Amino Acids (2015) 47:2359–2366
DOI 10.1007/s00726-015-2021-2

ORIGINAL ARTICLE

Synthesis and in vitro anti-cancer evaluation of luteinizing hormone-releasing hormone-conjugated peptide

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Received: 27 February 2015 / Accepted: 1 June 2015 / Published online: 10 June 2015

Abstract Luteinizing hormone-releasing hormone (LHRH) is a decapeptide hormone released from the hypothalamus and shows high affinity binding to the LHRH receptors. It is reported that several cancer cells also express LHRH receptors such as breast, ovarian, prostatic, bladder and others. In this study, we linked B1, an anti-cancer peptide, to LHRH and its analogs to improve the activity against cancer cells with LHRH receptor. Biological evaluation revealed that TB1, the peptide contains triptorelin sequence, present favorable anti-cancer activity as well as plasma stability. Further investigations disclosed that TB1 trigger apoptosis by activating the mitochondria–cytochrome c–caspase apoptotic pathway, it also exhibited the anti-migratory effect on cancer cells.

Keywords Anti-cancer · Apoptosis · Conjugation · Luteinizing hormone-releasing hormone · Peptide

Handling Editor: G. J. Peters.

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Introduction

Luteinizing hormone-releasing hormone (LHRH), also called gonadotropin-releasing hormone (GnRH), is a decapeptide hormone (primary sequence: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) released from the hypothalamus. LHRH can specifically bind to LHRH receptors on the surface of gonadotropins cells in pituitary and stimulates the synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Schally 1999). In addition, several cancer cells also express LHRH receptors on their membrane, including breast, ovarian, prostatic, bladder and others (Schally and Nagy 2004; Schally et al. 2001, 2011). The presence of specific binding site for LHRH on the membranes for cancer cells renders LHRH as well as its analogs present high affinity binding to these cells (Srkalovic et al. 1990; Emons et al. 1993). Thus, conjugating LHRH or its analogs with cytotoxic agents can directly target cancer cells with LHRH receptors, thereby increasing their concentration in tumor tissue while sparing benign cells from undesired damage (Schally and Nagy 2004).

Antimicrobial peptides (AMPs) are linear, cationic and amphipathic compounds in the host defense system of organisms (Yount et al. 2006; Splith and Neundorf 2011). The cytotoxic activity of AMPs against cancer cells has been described in several literatures (Mai et al. 2001; Hoskin and Ramamoorthy 2008). Briefly, AMPs can selectively disrupt the lipid bilayer structure of cancer cells, resulting in leakage of metabolites and depolarization, besides AMPs may also extend to the permeation and acting on mitochondria, which results in the release of cytochrome c and apoptosis. B1 is a 15-mer AMP derived from Cathelicidin-BF15 (BF-15) (Chen et al. 2011). In previous study, we found B1 might have certain anti-cancer

activities against several cancer cell lines while present relatively low toxicities to normal cells. Herein, we postulate that conjugates of B1 and LHRH or its analogs would target and selectively destroy cancer cells which express LHRH receptor.

In this study, we designed and synthesized a series of peptides by linking B1 to LHRH as well as two of its analogs, pGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2 (triptorelin) and Gln-His-Trp-Ser-Trp-Gly-Leu-Arg-Pro-Gly-NH_2 (W-LHRH) (Pazos et al. 1999; Yates et al. 2011). In vitro cytotoxicity evaluation revealed that all the peptides have improved anti-cancer effects on LHRH receptor-positive cancer cells. Among them, TB1, the conjugate of B1 and triptorelin, also exhibited superior plasma stability over other peptides. Further investigations were performed on TB1 and disclosed the possible anti-cancer mechanism of this peptide.

Materials and methods

Peptide synthesis, purification and analysis

The synthesis of LHRH-conjugated peptides was accomplished by solid-phase methodology on Fmoc Rink Amide-MBHA resin using a microwave synthesizer (CEM, NC, USA). The general procedure of peptide preparation under microwave irradiation was previously reported (Chi et al. 2008; Qian et al. 2010). The crude peptides were purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC; Shimadzu LC-10) using a C18 column (5 mm, 340 × 28 mm). Purity analysis and characterization were performed by ultra-high-performance liquid chromatography/mass spectrometry (UPLC/MS; Waters UPLC with the ACQUITY TQD; Waters Corporation, Milford, MA, USA) on a Waters ACQUITY UPLC BEH C18 column (1.7 × 50 mm, Waters). The purity of the peptides was above 95 %.

Cell cultures

The breast cancer cell line MCF-7, prostate cancer cell lines PC-3 and DU145, cervical cancer cell line HeLa, hepatic cancer cell line SMMC-7721, leukemia cell line K562, gastric epithelial cell line GES-1 and embryonic kidney cell line HEK-293 were used in this study. MCF-7, DU145 and GES-1 cell lines were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin antibiotics. SMMC-7721 and K562 cell lines were grown in RPMI 1640 medium supplemented with 10 % new born bovine serum (NCS) and antibiotics, HeLa and HEK-293 cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and antibiotics, and PC-3 cell lines were grown in DMEM/F12 medium supplemented with 10 % FBS and antibiotics. All the cell lines were cultured at 37 °C in a humidified atmosphere at 5 % CO_2 and 95 % air.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for LHRH receptor

mRNA expression of LHRH receptor in each cell lines was detected by RT-PCR. The quality control of RNA samples, synthesis of cDNA and target gene amplification, as well as PCR products analysis were as described (Kwok et al. 2014).

In vitro cytotoxic activity

The anti-proliferation activity of the newly synthesized peptides was determined by MTT assay. Cells were seeded in 96-well plates at a density of 5×10^3/well. Cells were cultured for 24 h and then treated with various concentrations of peptides while controls were not exposed to peptide. After incubating for 48 h, 20 μl MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37 °C. The MTT solution was replaced with 150 μl DMSO. The absorbance at 490 nm for each well was measured on a microplate reader (Bio-Rad, iMark 680). Survival rates were plotted against peptide concentrations and the IC_{50} value for each cell line was calculated based on the survival curve.

Hemolysis of rabbit red blood cells (rRBCs)

The assay was completed by a protocol described previously (Chen et al. 2011), with a final volume of 200 μl of PBS solution containing the peptides and rRBCs (final concentration 4 %). The release of hemoglobin was monitored by measuring the absorbance of supernatant at 540 nm. Controls for zero and 100 % hemolysis consisted of rRBCs suspended in PBS and 0.1 % Triton, respectively.

In vitro plasma stability

Plasma stability was assessed according to the process reported by Ritzel with minor revise (Ritzel et al. 1998). Peptides (2 mg/ml in PBS) were incubated with rat plasma (final volume 0.5 ml; final peptide concentration 20 μM) for 0, 15, 30 min, or 1, 2, 3, 4 or 6 h at 37 °C. The incubation was terminated by adding 1.5 ml acetonitrile with 0.1 % trifluoroacetic acid. After vortex, the samples were centrifuged at 10,000×*g* for 10 min. The supernatant was collected and analyzed using UPLC/MS.

LHRH receptor blocking experiments

To determine whether the anti-proliferation activity of the peptides was also mediated by LHRH receptor, an LHRH receptor blocking and competition study was carried out. Triptorelin is a kind of LHRH receptor agonist whose mechanism of action and LHRH receptor binding affinity are similar to natural LHRH. Triptorelin also shows favorable stability over natural LHRH, which would prevent the degradation of LHRH sequence during this experiment. Meanwhile, cetrorelix is a typical LHRH receptor antagonist. Therefore, we employed triptorelin and cetrorelix as the blocking molecules. Cells were seeded in 96-well plates as described above and pretreated with 10 μM triptorelin or cetrorelix for 1 h, then peptides of various concentration were added and cultured for another 48 h. The cell viability was then measured by MTT assay.

Annexin V-FITC/propidium iodide (PI) apoptosis assay

The DU145 cells were seeded in a 6-well microtiter plate at a density of 1×10^5 cells/well and cultured for 24 h. As shown in Table 2, the IC_{50} value of TB1 against DU145 cell line is 4.4 ± 1.0 μM, thus we chose the concentration of 4 μM in this assay as well as the following assays (except the wound-healing assay). After treating with 4 μM TB1 for 4 h, cells were collected and washed twice with PBS, then cells were treated with Annexin V-FITC/PI using the apoptosis detection kit (Nanjing Jiancheng Bioengineering Institute) according to the protocol. Annexin V-FITC/PI binding was analyzed by a flow cytometer.

Acridine orange/ethidium bromide (AO/EB) double staining

DU145 cells were grown in a 24-well plate at a density of 1×10^5 cells/well and incubated for 24 h. After treating with 4 μM TB1 for 30 min, the medium was removed and the cells were washed twice with PBS. Then cells were stained with 1 ml dye mixture, containing 10 μg/ml AO and 10 μg/ml EB in PBS. After staining for 10 min, excess AO/EB was washed off with cold PBS. Fluorescence was visualized immediately using fluorescence microscopy (OLYMPUS America, Melville, NY, USA).

Scanning electron microscopy (SEM)

DU145 cells were seeded at 1×10^5 cells/well into a 6-well microtiter plate containing sterilized coverslips. The plate was incubated for 24 h and then 4 μM TB1 was added for 30 min. The medium was removed and the cells were gently washed with PBS. 1 ml of 2.5 % glutaraldehyde solution was added to each well to fix the cells. After fixation, the samples were sent to the College of Life Science at Nanjing Agricultural University for further treatment and analysis on a Model S-3000N SEM (Hitachi High-Technologies Corporation).

Mitochondrial membrane potential ($\Delta\Psi_m$)

DU145 cells were plated at 1×10^5 cells/well in 24-well plate and incubated for 24 h. Then cells were treated with 4 μM TB1 for 4 h. The change of $\Delta\Psi_m$ was detected by flow cytometer using the fluorescent cationic dye 5,5′,6,6′-tetrachloro-l,l′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) as described by (Cossarizza et al. 1993).

Western blot analysis

After peptides treatment for 4 h, medium was removed and DU145 cells were harvested at 4 °C. Cytoplasmic and mitochondrial proteins were collected by an available kit (Nanjing Jiancheng Bioengineering Institute), respectively. Cytochrome c, caspase-9 and caspase-3 levels were then measured by western blot as described by Sun et al. (1999).

Wound-healing assay

DU145 cells were seeded in 6-well plates at 2×10^5 cells/well and incubated for 24 h. After the cells had grown to 90 % confluence, a cell-free gap was created and the cells were allowed to migrate in serum-free medium for 48 h. The cytotoxicity of TB1 against DU145 cells should be avoided here and we also found the highest nontoxic concentration of TB1 is about 1.0–1.3 μM (data not shown). Therefore, we used 1 μM TB1 in this assay and the wound closure or cell migration images were photographed after treatment with 1 μM TB1 at 0 h and 48 h.

Results

Structural characteristics of LHRH-conjugated peptides

The amino acid sequences and structural parameters of targeted peptides are shown in Table 1. Following the purification of the peptides by preparative HPLC, UPLC/MS was used for the analysis of the molecular weight values and purity of the peptides. The mass-to-charge ratio (m/z) was found nearly identical to the theoretical m/z in each case (Table 1).

Table 1 Structural characteristics of LHRH-conjugated peptides

Compound	Structure	Molecular formula	UPLC retention time (min)	Mass (Da)	
				Calculated	Observed
LB1	pGHWSYGLRPG-VKRFKKFFRKLKKSV-NH_2	$C_{149}H_{232}N_{44}O_{29}$	1.97	$[M + 2H]^{2+}$:1551.9	$[M + 2H]^{2+}$:1552.2
				$[M + 3H]^{3+}$:1034.9	$[M + 3H]^{3+}$:1035.3
				$[M + 4H]^{4+}$:776.5	$[M + 4H]^{4+}$:776.9
WB1	QHWSWGLRPG-VKRFKKFFRKLKKSV-NH_2	$C_{151}H_{236}N_{46}O_{28}$	1.92	$[M + 2H]^{2+}$:1571.9	$[M + 2H]^{2+}$:1571.9
				$[M + 3H]^{3+}$:1048.3	$[M + 3H]^{3+}$:1048.4
				$[M + 4H]^{4+}$:786.5	$[M + 4H]^{4+}$:786.7
TB1	pGHWSYwLRPG-VKRFKKFFRKLKKSV-NH_2	$C_{158}H_{239}N_{45}O_{29}$	2.16	$[M + 2H]^{2+}$:1616.4	$[M + 2H]^{2+}$:1616.4
				$[M + 3H]^{3+}$:1078.0	$[M + 3H]^{3+}$:1078.4
				$[M + 4H]^{4+}$:808.7	$[M + 4H]^{4+}$:809.1

pG pyroglutamic acid, *w* D-Trp

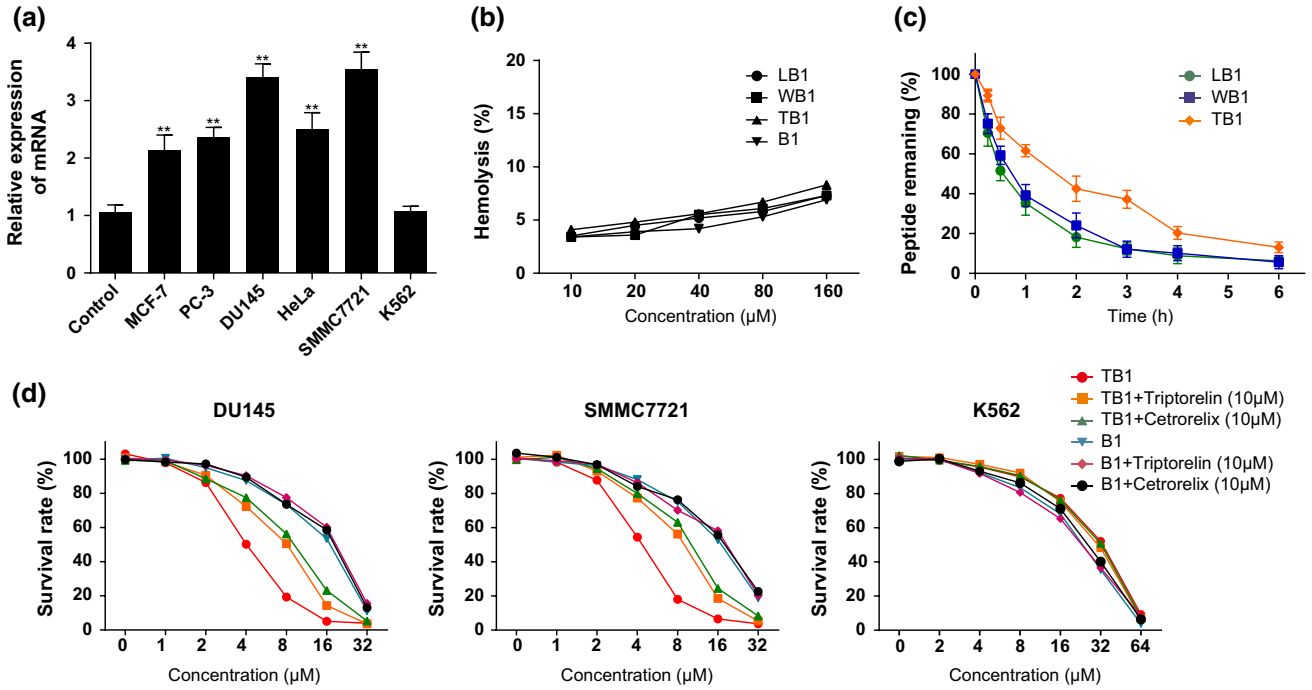

Fig. 1 **a** The expression of mRNA for LHRH receptor in each cancer cell lines; **b** the *curves* of the hemolytic activity of the peptides; **c** degradation of the peptides by rat plasma; **d** LHRH receptor blocking assays

Table 2 In vitro cytotoxicity of the peptides

Compounds	IC_{50} (μM)							
	MCF-7	PC-3	DU145	Hela	SMMC-7721	K562	HEK293	GES-1
LB1	5.8 ± 1.1	13.1 ± 0.6	4.8 ± 0.3	11.2 ± 1.0	4.9 ± 0.3	24.5 ± 3.8	81.9 ± 9.1	89.3 ± 10.3
WB1	4.7 ± 0.8	10.4 ± 1.1	3.8 ± 0.5	10.1 ± 2.5	4.0 ± 0.6	28.8 ± 2.6	78.1 ± 8.6	85.2 ± 12.1
TB1	5.5 ± 0.6	11.6 ± 2.8	4.4 ± 1.0	12.4 ± 1.3	4.4 ± 0.2	25.8 ± 4.1	85.3 ± 5.6	79.3 ± 6.4
B1	23.5 ± 3.8	33.1 ± 4.2	18.1 ± 3.7	36.3 ± 4.2	24.7 ± 3.3	26.3 ± 5.2	74.3 ± 9.1	96.5 ± 8.1
Triptorelin	75.9 ± 7.1	70.8 ± 6.9	67.4 ± 8.2	73.4 ± 6.8	146.2 ± 19.3	151.4 ± 22.7	156.6 ± 18.1	148.6 ± 19.8
Adriamycin	0.46 ± 0.05	3.2 ± 0.1	0.11 ± 0.07	4.4 ± 0.4	0.81 ± 0.11	0.48 ± 0.05	0.46 ± 0.15	0.60 ± 0.22

Expression level of mRNA for LHRH receptor in each cells

The expression of mRNA for LHRH receptors was accomplished by RT-PCR analysis. As presented in Fig. 1a, all the tested cancer cell lines but K562 exhibited mRNA expression for LHRH receptors. Additionally, DU145 and SMMC-7721 had more expression quantities than other cells (Fig. 1a).

Cytotoxicity, hemolysis and in vitro plasma stability of the peptides

The cytotoxicity activity of the series of peptides was screened against various cell lines (Table 2). All the conjugated peptides exhibited improved cytotoxic effect on the cancer cells expressing LHRH receptor, especially for the DU145 and SMMC-7721, while less toxic to the benign cell lines (Table 2). Hemolysis assay also revealed that all the tested peptides had little hemolytic activity even at the concentration of 160 μM (as shown in Fig. 1b). Meanwhile, plasma stability assay was performed to evaluate the in vitro stability of LHRH-conjugated peptides. As shown in Fig. 1c, the peptide conjugated with triptorelin (TB1) displayed prominent plasma stability over LB1 as well as WB1.

LHRH sequence ameliorate anti-proliferation activity of TB1

In the control cell line K562, which does not express LHRH receptors, no reduction of anti-proliferation activity of TB1 was observed when pretreated with triptorelin or cetrorelix. On the other hand, the anti-proliferation effects of TB1 in triptorelin or cetrorelix pretreated DU145 and SMMC-7721 cells were weakened (Fig. 1d). This finding suggests that triptorelin or cetrorelix has competitive effect with TB1 in the presence of LHRH receptor. In other words, the cell growth inhibitory effect of TB1 was LHRH receptor dependent.

Membrane-disruptive activity of the TB1

AO and EB are both fluorescent dyes: AO penetrates the cell membrane, incorporates into DNA and makes the cells appear green; while EB only penetrates cells with disrupted cell membranes before inserting into DNA and making the cells appear red. As shown in Fig. 2a, b, DU145 cells turned red after treating with 4 μM TB1 for 30 min compared with the control.

Moreover, the membrane-disruptive effect of the TB1 was visualized by SEM. After incubation with 4 μM TB1, significant variations of membrane morphology were observed when compared to the control group. Figure 2c shows that cells treated with PBS displayed a normal smooth surface and the cells were intact, but the cancer cells treated with the peptides had disrupted cell membranes.

The pro-apoptotic activity and mechanism of TB1

First, the apoptotic effect of TB1 was evaluated by Annexin-FITC/PI staining. The flow cytometric analysis showed that DU145 cells presented a significant increase in the percent of Annexin-FITC/PI-positive apoptotic cells after treated with TB1 (Fig. 3a).

As we know, the leakage of cytochrome c into cytoplasm will trigger activation of caspase proteases leading to apoptosis. Generally, cytochrome c is stored in mitochondria and will be released into cytoplasm following the induction of apoptosis by stimuli. In cytoplasm, cytochrome c triggers the activation of caspase-9, a critical upstream activator of the caspase cascade, which can then activate the effector caspases-3 (Sun et al. 1999). Thus, the loss of $\Delta\Psi_m$ was thought to be important in the activation of apoptosis (Kroemer et al. 1997). JC-1 is a $\Delta\Psi_m$-sensitive dye. In healthy cells, the intact $\Delta\Psi_m$ allows JC-1 to accumulate in mitochondria, where it forms J-aggregates and emits red florescence. While the mitochondrial membrane is depolarized, the collapse of $\Delta\Psi_m$ causes JC-1 remains in cytoplasm in a green florescent monomeric form. As shown in Fig. 3b, the augment of green fluorescence (LR) as well as the decrease of red fluorescence (UR) was observed after the treatment by TB1.

Furthermore, the western blot was used to evaluate the alteration of cytochrome c, caspase-9 and caspases-3 in DU145 cells after treating with 4 μM TB1 for 4 h. As shown in Fig. 3c, the contents of cytochrome c, caspase-9 and caspases-3 in cytoplasm were significantly increased after treatment with TB1.

The anti-migratory effect of TB1

The wound-healing assay was performed to assess the anti-migratory effect of TB1. The migratory ability of DU145 cells was significantly inhibited when treated with TB1 (1 μM) (Fig. 3d).

Discussion

Theoretically, ideal anti-cancer drugs would be those that eradicate malignant cells without harming normal tissues. Hormonal peptide or its analogs carrying cytotoxic agents could be used for targeted chemotherapy of cancers which express receptors for the peptide moieties on

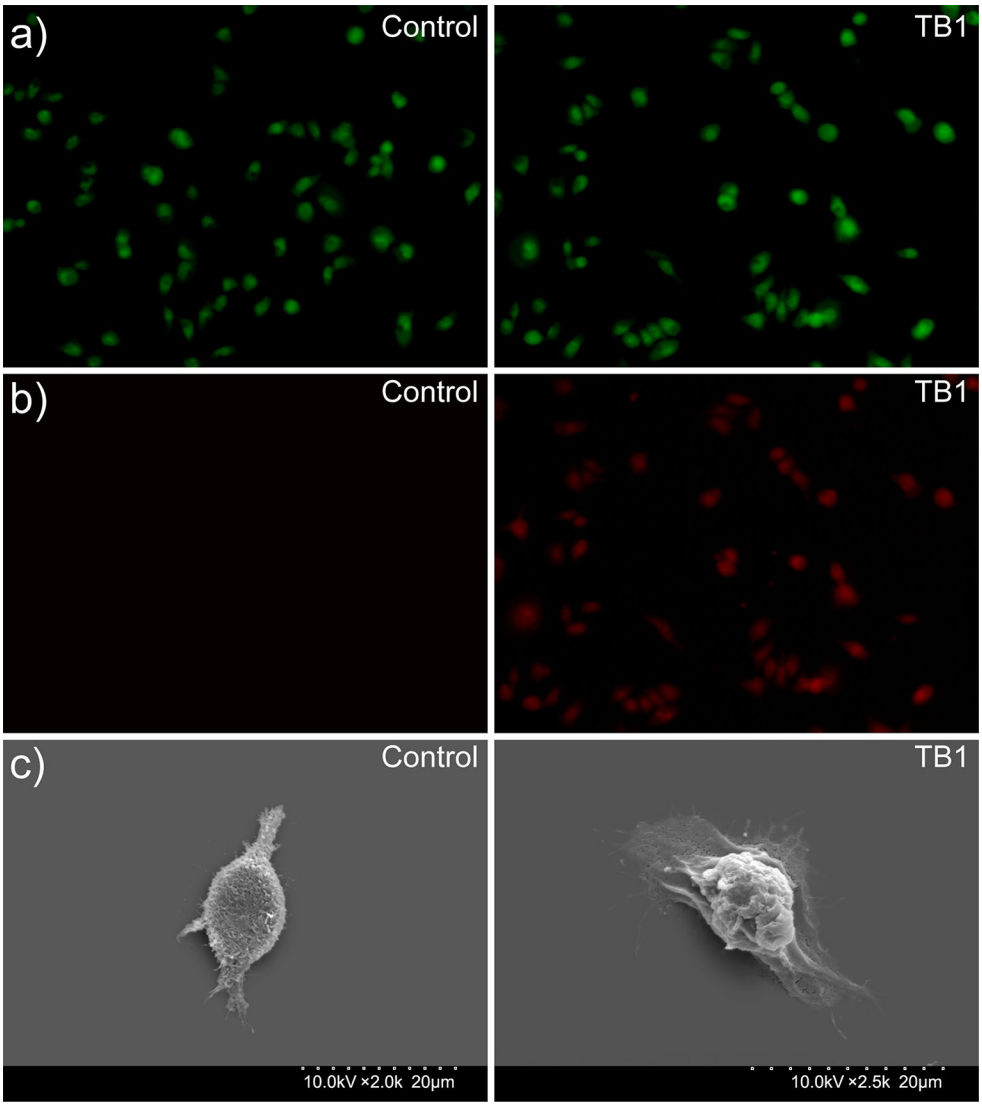

Fig. 2 **a–c** AO/EB staining and SEM images of DU145 cells after treatment with TB1

cell membranes (Janaky et al. 1992). Thus, we synthesized a series of LHRH-conjugated peptides by attaching an AMP sequence with LHRH as well as modified LHRH sequences, which were utilized to target and destroy cancer cells that possess LHRH receptor. In vitro anti-proliferation assay revealed that addition of LHRH sequences lowered the IC_{50} value of the peptides against the cancer cells which express LHRH receptor. Meanwhile, the peptides present minimal cytotoxic effects on the non-malignant cells such as GES-1 and HEK-293. These peptides also had little hemolytic activity against the rRBCs. Therefore, the conjugation of LHRH sequences increased the anti-cancer activity of B1 against the LHRH receptor-positive cancer cells while maintained the selectivity of B1.

The degradation of native LHRH can be easily occurred at glycine residue in position 6, which makes it a short half-life of 2–5 min (Rick et al. 2013). While triptorelin, a LHRH analog with D-tryptophan substitution in position 6, has longer plasma half-life than LHRH with an elimination half-life superior to 80 min (Ezan et al. 1986). Therefore, it might somehow explain that TB1, the conjugated peptide composed of B1 and triptorelin, presents favorable metabolic stability over others. Meanwhile, the results of LHRH receptor blocking assay showed that triptorelin or cetrorelix will reduce the anti-proliferation activity of TB1 against the LHRH receptor-positive cancer cells. This finding implied that the peptide without conjugating LHRH showed substantial activity and selectivity for the cancer cells, while the LHRH sequence enhances their anti-cancer effect on the cancer cells containing LHRH receptor.

The Annexin-FITC/PI staining proved that TB1 presents apoptosis-promoting effect on cancer cells. Thus, further

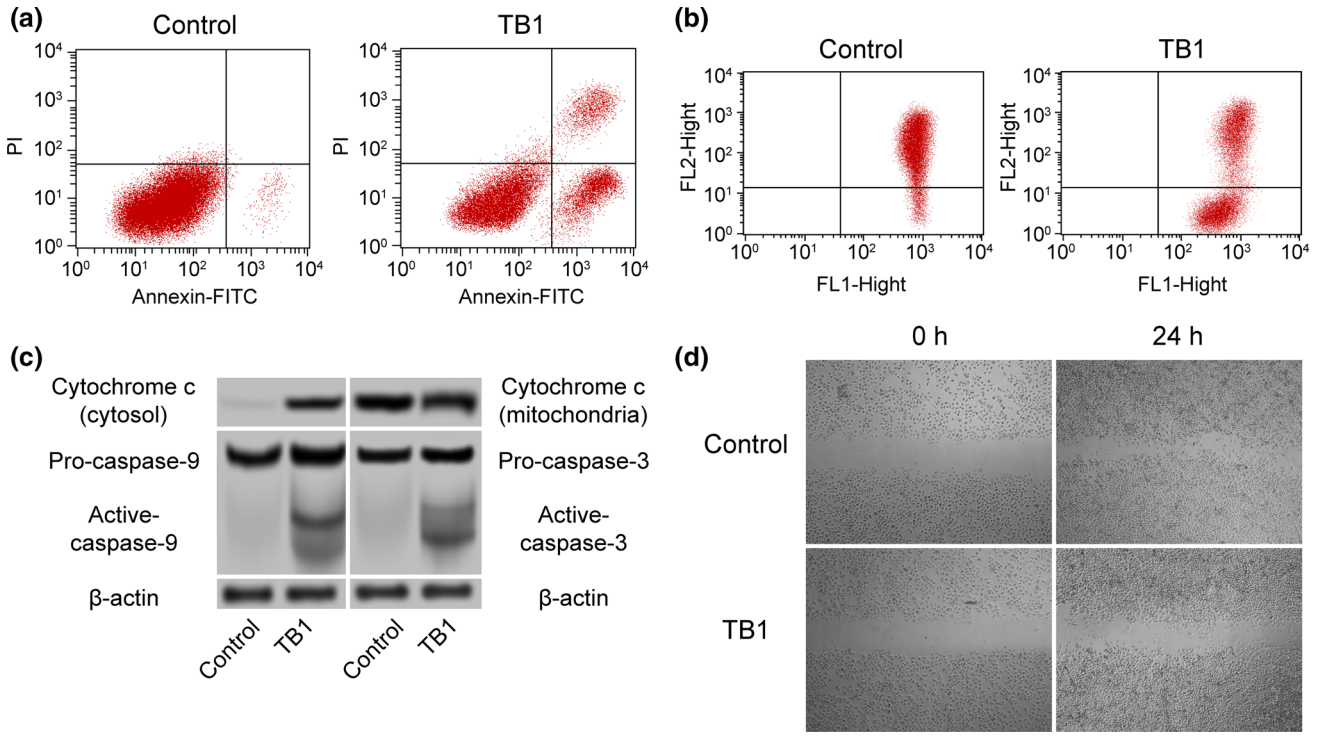

Fig. 3 **a** The effect of TB1 on DU145 cell apoptosis using Annexin-FITC/PI staining; **b** the effect of TB1 on $\Delta\Psi_m$ of DU145 cells; **c** the western blot analysis of cytochrome c, caspase-9 and caspase-3 for DU145 cells; **d** the anti-migratory assay of TB1

investigation was carried out to clarify the possible anti-cancer mechanism of TB1. The results from AO/EB double staining test suggested that TB1 presents membrane-disrupting activity against cancer cells. Moreover, the SEM visualization of cell morphology also corroborated this suggestion, as the images showed impaired cell membrane of DU145 after treatment with TB1.

It is reported that the release of cytochrome c from mitochondria can stimulate cell apoptosis by activating pro-caspase-9, an initiator factor in caspase apoptosis pathway. The active caspase-9 in turn cleaves and activates caspase proteases in downstream, such as caspase-3 (Goldstein et al. 2000; Li et al. 1997). According to the JC-1 assay and western blot analysis, we proved that TB1 can promote cell apoptosis by initiating mitochondria–cytochrome c–caspase apoptotic pathway.

Besides, the wound-healing assay was applied to appraise the anti-migratory effects of TB1 at maximal non-toxic concentrations and verified the inhibitory effects of migration of TB1 on DU145 cells.

In conclusion, we designed and synthesized a series of anti-cancer peptides by conjugating LHRH sequences to the AMP, B1. All the LHRH-conjugated peptides showed significantly enhanced anti-cancer effects on cancer cell lines with LHRH receptor. Moreover, these peptides present less toxic to the benign cells as well as little hemolytic activity for rRBCs. Further investigation revealed that TB1, the peptide containing triptorelin sequence, exhibits favorable plasma stability over other peptides. Therefore, we suggest that TB1 might be a promising candidate for cancer therapy.

Acknowledgments The work was supported by the National Natural Science Foundation of China (No. 81172932 and 81273376), the Natural Science Foundation of Jiangsu Province (No. BK2012356).

Conflict of interest The authors have no conflicts of interest to declare.

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