Targeted Proapoptotic LHRH-BH3 Peptide

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Purpose. The purpose of this work was to construct and evaluate a novel targeted proapoptotic peptide for cancer treatment.

Methods. The peptide consisted of luteinizing hormone-releasing hormone (LHRH) as a targeting moiety specific to LHRH receptors and a synthetic BCL-2 homology 3 (BH3) domain peptide as an apoptosis inducer and a suppressor of antiapoptotic cellular defense. Anticancer activity of the peptide was evaluated on different cancer cell lines.

Results. The targeting receptor to LHRH peptide is overexpressed in several cancer cell lines but is not expressed in healthy human visceral organs. LHRH and BH3 peptides when applied separately did not demonstrate cellular toxicity. In contrast, the LHRH–BH3 peptide was toxic in several cancer cell lines. Coincubation of LHRH and LHRH–BH3 peptides significantly decreased cytotoxicity of the latter. It was found that the LHRH–BH3 peptide induced apoptosis by simultaneous inhibition of the antiapoptotic function of BCL-2 protein family and activation of caspase-dependent signaling pathway.

Conclusions. The proposed anticancer proapoptotic LHRH–BH3 peptide simultaneously affects two molecular targets: 1) extracellular cancer-specific LHRH receptors and 2) the intracellular controlling mechanisms of apoptosis. The results of this work may be used to design novel approaches for the treatment of various cancers.

KEY WORDS: LHRH peptide; BH3 peptide; apoptosis; cancer.

INTRODUCTION

Chemotherapeutic agents are known to induce cell death. The activation of cellular antiapoptotic defense that prevents the translation of drug-induced damage into cell death is the key factor in cellular resistance to a broad spectrum of anticancer drugs (1–4). Thus, a net increase in apoptosis induction resulting from the treatment of cancer should significantly increase cancer cell death and the efficacy of chemotherapy. It is known that the upregulation of the cellular antiapoptotic system plays the main role in this defense and the BCL-2 family proteins are key players (2,3,5–7). It was previously found that short peptides, corresponding to the minimal sequence of BCL-2 homology 3 (BH3) domain when bound to the antiapoptotic BCL-2 family proteins, suppress cellular antiapoptotic defense (8–10). Therefore, synthetic peptides homologous to BH3 domain can potentially improve traditional cancer chemotherapy by decreasing the resistance of cancer cells to the chemotherapeutic agent. This decrease in resistance is achieved via suppression of the normal ability of the antiapoptotic BCL-2 family of proteins to counteract apoptosis. Moreover, recently we found that BH3 peptide delivered by the internalization sequence of the antennapedia protein (Ant-BH3) exhibits cytotoxicity in cancer cells and showed an ability to induce apoptosis in human ovarian carcinoma cells (8,11). However, the practical use of the BH3 peptide is limited by its low capacity to permeate cells thus requiring a carrier that is capable of delivering it into cancer cells.

Targeting drug therapy specifically to cancer cells has numerous benefits including the maintenance of low plasma to cell concentrations ratio reducing side effects and increasing anti-cancer effectiveness. A targeted anticancer drug must achieve high cell permeability and retention by the specific cell population. Target cell specificity might be achieved by attaching molecular targeting moieties to the drug that interact precisely with cell surface specific receptors, minimizing adverse side effects on healthy tissues. Luteinizing hormone-releasing hormone (LHRH) receptor is expressed in several types of cancer cells (ovarian, breast, prostate) and is not normally expressed in healthy human visceral organs (12,13). Normally LHRH is released from the hypothalamic region of the brain into the pituitary portal circulation. From there, it is transported to the adenohypophysis, where it triggers the release of the luteinizing hormone into the systemic circulation. Therefore, the LHRH decapeptide can be used to target cell surface receptors to deliver BH3 peptide specifically to cancer cells expressing these receptors and facilitate cellular uptake of BH3 peptide.

We hypothesized that a combined LHRH–BH3 peptide would significantly increase the efficacy of cancer treatment by the concurrent targeting of cancer cells, induction of programmed cell death, and the suppression of the main antiapoptotic cellular defense mechanisms. Therefore, the proposed targeted proapoptotic peptide used a novel two-tier approach, simultaneously targeting two molecular targets: 1) extracellular cancer-specific receptor to increase cancer specificity of drug, limit adverse drug side effects, and enhance uptake of the peptide by cancer cells and 2) intracellular controlling mechanisms of apoptosis to suppress cellular antiapoptotic defense. Our main objective was to develop, synthesize, characterize, and evaluate a novel two-tier targeted proapoptotic anticancer peptide, which includes the LHRH peptide as a cell surface-targeting moiety and BH3 peptide as an inducer of apoptosis and a suppressor of antiapoptotic cellular defense.

METHODS

Peptides and Chemicals

The following peptides were used in the present study: 1) LHRH peptide (Gln-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly); 2) BH3 peptide (Met-Gly-Gln-Val-Gly-Arg-Gln-Leu-Ala-Ile-Ile-Gly-Asp-Asp-Ile-Asn-Arg-Arg-Tyr); 3) LHRH–BH3 peptide (Gln-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Met-Gly-Gln-Val-Gly-Arg-Gln-Leu-Ala-Ile-Ile-Gly-Asp-Asp-Ile-Asn-Arg-Arg-Tyr); 4) scLHRH–BH3 peptide with scrambled sequence of LHRH peptide (His-Gln-Ser-Trp-Gly-Tyr-Arg-Leu-Gly-Pro-Met-Gly-Gln-Val-Gly-Arg-Gln-Leu-Ala-Ile-Ile-Gly-Asp-Asp-Ile-Asn-Arg-Arg-Tyr); and 5) LHRH–scBH3 peptide with scrambled sequence of BH3 pep-

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tide (Gln-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Gly-Met-Val-Gln-Gly-Gln-Arg-Leu-Ile-Ala-Ile-Asp-Gly-Ile-Arg-Asp-Asn-Tyr-Arg). Peptides were synthesized according to our design by American Peptide Company, Inc (Sunnyvale, CA, USA). The purity of peptides was higher than 85% as determined by reverse-phase high-performance liquid chromatography. Peptides with scrambled sequences were used as negative controls. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Fisher Scientific (Fair Lawn, NJ, USA) and used as received.

Cell Lines

Four cancer cell lines with different level of expression of LHRH receptors were used. The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center), the human breast cancer MCF-7 cells were obtained from Dr. W. Hait (The Cancer Institute of New Jersey), and prostate cancer PC-3 cells and LHRH-negative ovarian SKOV-3 cancer cells were obtained from the ATCC (Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. All experiments were performed on cells in the exponential growth phase.

Cytotoxicity Assay

The cytotoxicity of peptides was assessed using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described (14). To measure cytotoxicity, cells were separately incubated in a 96-well microtiter plate with 45 different concentrations of LHRH, BH3, and LHRH-BH3 peptides in the cell growth medium. Six wells were used for control cells receiving an equivalent volume of fresh medium. The duration of incubation was 48 h. Based on these measurements the IC_{50} doses of peptides (the concentration of peptide necessary to inhibit the cell growth by 50%) were calculated as previously described (14). In part of the experiments the IC_{50} doses of LHRH–BH3 peptide were measured in human A2780 ovarian cells in the presence of 45 different concentrations of LHRH peptide. The IC_{50} data then were plotted vs. LHRH concentration and the concentration of LHRH peptide necessary to decrease the toxicity of LHRH–BH3 peptide by 50% (EC_{50}) was calculated by logarithmic regression analysis.

Apoptosis Detection

Apoptosis induction was analyzed in A2780 human ovarian carcinoma cells incubated with 4 ng/mL of LHRH–BH3 peptide. This dose is close to the mean IC_{50} dose of LHRH–BH3 peptide. Two approaches were used to assess apoptosis induction. The first approach was based on measuring the enrichment of cell cytoplasm by histone-associated DNA fragments (mono- and oligo-nucleosomes) using anti-histone and anti-DNA antibodies (Cell Death ELISA Plus kit, Roche Diagnostics) as previously described (15–17). The method was used to analyze time-dependent (0, 18, 24, and 48 h) apoptosis induction in A2780 cells. The second approach was based on the detection of single- and double-stranded DNA breaks (nicks) by *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany) using terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) method as previously described (15,17). After 48 h of incubation with 4 ng/mL of LHRH–BH3 peptide, cells were fixed, permeabilized, and incubated with the TUNEL reaction mixture. The label incorporated at the damaged sites of the DNA was visualized by a fluorescence microscope.

Gene Expression

Combination of reverse transcription (RT) and polymerase chain reaction (PCR) was used for the analysis of gene expression in control and cells separately incubated with LHRH, BH3, and LHRH–BH3 peptides in doses corresponding to mean IC_{50} dose LHRH–BH3 peptide after incubation for 48 h. Total cellular RNA was isolated using an RNeasy kit (QIAGEN, Valencia, CA, USA). First-strand cDNA was synthesized by Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ, USA) with 2 μg of total cellular RNA (from 1×10^7 cells) and 100 ng of random hexadeoxynucleotide primer (Amersham Biosciences, Piscataway, NJ, USA). After synthesis, the reaction mixture was immediately subjected to PCR, which was performed using GenAmp PCR System 2400 (Perkin–Elmer Instruments, Shelton, CT, USA). The pairs of primers used to amplify cDNA are shown in Table I. The PCR regimen was: 94°C/4 min, 55°C/1 min, 72°C/1 min for 1 cycle; 94°C/1min, 55°C/50 s, 72°C/1 min for 28 cycles; and 60°C for 10 min. The gene encoding β_2-microglobulin (β_2-m) was used as an internal standard. PCR products were separated in 4% NuSieve 3:1 Reliant® agarose gels (BMA, Rockland, ME, USA) in 1× TBE buffer (0.089 M Tris/Borate, 0.002 M EDTA, pH 8.3; Research Organics, Inc., Cleveland, OH, USA) by submarine

Table I. List of Primers Used in Reverse-Transcription Polymerase Chain Reaction

Genes	Primers (5′ to 3′)		Product size (bp)
	Sense primer	Antisense primer	
LHRHR	gaccttgtctggaaagatcc	caggctgatcaccaccatca	319
BCL-2	ggattgtggccttctttgag	ccaaactgagcagagtcttc	233
BCL-XL	atgaactcttccgggatgg	tggatccaaggctctaggtg	166
APAF-1	gggtttcagttgggaaacaa	cacccaagagtcccaaacat	200
SMAC	ggagccagagctgagatgac	cagcttggtttctgctttcc	213
Caspase 3	tggaattgatgcgtgatgtt	ggcaggcctgaataatgaaa	201
Caspase 9	tgactgccaagaaaatggtg	cagctggtcccattgaagat	196
β_2-*m,* internal standard	accccactgaaaaagatga	atcttcaaacctccatgatg	114

electrophoresis. The gels were stained with ethidium bromide, digitally photographed and scanned using Gel Documentation System 920 (NucleoTech, San Mateo, CA, USA).

Protein Expression

To confirm RT-PCR data, we measured the expression of BCL-2 and BCL-XL proteins and Caspase 9. The identification of the above proteins was made by Western immunoblotting analysis and processed using the scanning densitometry (Gel Documentation System 920, NucleoTech, San Mateo, CA, USA) to quantify the expressed protein. To this end, harvested cells were lyzed in Ripa buffer (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) using a needle and syringe. After incubation on ice for 45 min, the cells were centrifuged at 10,000 g for 10 min. Protein content in the supernatant was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and 50 μg of protein was run on a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel immersed in Tris/Glycine/sodium dodecyl sulfate buffer (Bio-Rad, Hercules, CA, USA) for 90 min at 70 V. Proteins were transferred to an Immobilon-P nitrocellulose membrane (Millipore, Bedford, MA, USA) in a Tris/Glycine buffer (cat no. 161-0771, Bio-Rad) for 90 min at 100 V. The membrane was blocked in nonfat milk for 30 min at room temperature on a rotating shaker to prevent nonspecific binding, washed, and incubated overnight with the rabbit primary antibody, anti-BCL-2 (cat no. AAP 070, 1:280 dilution, Stress Gen Biotechnologies, Victoria State, BC, Canada), anti BCL-XL (cat no. 3041-100, 1:2000 dilution, Biovision, Mountain View, CA, USA), and anti-Caspase 9 (cat no. AAP 070, 1:2000 dilution, Stress Gen Biotechnologies, Victoria State, BC, Canada) at 4°C. After further washing, the membrane was immersed in a goat anti-rabbit IgG biotinylated antibody (cat no. 1706412, 1:3000 dilution, Bio-Rad) at room temperature for 1 h on a rotating shaker. Bands were visualized using an alkaline phosphatase color development reagent (cat no. 1706412, Bio-Rad). The bands were digitally photographed and scanned using Gel Documentation System 920 (NucleoTech).

Caspase Activity

The direct measurements of caspase activity were made using colorimetric protease assay kits (MBL International, Watertown, MA, USA) as previously described (16) after the incubation of cells separately with IC_{50} dose of LHRH–BH3 peptide within 48 h. The concentration of the peptide corresponded to its IC_{50} dose. The assay is based on the spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the substrates X-*p*NA, where X stands for amino acid sequence recognized by the specific caspase (DEVD and LEHD for caspases 3 and 9, respectively). The increase in the caspase activity was determined by comparing these results with the level of the untreated control.

Statistical Analysis

Statistical analysis of data included calculations of mean values and standard deviation based on four to eight independent measurements. Significance of the difference between series was analyzed using two-way nonparametrical Student's *t*-test. The difference between series was considered statistically significant if $p < 0.05$.

RESULTS

Targeting Receptor to LHRH Peptide Overexpression in Several Cancer Cells and Nonexpression in Normal Cells

To determine the expression of the gene encoding LHRH receptor, we used RT-PCR with pairs of primers specific to human LHRH receptor. To show whether normal cells express the receptor to LHRH, we repeated the above-described PCR procedure to cDNA isolated from different healthy human organs (Multiple Tissue cDNA Panels, Clontech, Palo Alto, CA). Figure 1A shows a typical image of the RT-PCR products obtained in this series of experiments. It

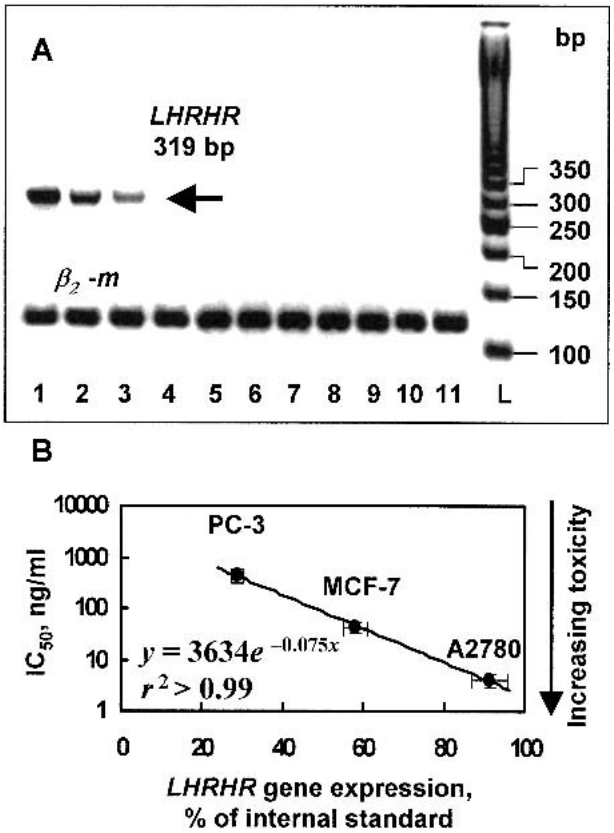

Fig. 1. (A) Typical image of reverse-transcription polymerase chain reaction (RT-PCR) product of gene encoding luteinizing hormone-releasing hormone receptor (LHRHR) in cancer cells and cDNA isolated from healthy human organs. A 50-bp ladder (Pharmacia) was used as size marker (L). Predicted size of RT-PCR product was 319 bp. β_2-microglobulin (β_2-*m*) was used as an internal standard and to confirm the efficacy of PCR. The *LHRHR* gene was expressed in human ovarian carcinoma cells. The expression of the *LHRHR* gene in healthy human organs was below the detection limits of PCR, while the β_2-*m* gene was expressed in all studied tissues. (B) The correlation between LHRH receptor expression and cytotoxicity of LHRH-BH3 peptide. A strong exponential correlation was found between the IC_{50} dose of LHRH-BH3 peptide and the expression of the *LHRHR* gene in cancer cells with the coefficient correlation $r^2 > 0.99$ was found. In (A), 1, A2780 cells; 2, MCF-7 cells; 3, PC-3 cells; 4, SKOV-3 cells; 5, liver; 6, lung; 7, heart; 8, kidney; 9, spleen; 10, muscle; and 11, thymus.

can be seen that LHRH receptor is overexpressed in human A2780 ovarian cancer cells and expressed to a lesser extent in MCF-7 breast and PC-3 prostate cancer cells and is not expressed in LHRH receptor-negative human SKOV-3 ovarian cancer cells and healthy human visceral organs (lung, liver, kidney, spleen, muscle, heart, thymus), whereas the internal standard (the β_2-*m* gene) was expressed in both cancer cells and the studied organs.

LHRH as a Targeting Moiety Significantly Enhanced Cellular Uptake of BH3 Peptide

The feasibility of using LHRH peptide to target the BH3 peptide to cancer cells was investigated. The cytotoxicity of BH3, LHRH, and LHRH–BH3 peptides was assessed by the modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Studied A2780 human ovarian carcinoma cells were incubated separately with 45 different concentrations of peptides. The results of the measurements are shown on Fig. 2. We found that BH3 peptide alone was not toxic up to the maximal possible concentration limited by its solubility (1 mg/mL). Further analysis showed that LHRH peptide alone also did not show any significant toxicity up to highest possible concentration. Similarly, LHRH–scBH3 and scLHRH–BH3 peptides with scrambled LHRH or BH3 sequences (negative controls) were not toxic up to the highest possible concentrations (1 mg/mL). In contrast, LHRH–BH3 peptide was toxic in human ovarian cancer cells. The mean IC_{50} value, drug concentration that inhibits growth by 50% relative to nontreated control cells, was 3.97 ± 0.33 ng/mL for A2780 human ovarian carcinoma cells overexpressing LHRH receptor.

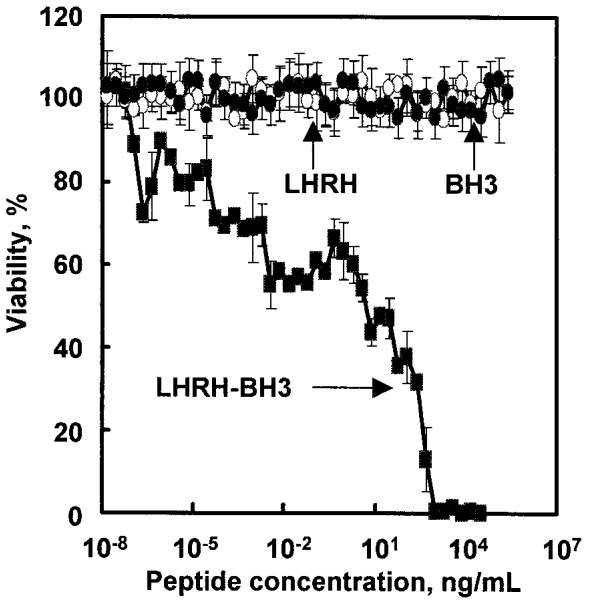

Fig. 2. Cytotoxicity of luteinizing hormone-releasing hormone (LHRH) and BH3 peptides separately and BCL-2 homology 3 (BH3) peptide in A2780 human ovarian carcinoma cells. Means ± SD are shown. Open circles, LHRH peptide; closed circles, BH3 peptide; closed squares, LHRH-BH3 peptide.

True Targeting: Cytotoxicity of the LHRH–BH3 Peptide Depends on the Expression of the Gene Encoding LHRH Receptor

To evaluate whether the cytotoxicity of LHRH–BH3 peptide depends on the expression of targeted LHRH receptor gene (*LHRHR*), we measured IC_{50} doses of the peptide and the expression of the *LHRHR* gene in human A2780 and SKOV-3 ovarian, MCF-7 breast and PC-3 prostate cancer cells. The measurement of the expression showed that SKOV-3 cells did not express the *LHRHR* gene (Fig 1A). The expression of the target gene was 1.6-fold and 3.2-fold less in MCF-7 and PC3 cells, respectively, when compared with A2780 cells. Correspondingly the toxicity of LHRH–BH3 peptide in MCF-7 and PC-3 cells was more than 10-and 100-fold, respectively, less when compared with A2780 ovarian carcinoma cells. In addition, the toxicity of LHRH–BH3 peptide in *LHRHR*-negative SKOV-3 ovarian carcinoma cells was not detectible. Further analysis revealed a strong exponential correlation between the IC_{50} dose of LHRH–BH3 peptide and the expression of the *LHRHR* gene in cancer cells (Fig 1B) with the coefficient correlation $r^2 > 0.99$.

Cytotoxicity of the LHRH-BH3 Peptide Was Significantly Reduced by Coincubation of Cells with LHRH Peptide

To show that cytotoxicity of LHRH–BH3 peptide is associated with its binding to LHRH receptor, we incubated A2780 human ovarian carcinoma cells with 45 different concentrations of LHRH peptide (ligand for LHRH receptor) and then measured cytotoxicity of LHRH–BH3 peptide. Data obtained showed that LHRH receptor ligand limited cytotoxicity of LHRH–BH3 in concentration-dependent manner (Fig. 3). Logarithmic regression analysis showed that the concentration of 7.3×10^{-5} ng/mL LHRH decreased cytotoxicity of LHRH–BH3 peptide by 50% (EC_{50} dose on Fig. 3).

Inhibition of Cellular Antiapoptotic Defense by LHRH-BH3 Peptide

To examine an influence of the peptide on the antiapoptotic cellular defense we studied the expression of genes encoding antiapoptotic members of BCL-2 protein family. It was found that after incubation of A2780 human ovarian carcinoma cells with LHRH–BH3 peptide the expression of genes encoding BCL-2 and BCL-XL proteins was significantly downregulated (Fig. 4). In contrast, LHRH or BH3 peptides when used separately did not change the expression of studied genes significantly (data not shown). The direct measurement of the expression of BCL-2 and BCL-XL proteins by Western blotting (Fig. 5) showed that LHRH–BH3 peptide suppressed the expression of BCL-2 protein and significantly decreased the expression of BCL-XL protein (Fig. 5).

LHRH-BH3 Peptide Activates Caspase-Dependent Signaling Pathway of Apoptosis

Human caspases were analyzed using three methods: 1) RT-PCR to measure the expression of genes encoding caspases 3 and 9; 2) Western blotting to analyze proteins; and 3) specific caspase substrates to measure the activity of caspases. The incubation of A2780 human ovarian carcinoma

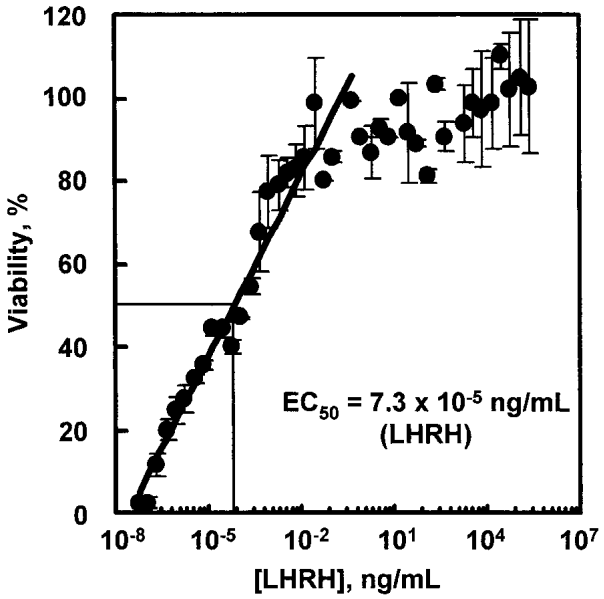

Fig. 3. Competitive inhibition of the cytotoxicity of luteinizing hormone-releasing hormone–BCL-2 homology 3 (LHRH-BH3) peptide by coincubation with LHRH peptide. Human A2780 ovarian carcinoma cells were incubated simultaneously with 45 different concentrations of LHRH peptide and 40 ng/mL of the LHRH-BH3 peptide. Cellular viability was measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide MTT assay. Means ± SD are shown. Effective concentration of LHRH peptide that decreases toxicity of LHRH–BH3 peptide by 50% (EC_{50}) was calculated using logarithmic regression.

cells with the LHRH–BH3 peptide led to the upregulation of the expression of genes encoding caspase-activating proteins: apoptotic protease activating factor-1 (APAF-1) and second mitochondria-derived activator of caspase (SMAC; Fig. 4A and B). The upregulation of APAF-1 and SMAC converted the inactive procaspase 9 into active caspase 9. Western blotting analysis of caspase 9 showed that the exposure to LHRH–BH3 peptide led to the appearance of an active form of this caspase almost completely absent in the control (Fig. 5C). Active caspase 9 triggers a cascade of downstream caspases, which in turn leads to apoptosis induction. The direct measurements of caspase 3 and 9 activity (Fig. 6) confirmed this observation and showed that LHRH–BH3 peptide activated both studied caspases in A2780 human ovarian carcinoma cells.

LHRH–BH3 Peptide Induces Apoptosis

Two methods were used to detect apoptosis. The first approach was based on measuring the enrichment of cell cytoplasm by histone-associated DNA fragments (mono- and oligo-nucleosomes) using anti-histone and anti-DNA antibodies. The second method of apoptosis evaluation was based on the detection of single- and double-stranded DNA breaks (nicks) occurring at early stages of apoptosis using a TUNEL method. The measurement of apoptosis showed that incubation of A2780 human ovarian carcinoma cells with LHRH–BH3 peptide induced apoptosis (Fig. 7). The degree of apoptosis induction was directly proportional to the time of incubation of cells with the peptide.

DISCUSSION

In contrast with traditional anticancer drugs that in most cases have only one primary cellular target, the proposed LHRH–BH3 peptide represents a novel two-tier molecular targeting approach, simultaneously targeting the following two molecular targets: 1) extracellular cancer specific receptor and 2) intracellular controlling mechanisms of apoptosis to suppress cellular antiapoptotic defense.

The first molecular target is the plasma membrane LHRH receptor, which is overexpressed in ovarian carcinoma cells, slightly expressed in breast and prostate cancer cells, and is not expressed in cells from other healthy organs. Targeting of peptide to this receptor fulfills two major tasks: 1) it increases the bioavailability of active ingredient—BH3 peptide—to cancer cells and 2) it targets BH3 peptide specifically to the cancer cells. The latter should prevent accumulation of whole peptide in healthy organs resulting in minimal adverse side effects and damage to healthy organs. We, as well as others, have observed poor bioavailability of the BH3 peptide and therefore used internalization sequence of the antennapedia protein (Ant) to deliver BH3 peptide into cancer cells (8–11,18,19). However, Ant-BH3 peptide is expected to increase bioavailability and cellular uptake of BH3 peptide not only for cancer cells, but also for cells in normal healthy organs. To target cancer cells and enhance the peptide uptake specifically by ovarian cancer cells we used the LHRH decapeptide instead of Antennapedia internalization sequence to deliver BH3 peptide. Our data on the expression of LHRH receptor in ovarian, breast, and prostate cancer cells and absence of the expressed receptor in healthy human visceral

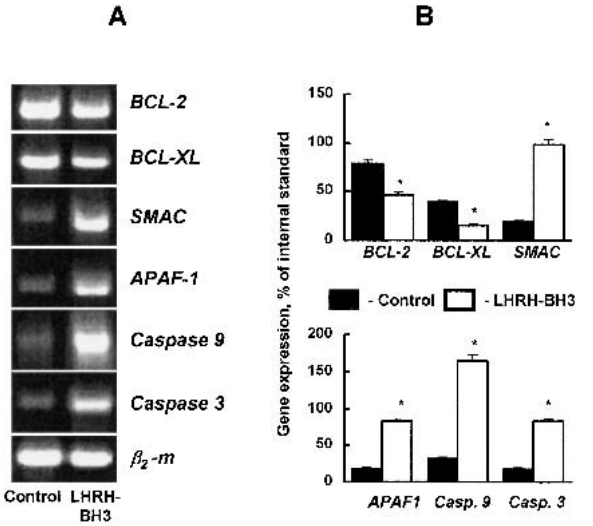

Fig. 4. Influence of the luteinizing hormone-releasing hormone–BCL-2 homology 3 (LHRH–BH3) peptide on the caspase-dependent signaling pathways of apoptosis in A2780 human ovarian carcinoma cells. (A) Typical images of gel electrophoresis of reverse-transcription polymerase chain reaction products of genes encoding BCL-2, BCL-XL, APAF-1, and SMAC proteins and caspases 3 and 9. (B) Densitometry analysis of gene expression. Gene expression was calculated as the ratio of band intensity of gene of interest to that of internal standard (β_2-microglobulin, β_2-*m*). Cells were incubated for 48 h with 4 ng/mL of the LHRH–BH3 peptide. Means ± SD are shown. *$p < 0.05$ when compared with control.

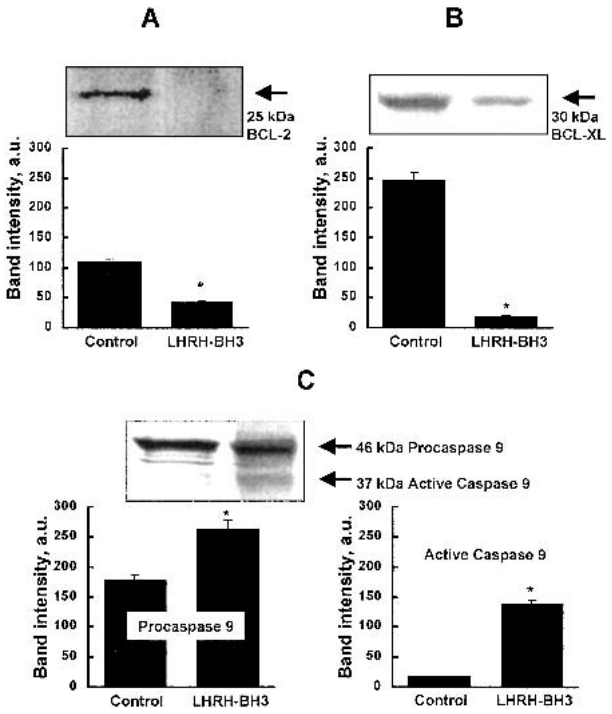

Fig. 5. Typical images of Western blots of BCL-2 (A), BCL-XL (B), and caspase-9 (C) proteins and densitometry analysis of bands. Bands intensities are shown in arbitrary units. A2780 human ovarian carcinoma cells were incubated 48 h with 4 ng/mL of the LHRH-BH3 peptide. Means ± SD are shown. *p < 0.05 when compared with control.

organs are supported by recent results obtained by other investigators (12,13).

Present data support the concept and show that LHRH peptide can be used to target expressed LHRH receptor in cancer cells. Several findings confirm the feasibility of the idea. First, we found that targeted BH3 peptide (LHRH–BH3) was highly toxic to ovarian cancer cells, which overexpressed this receptor and relatively nontoxic to other cancer cells with a limited expression of this receptor. At the same time, free BH3 peptide did not show measurable toxicity up to maximal available concentrations limited by the solubility of the peptide. Second, the toxicity of the peptide was highly correlated with the expression of LHRH receptors. Using cells with different levels of receptor expression we found very strong correlation ($r^2 > 0.99$) between the expression and the toxicity. Moreover, the toxicity declined 100 times if gene expression decreased three times. In contrast the LHRH–BH3 peptide was not toxic in ovarian carcinoma cells that do not express LHRH receptors. Third, competitive binding of LHRH peptide to LHRH receptor in LHRHR positive ovarian carcinoma cells significantly limited toxicity of the combined LHRH–BH3 peptide. It should be stressed that the effective concentration of LHRH peptide that decreased the toxicity by 50% was extremely low. Taken together these findings clearly show that enhancement of the BH3 peptide toxicity by its fusion with the LHRH peptide was achieved by targeting the peptide to LHRH receptor, overexpressed in LHRH sensitive human ovarian carcinoma cells and expressed in other types of cancer cells.

The second molecular target of proposed peptide is cellular antiapoptotic defense. In the LHRH–BH3 peptide the LHRH peptide mediated intracellular delivery of the whole construct while the synthetic BH3 peptide modulated its anticancer activity. During the development of the targeted proapoptotic peptide, initially we planned to limit the role of BH3 peptide only to a suppressor of antiapoptotic cellular defense and combine it in the future with one of the traditional anticancer drug as an apoptosis inductor (8,11). The results of the present study showed that BH3 peptide could also efficiently induce apoptosis. Therefore, in the proposed complex peptide BH3 peptide plays both roles, inducing apoptosis and simultaneously suppressing cellular antiapoptotic defense. This two-pronged action of BH3 peptide provides for a significant advantage over the traditional anticancer drugs, which also induce apoptosis and at the same time activate cellular antiapoptotic defense (8,11,15–17,20). However, the combination of LHRH–BH3 peptide with an anticancer drug in one drug delivery system might have certain advantages decreasing the amount of expensive peptides by enhancing cytotoxicity of whole system (11).

Although the mechanism of action of the synthetic BH3 peptides as well as BH3-only proteins remains unclear, recent studies on the regulation of critical protein—protein interactions and activity of BAD protein by phosphorylation in response to growth factor signaling suggest that the active state of BH3-only proteins may be regulated by post-translational modification (21,22). Additional modes of regulation, such as transcriptional, translational, and subcellular localization, are also likely to be important (6). The present data on the suppression of BCL-2 protein and genes encoding antiapoptotic BCL-2 and BCL-XL proteins support this conception. Normally, proapoptotic members of the BCL-2 protein family are dormant in the cytosol of normal cells and are activated upon induction of apoptosis (3,5). In apoptotic cells, these proteins may translocate to mitochondria, insert into the outer membrane, oligomerize and trigger the release of cytochrome *c*, possibly by channel formation. The BH3 domain-only proteins can induce a conformational change in proapoptotic members of BCL-2 protein family before their insertion into

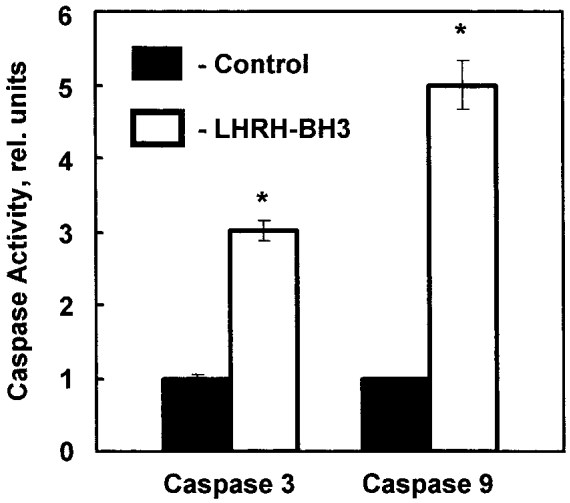

Fig. 6. Activity of caspases 3 and 9. Activities are expressed as relative units setting untreated cells as 1 U. A2780 human ovarian carcinoma cells were incubated for 48 h with 4 ng/ml of the luteinizing hormone-releasing hormone–BCL-2 homology 3 (LHRH-BH3) peptide. Means ± SD are shown. *p < 0.05 when compared with control.

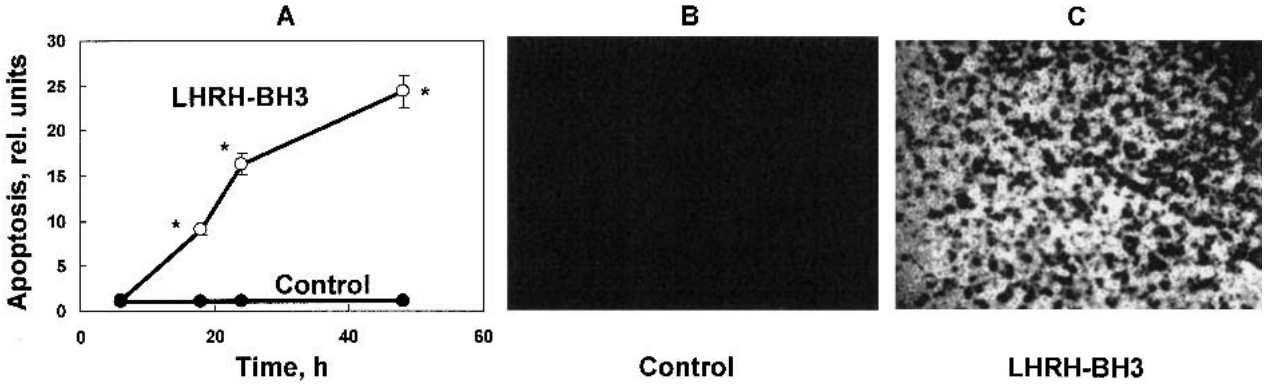

Fig. 7. Apoptosis induction by the luteinizing hormone-releasing hormone–BCL-2 homology 3 (LHRH–BH3) peptide. A2780 human ovarian carcinoma cells were incubated with 4 ng/mL of the LHRH–BH3 peptide. (A) Time-dependent apoptosis induction. The enrichment of control cells by histone-associated DNA fragments (mono- and oligonucleosomes) was set to 1 U and the degree of apoptosis was expressed in relative (to control) units. Means ± SD are shown. *$p < 0.05$ when compared with control. (B, C) Typical fluorescence microscopy images of terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end-labeled (TUNEL) cells in control (B) and after 48 h exposure to 4 ng/mL of the LHRH–BH3 peptide (C).

the outer mitochondrial membrane (6,9). The mechanism by which they promote activation of proapoptotic proteins is not understood. However, it was reported recently that short form of BH3 only BID protein cut with caspase 8 stimulated BAX protein to form ionic channels in liposomes and planar bilayers that were cytochrome c-permeable (23). Upon its release to the cytosol, cytochrome c binds to apoptotic protease activating factor-1 (APAF-1) in the presence of dATP or ATP. Cytochrome c/APAF-1 complex then recruits procaspase-9, inducing its autoactivation and leading to the activation of downstream caspases. It was recently found that other caspase activators, SMAC and direct IAP-binding Protein with low pI, might be involved in this process (24,25). The results of the present experiments support this finding and show that incubation of human ovarian carcinoma cells with LHRH–BH3 peptide led to apoptosis induction through the overexpression of genes encoding APAF-1 and SMAC proteins and activation of apoptosis caspase-dependent signaling pathway. However, it is possible that BH3 peptide antagonized BCL-XL functions and induced apoptosis through cytochrome c-independent activation of caspases (9). Whether or not cytochrome c release was involved in the induction of apoptosis by LHRH–BH3 peptide, present data clearly demonstrated that LHRH–BH3 peptide induced apoptosis by the activation of caspase-dependent signaling pathway.

It should be mentioned that we and other investigators already have shown that a synthetic BH3 peptide can be used to suppress antiapoptotic defense in cancer cells (8–11). In addition, LHRH agonists are being used for treatment of certain cancers (26–29). However, our approach is dramatically different from previous approaches even though known elements such BH3 and LHRH peptides are used. There are no previous attempts to use peptides, which combine in one complex system an apoptosis inducer/a suppressor of antiapoptotic cellular defense and a targeting penetration enhancer. This is a cellular-molecularly based three-pronged attack on cancer. It targets, prevents resistance, and promotes the cellular pathways leading to death in cancer cells. We expect that the use of this novel targeted proapoptotic peptide will increase the efficacy of ovarian cancer therapy to an extent that cannot be achieved by individual components applied separately.

This LHRH–BH3 peptide can serve as a model for a more advanced drug delivery systems (11), which in addition to a targeting moiety and a suppressor of antiapoptotic cellular defense (nonpump resistance), will also include an anticancer drug and a suppressor of drug efflux pumps (pump resistance). These advanced targeted proapoptotic drug-delivery systems are currently being developed in our laboratory. However, even this simple LHRH-BH3 two-tier targeted system might be effectively used for treatment of LHRH positive ovarian cancer cells and to a lesser extent for breast and prostate cancer.

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