as a targeting moiety is its small size. Fusion of wild-type 4EBP1 peptide to [DLys6]GnRH did not interfere with its ability to bind eIF4E and to prevent eIF4E from binding eIF4G. A third factor is the capability of GnRH agonists to facilitate cellular uptake. Folic acid has been studied as a targeting moiety, but one disadvantage is the low efficiency of folate receptor internalization (38). On the other hand, GnRH agonists are efficiently internalized by receptor-mediated endocytosis and, shortly thereafter, dissociate from the receptor (28). GnRH agonists have been used as delivery vehicles for peptides that inhibit cytoplasmic proteins. These include Pseudomonas exotoxin that inactivates elongation factor-2 through ADP ribosylation (17), and pokeweed antiviral protein, an RNA N-glycosidase that inactivates ribosomes by inducing conformational change (39). The mechanism by which these peptides exit endosomes is unclear. In our study, an appreciable proportion of internalized [DLys6]GnRH-4EBP1 WT peptide did not localize to late endosomes and lysosomes. Moreover, binding of the peptide to eIF4E was strongly detected in GnRH-RI-expressing tumor cells that had been treated with this peptide, and binding of eIF4E to eIF4G was inhibited in these cells. The possibility that wild-type peptide might inhibit growth in part by mechanisms unrelated to inhibiting eIF4E activity cannot be totally excluded, but seems neither to involve alteration of GnRH-RI signaling in tumor cells nor a suppression of LH levels.

Seventy percent of patients diagnosed with epithelial ovarian cancer present with disseminated disease (9). For these patients, the 5-year survival rate is only 30% and most will eventually die of the disease. For many years, tumor-debulking surgery and taxane-platinum combination therapy have remained the standard-of-care. Relapse is frequent and the high initial response rate does not translate to a high cure rate. New-generation agents that target molecular focal points that drive tumor growth and metastasis are therefore essential. Several parameters need to be improved for our [DLys6]GnRH-4EBP1 WT peptide, in particular cytoplasmic delivery. This might be achieved by conjugating viral peptides that facilitate peptide exit from endosomes (40). Improved peptide delivery at early stages of the regimen might also curtail possible loss of potency stemming from desensitization of GnRH-RI after extended treatment. In addition, antitumor activity might be enhanced by combining the fusion peptide with conventional chemotherapeutic agents. The proof-of-concept shown in this study merits further investigation in epithelial ovarian cancer and also other endocrine tumors that express GnRH-RI.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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