Journal of Steroid Biochemistry and Molecular Biology 200 (2020) 105651

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Breast cancer targeted/ therapeutic with double and triple fusion Immunotoxins

Zoleikha Goleij[a], Hamideh Mahmoodzadeh Hosseini[a], Hamid Sedighian[a], Elham Behzadi[b], Raheleh Halabian[a], Rahim Sorouri[a], Abbas Ali Imani Fooladi[a,*]

[a] Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
[b] Department of Microbiology, College of Basic Sciences, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran

ARTICLE INFO

Keywords:
PE38
Herceptin
HER2
Breast cancer
Apoptosis

ABSTRACT

Target-specific transport of therapeutic agents holds promise to increase the efficacy of cancer treatment by decreasing injury to normal tissues and post treatment problems. HER2 is a tumor cell surface marker that is expressed in 25–30 % of breast cancer patients. The significant role of HER2 in cancer development and its biological feature makes it a highly appealing goal for the therapeutic treatment of cancer targeted therapy using HER2 monoclonal antibody. This approach is currently used as a special treatment against breast cancer in some research. In the present study, HER2 monoclonal antibody (mAb), (Herceptin) fused to PE38 by recombinant DNA technology and a new recombinant IT was developed. The scFv(Herceptin)-PE-STXA and scFv(Herceptin)-PE fusions cloned in pET28a and recombinant protein expression was carried out and then the proteins were purified. MCF-7 and SKBR-3 cells were used as HER2-negative and HER2-positive breast cancer cells, respectively. The cytotoxicity of its evaluated using MTT assay. The cell ELISA was used to determine the binding ability of immunotoxins (ITs) to the cell receptor and internalization and apoptosis were also assessed. The results revealed that cell cytotoxicity occurred in SKBR-3 cells in a dose-dependent manner but not in MCF-7 cells. It is possible that this ITs can attach to HER2-positive breast cancer cells and then, internalize and eradicate cancer cells by apoptosis. Here, we concluded that the recombinant ITs have therapeutic potential against HER2-positive breast cancer.

1. Introduction

Despite major progresses in cancer therapy research, breast cancer is still the most commonly recognized cancer and one of the chief causes of mortality in females, worldwide. One of the major problems in breast cancer treatment is the heterogeneous nature of the disease which consist of at least five subtypes. The evidences show that 25–30 % of breast cancer cases are human epidermal growth factor receptor 2 (HER2)positive, which in comparison with other breast cancer subtypes that are associated with reduced disease-free survival, tend to be more aggressive.The HER2 (also known as ERBB2) belongs to a class of proteins involved in signal transduction pathways and is a member of the human epidermal growth factor receptor family with intracellular domains that have tyrosine kinase activity. These receptors are associated with the morphogenesis, normal regulation of cell growth and differentiation of cells. Generally, HER2 or ERBB2 is expressed in epithelial cells [1]. The significant role of HER2 in cancer development and its biological features make it a highly appealing goal for the therapeutic treatment of the cancer. HER2 targeted therapies including HER2 monoclonal antibody (mAb), (Herceptin) have considerably improved the consequences in HER2-positive cancers; but the consumption of these agents can be restricted due to the tolerability and the development of resistance in cancer patients. Hence, there is a need for new and advanced therapeutic methods targeting HER2 [2]. Immunotoxins (ITs) are a class of the targeted therapies that are composed of two functional proteins, a mAb and a toxin that are covalently attached. The targeting moieties identify and deliver the complete molecule to the specific receptors on the surface of the target cells. After internalization, the toxin initiates signaling cascades leading to the cell death, either by entering the cytosol and catalytically deactivating fundamental cell processes or by changing the tumor cell membrane properties, permitting the selective removal of the cells that express the antigens identified by the mAbs [3,4].

In general, ITs have developed over time and by significant

* Corresponding author at: Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Vanak Sq. Molasadra St., P.O. Box 19395-5487, Tehran, Iran.
E-mail address: imanifouladi.a@gmail.com (A.A. Imani Fooladi).

https://doi.org/10.1016/j.jsbmb.2020.105651
Received 1 June 2019; Received in revised form 29 January 2020; Accepted 4 March 2020
Available online 05 March 2020

advances in vaccine technology can be separated into three generations. First generation ITs were produced by chemical binding of the complete toxins to the antibodies, second generation ITs were produced by chemical conjugation of altered toxins lacking receptor binding domains and third-generation ITs were prepared by recombinant DNA methods under a group called recombinant immunotoxins (RITs) [5,6]. One of the toxins that are usually used in IT structure is *pseudomonas exotoxin* (PE). The crystallography of the native PE revealed that it is composed of three separate structural regions recognized as domain I (subdivided into discontinuous domains Ia, residues 1–252 and Ib, residues 365–404), domain II (residues 253–364) and domain III (residues 405–613) [7]. Subsequent interaction between domain I and _2-macroglobulin receptor/low-density lipoprotein receptor-related protein 1 (LRP1), PE enters the cells via receptor-mediated endocytosis. The internalized toxin transfers through the cell into the endocytic vesicles and undergoes numerous processing steps before passing endoplasmic reticulum (ER) membrane into the cytosol [8]. Domain II of PE plays an important role in the translocation of enzymatic domain III into the cytosol [9]. Domain III inhibits protein synthesis by ADP-ribosylation and inactivation of elongation factor 2 which leads to cell death [10].

In the PE-based ITs, domain Ia of toxin is replaced by the variable fragment (Fv) of an antibody. In addition, residues 365–384 of domain Ib is eliminated. This replacement, changes the property of the toxin and identifies target antigens for immune-based therapy in cancer patients. In this study, the catalytic domain of the shiga toxin was also used to design the immunotoxin. Shiga toxin (STX) is extensively employed for immunotoxin construction. This toxin is produced by *Shigella dysenteriae* and certain strains of *Escherichia coli*. STX is a member of the AB5 toxin family composed of two non-covalently attached subunits: the A subunit (STXA) consists of an enzymatically active (A1) fragment and the subunit B (STXB) is a non-toxic, pentameric binding (B) subunit. STX exactly binds to the glycosphingolipid globotriaosylceramide (Gb3) at the surface of target cells and subsequently is internalized by endocytosis. In the target cells, the STX/Gb3 complex is transported in the retrograde direction via the Golgi apparatus to the endoplasmic reticulum (ER), then, the active fragment of STX is translocated into the cytosol that inhibits the synthesis of protein by the alteration of ribosomal 28S RNA [11]. In a study conducted by Habibi et al. a chimeric protein constructed by fusing Shiga toxin tothe granulocyte macrophage colony stimulating factor (GM-CSF) to induce colon cancer cell line to express GM-CSF receptors (GM-CSFRs). This chimeric protein had selective cytotoxic effect on the colon cancer cells expressing GM-CSFRs and also caused apoptosis in this cell lines [12]. In another study, the antitumor activity of Shiga-like toxin (SLT)–vascular endothelial growth factor (VEGF) fusion protein on pancreatic cancer that overexpressed VEGFR-2, was evaluated. VEGF can conduct toxins to tumor vessels by VEGFR-2 for antiangiogenic therapy. SLT-VEGF did not effect proliferation of pancreatic cancer cells, human umbilical vein endothelial cells (HUVECs (low-level VEGFR-2)) decreased their proliferation level and the tube formation but not their viability. These fusion protein decreased tumor growth and propagation and improved survival rate [13].

In the current study, the scFv (Herceptin)-PE IT was produced by combining the single-chain variable fragment (scFv) of Herceptin monoclonal antibody with PE38(38-kDa truncated form of PE) [8] and the scFv(Herceptin)-PE-STXA IT was constructed by elimination of domain III of PE and replacing with STXA using recombinant DNA technology. STXA was transported across the cell membrane using domain II of PE. The catalytic domain of exotoxin A from *Pseudomonas aeruginosa* has been revealed to facilitate the internalization of extracellular compounds into the cytosol and can translocate heterologous molecules into the target cells [14]. Therefore, PE II and STX are linked to fragment variable (Fv) domains of Herceptin and construct a (single chain) scFv immunotoxin [15].

Bioiformatics is the branch of science that utilizing different methods for solving of biological problem especially on molecular levels [16]. In our previous bioinformatics studies, related methods were used to analyze DNA/protein sequences and to predict secondary and tertiary structures of protein molecules, protein– protein interactions and etc. on these two chimeric proteins [17].

2. Materials and methods

2.1. Cell lines and reagents

Breast adenocarcinoma cell line SKBR-3 (RRID: CVCL-0033, lot number cl0682, passage number p + 13) and breast adenocarcinoma cell line MCF-7 (RRID: CVCL-0031, lot number cl0147, passage number p + 12) cell lines were purchased from Irish Bank Resolution Corporation (IBRC) of Iran (Tehran, Iran) on 12. October 2016. These cells were negative for *mycoplasma* infection (using PCR and direct culture).

Restriction enzyme was obtained from FermentasThermo Fisher Scientific (Massachusetts, USA). Isopropyl-beta-D-thiogalactopyranoside (IPTG) and protein marker/ladder were provided by ThermoFisher Scientific (Bremen, Germany). Kanamycin supplied by Sigma-Aldrich (Missouri, USA). The poly (vinylidene fluoride) (PVDF) membrane and diaminobenzidine (DAB) were purchased from Amersham (London, UK) and Sigma-Aldrich (Missouri, USA), respectively. Nickel-nitrilotriacetic acid (Ni-NTA) column and plasmid extraction kit were purchased from Qiagen GmbH (Hilden, Germany), Metabion (Munich, Germany) and Bioneer (Daejeon, South Korea), respectively. Dithiothreitol (DTT) was obtained from Merck (Darmstadt,Germany). FBS was obtained from Gibco (Paisley, Scotland), RPMI 1640 medium, penicillin, streptomycin, trypan blue (TB) and dimethylthiazoldiphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (MO, USA) and were at least of analytical grade. *in situ* cell death detection kit (Annexin-V FITC) was purchased from IQ products (Groningen, The Netherlands). Flasks, tubes and culture plates were obtained from Greiner Bio-One (Frickenhausen, Germany). All solutions were prepared in deionized double distilled water.

2.2. Design and construction of chimeric genes

scFv-(Herceptin)-PE and scFv(Herceptin)-PE-STXA chimeric constructs were generated by fusing toxins with single chain fragment variable of Herceptin using a linker. For this purpose, PE38 was fused with VL-VH of Herceptin, by ASGGPE linker and PE-STXA was connected to VL-VH of Herceptin by ASGGPE linker (Fig. 1). These chimeric constructs were synthesized by Biomatik company (Ontario, Canada).

2.3. Transformation and expression of recombinant protein

Recombinant *pET28a-* scFv(Herceptin)-PE and *pET28a-* scFv (Herceptin)-PE-STXA vectors were digested by *BamH*I and *Xho*I restriction enzymes according to manufacturer's instruction. Then, the constructs were transferred into *E. coli* BL21(DE3) Pasteur Institute of Iran (Tehran, Iran)) as expression host. Transformed bacteria were cultured in 5 ml LB (Luria-Bertani) medium containing 20 mg/mL kanamycin and incubated at 37 ˚C overnight. When OD600 reached 0.6–0.8, the bacterial cultures were induced by 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG).

Subsequently, the cultures were incubated again at 37 ˚C overnight. Then, the expression of scFv(Herceptin)-PE and scFv(Herceptin)-PE-STXA were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE). The different concentrations of IPTG (0.5, 0.6, 0.7,0.8, 0.9, and 1 mM) with various time durations (2, 5, 7, 16 h) were examined to optimize protein expression.

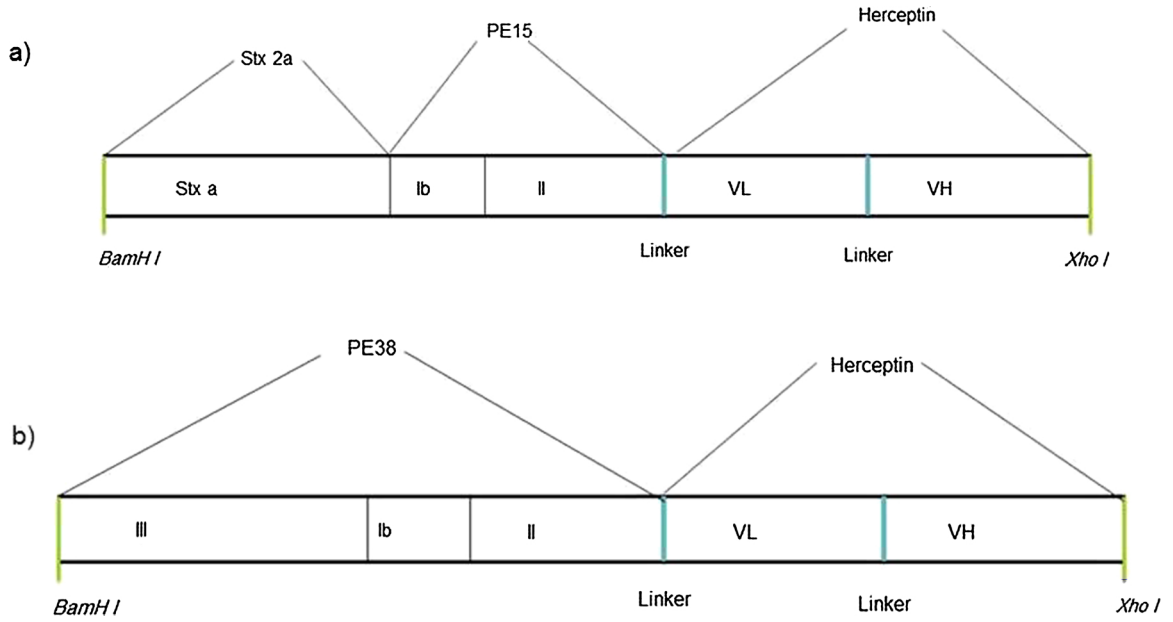

Fig. 1. Schematic Model of Fusion Proteins.
A. This model displays the construction of PE and scFv(Herceptin) bound together with the ASGGPE linker, VL and VH bound together with GGSGSGGG linker.
B. The construction of scFv(Herceptin) and PE-STXA bound together by the ASGGPE linker, also VL and VH connected with the GGSGSGGG linker.

2.4. Isolation and purification of chimeric recombinant proteins

For isolating the recombinant proteins, the bacteria were gathered 16 h after IPTG treatment. The pellets were washed twice with phosphate-buffered saline (PBS), pH 7.4. The bacterial pellets were (4 g) resuspended in 20 ml of lysis buffer (50 mMTris－HCl containing 1 mM EDTA, 5 mM DTT) and the cells were disrupted by sonication five times for 30 s at 200 W. The resulting solutionswere centrifuged at 6000 × g at 4 ˚C for 30 min. The supernatant was discarded and the pellets were washed twice with washing buffer (1 M urea in 50 mMTris－HCl, pH 8.0, containing 5 mM EDTA and 1 mM DTT), then centrifuged at 11000 × g at 25 ˚C for 15 min.To extract proteins from washed inclusion bodies, urea buffer (0.01 M Tris/base,0.1 M NaH_2PO_4, 8 M Urea, pH:8) was used. After incubation at room temperature for 16 h, the washed pellets were centrifuged for 20 min and the supernatant was analyzed by SDS/PAGE. Subsequently, the supernatant that contained recombinant fusion proteins was loaded onto Ni-NTA affinity column. The column washed with washing buffer (50 mm NaH_2PO_4, 20 mm imidazol, 300 mm NaCl, pH 8) and unbound proteins were removed. Recombinant proteins attached to the column were eluted with elution buffer (50 mM NaH_2PO_4, 250 mM imidazol, 300 mM NaCl pH 8).

2.5. Western blotting of recombinant proteins

The expressed recombinant proteins were analyzed by western blotting. Recombinant proteins were solubilized in reducing sample buffer. The SDS-PAGE was carried out using 12.5 % gels at a constant voltage of 100 V for 1–2 h.The gel was blotted onto nitrocellulose membrane.

The membrane was blocked with 5% skim milk in 0.1 mol/L PBS containing 0.05 % Tween 20 (PBS-T) at 4˚Ċ for 16 h. Then, the membrane was incubated with horseradish peroxidase (HRP)-conjugated mice anti-His-tag antibody (diluted 1:2000 in PBS-T) at room temperature for 1 h. After washing five times with PBS-T, the membrane was stained using (0.5 mg/ml 3,30-diaminobenzidine (DAB), 0.1 % H_2O_2).

2.6. Analysis of the cytotoxicity of e recombinant proteins

The MTT assay was used to determine the cytotoxicity of recombinant proteins. SKBR-3, the human breast adenocarcinoma cell line that overexpresses the antigen HER2 (4^+) and MCF-7, the human breast adenocarcinoma cell line that does not overexpress the antigen HER2 (1^+) [18] and HEK-293(human embryonic kidney 293) were cultured in 96-well plates at 1×10^4 cells/well in RPMI 1640 (supplemented with 10 % fetal bovine serum (FBS), 100 U/ml of penicillin and 100 µg/ml of streptomycin), incubated at 37 ˚C in a humidified atmosphere of 95 % air and 5% CO_2. After 24 h, the cells were treated with different concentrations of recombinant Its (0.1, 1, 10, 25, 50, 75 and 100 µg/ml) for 48 h. Each assay was carried out in triplicate. After the exposure time has elapsed, the medium was exchanged with fresh medium consisting of 5 mg/ml MTT and the cells were incubated at 37 ˚C for 4 h. The supernatant of each well was removed and the created formazan crystals were dissolved in 100 µl DMSO (Dimethyl sulfoxide) and the absorbance was measured at 570 nm by a microplate reader (Bio-Rad, Hercules, CA, USA) [19].

2.7. Binding affinity of recombinant proteins to the receptors

The antigen binding ability of purified fusion proteins was evaluated by an ELISA-based binding assay. SKBR-3 and MCF-7cells were seeded in a 96-well culture plates at a density of 1.5 × 10 [4] cells/well, separately. After 24 h, the culture media were discarded and cells were washed with PBS. In the next step, the cells were fixed with 10 % paraformaldehyde and blocked with 3% bovine serum albumin (BSA), which subsequently, were treated with various concentrations of purified recombinant proteins (1, 10, 25, 50 and 75 µg/ml) for 1 h. Then, cells were washed 5 times with PBS-T and afterward, incubated with anti-hexa histidine HRP-conjugated antibody (1:2000) at room temperature for 1.5 h. Ultimately, tetramethylbenzidine (TMB) solution with 1.5 % H_2O_2 was added to each well and the color was developed after adding stop solution (H_2SO_4,). The optical density was measured at 450 nm using a ELISA plate reader [20].

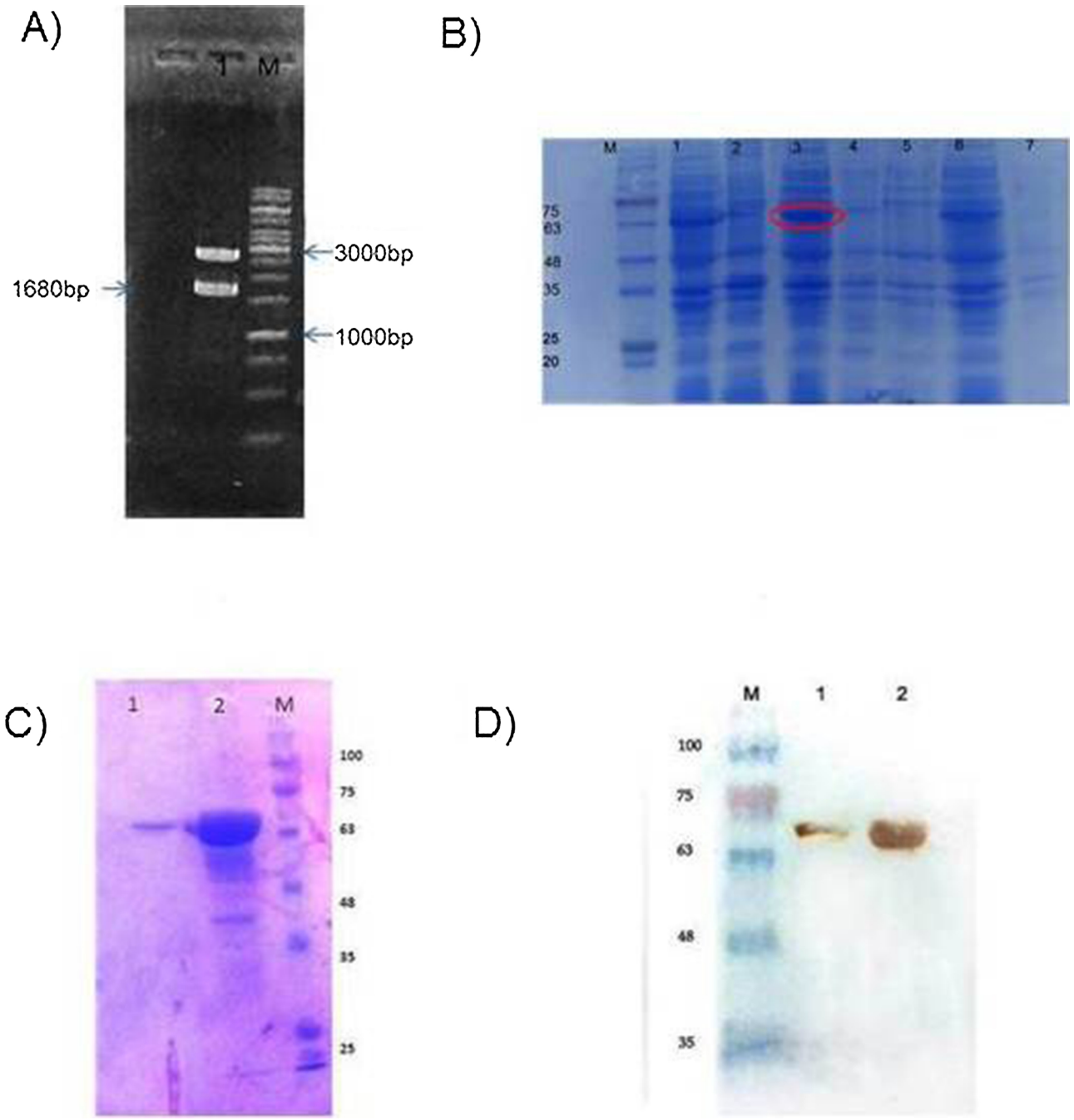

Fig. 2. A) Double digestion of scFv(Herceptin)-PE-STXA–PET-28 by BamHI & XhoI. Lane 1: scFv(Herceptin)-STXA fusion gene. M: 1 kb DNA ladder. B) Optimization of scFv(Herceptin)-PE-STXA expression was done at different concentrations of IPTG. Lane 1: The supernatant of bacteria was induced at a concentration of 1 mmol/L IPTG at 37 ˚C, Lane 2: The supernatant of bacteria was induced at a concentration of 0.9 mmol/L IPTG at 37 ˚C, Lane 3: The supernatant of bacteria was induced at a concentration of 0.8 mmol/L IPTG at 37 ˚C, Lane 4: The supernatant of bacteria was induced at a concentration of 0.7 mmol/L IPTG at 37 ˚C, Lane 5: The supernatant of bacteria was induced at a concentration of 0.6 mmol/L IPTG at 37 ˚C, Lane 6: The supernatant of bacteria was induced at a concentration of 0.5 mmol/L IPTG at 37 ˚C, Lane 7: The supernatant of bacteria remained uninduced.
C) Purification of scFv(Herceptin)-PE-STXA fusion protein. Lane 1: The purified protein of scFv(Herceptin)-PE-STXA by Ni-NTA column; Lane 2: Unpurified protein of scFv(Herceptin)-PE-STXA; M: protein marker. D) Characterization of scFv(Herceptin)-PE-STXA fusion protein by western blot analysis. Lane M: protein marker; Lane 1: The purified scFv(Herceptin)-PE-STXA fusion protein; Lane 3: Unpurified scFv(Herceptin)-STXA fusion protein.

2.8. Internalization assay of recombinant proteins

The ability of the recombinant proteins to be internalized by cells was assessed in HER2-overexpressing SKBR-3 and HER2–negative MCF-7 cells. For this purpose, 3 × 10 [5] cells were cultured in 6-well plates and incubated at 37 ˚C in a humidified atmosphere of 95 % air and 5% CO_2. After 24 h, the culture medium was replaced with 1 ml fresh RPMI 1640 medium, containing 25 and 50 μg/ml recombinant proteins and the plates were incubated at 37 ˚C for 2 h. In the next step, the cell culture supernatant was removed and the cells were washed twice with PBS, trypsinized at 37 ˚C for 5 min and then were collected. Harvested cells were lysed with RIPA buffer (25 mM Tris−HCl, 140 mM NaCl, 2 mM EDTA, 0.1 ml Triton X 100 0.5 M, sodium deoxycholate [pH 7.4]) The cell lysate was run on a SDS-PAGE gel followed by western blot

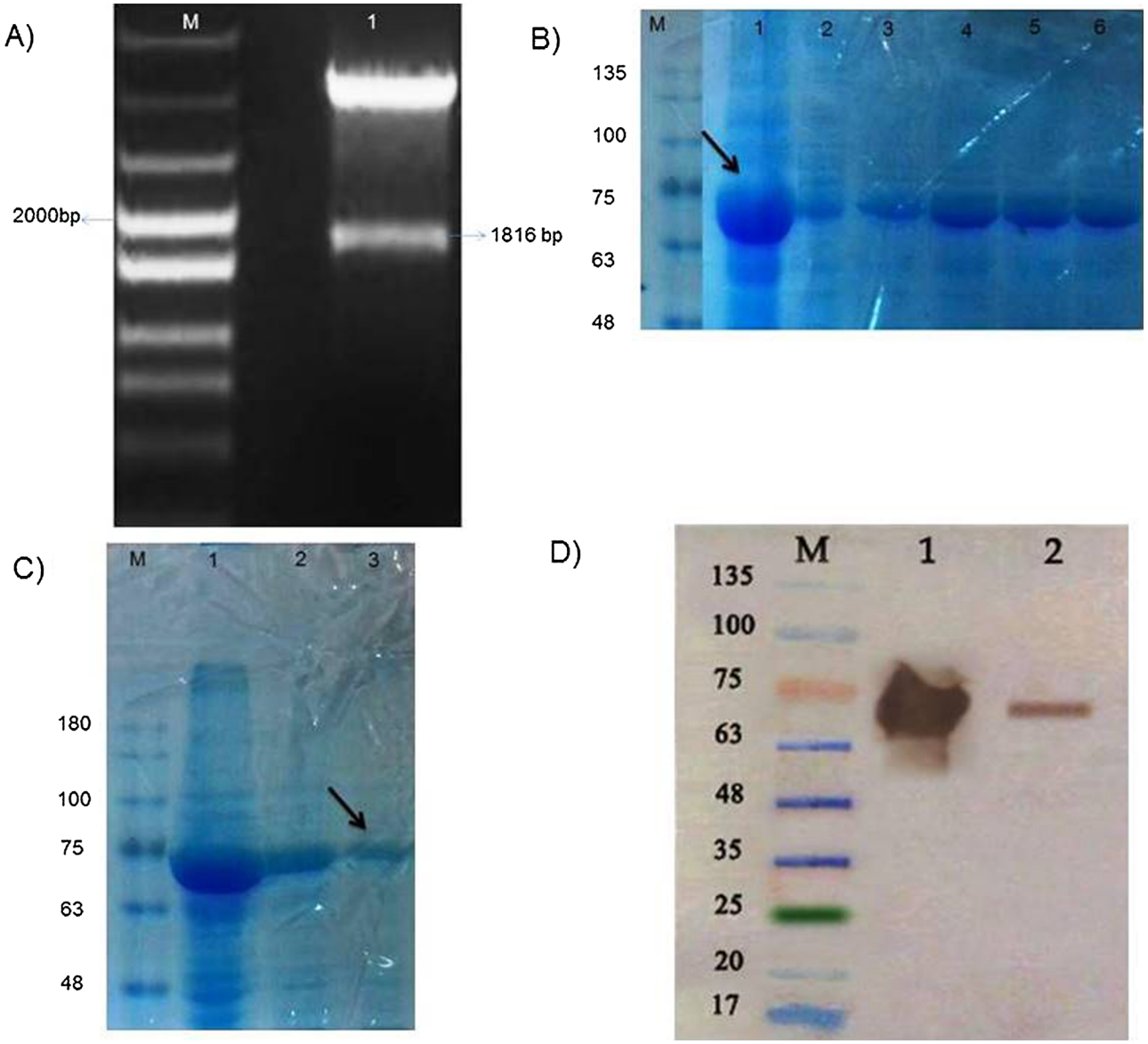

Fig. 3. A, The study of expression plasmid (PET-28a- scFv(Herceptin)-PE) using restriction enzyme. The plasmid was digested with *Bam H*-I and *Xho*-I and the 1816-bp fragment was achieved. Lane: (M), 1 kbDNA ladder. 1: scFv(Herceptin)-PE fusion gene. B, IPTG optimization of recombinant scFv(Herceptin)-PE: Expression at the concentrations of 1, 0.9, 0.8, 0.7, 0.6 and 0.5 mM IPTG (lanes 1-6) M; Protein size marker. C, Protein purification of scFv(Herceptin)-PE fusion protein. Induction was performed by 1 mM IPTG at 37 °C. Lane 1: Before purification. Lane 2: Flow. Lanes 3: Purified protein (70 kDa) eluted in buffer containing 250 mM imidazole. M: protein ladder. D, Western blot analysis of scFv(Herceptin)-PE using anti-6X-His-tag antibodies. M; Protein weight marker. Lane 1: unpurified scFv(Herceptin)-PE protein before column. Lane 2: purified scFv(Herceptin)-PE protein.

analysis of the cellular protein extracts [21].

2.9. Apoptosis assay

For flow cytometry analysis, annexin V-FITC apoptosis detection kit was used. SKBR-3 and MCF-7 cells were seeded (5×10^5 cells/well) in a 6-well plate and incubated at 37 °C in a humidified atmosphere of 95 % air and 5% CO_2. After 24 h of incubation, cells were treated with various concentrations of recombinant proteins (10, 25 and 50 μg/ml) for 48 h. After 48 h, the culture medium was removed and the treated cells were trypsinized at 37 °C for 5 min followed by washing with PBS. The collected cells resuspended in 1 ml of annexin V-binding buffer and then, 5 μl annexin V-coupled FITC and propidium iodide was added to 1 $\times$ 10^5 cells. The samples were mixed slightly, incubated at room temperature, in the dark, for 15 min and then, analyzed by flow cytometry for apoptosis [22].

2.10. Statistitical analysis

All experiments were performed in triplicates, repeated three times and the results were reported as the mean ± standard deviation (SD). Mean difference among groups was calculated by one- and two-way variance analysis (one- and two-way ANOVA). A p value of <0.05 was considered statistically significant.

3. Results

3.1. Transformation and expression of recombinant proteins

Recombinant protein constructs were synthesized and subcloned into *pET28a* vector by Biomatik company (Ontario, Canada). After double digestion with *BamH*I and *Xho*I restriction enzymes, the 1683-pb and 1816-bp fragments were observed on agarose gel electrophoresis

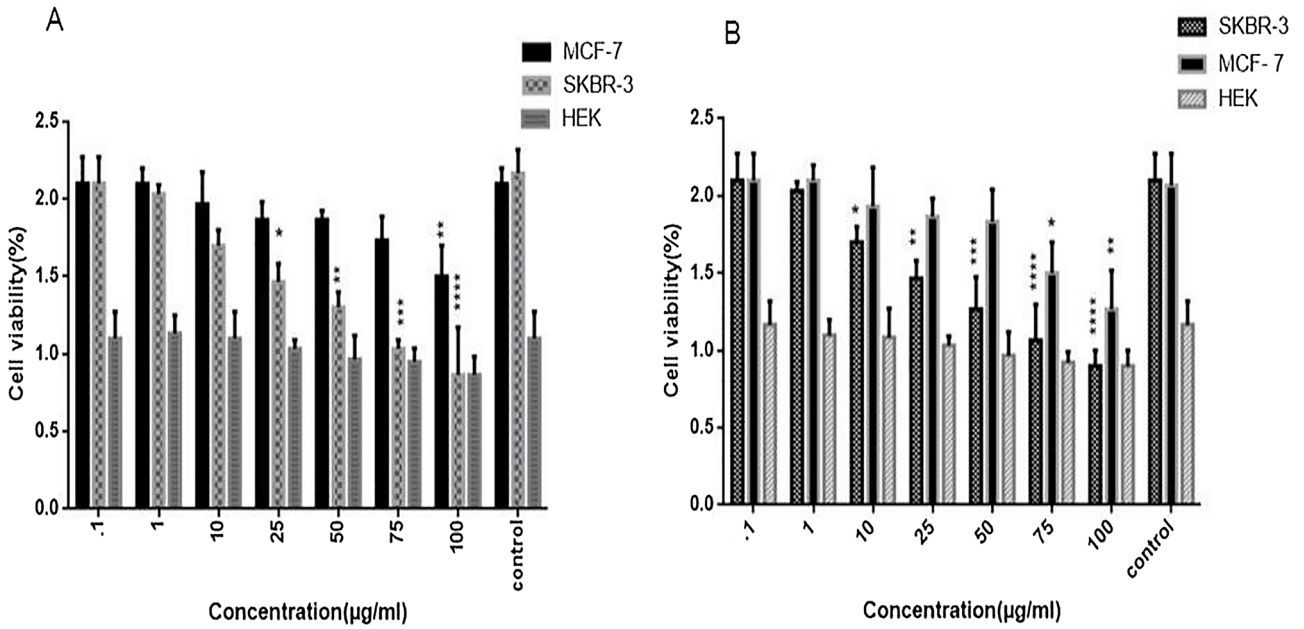

Fig. 4. scFv(Herceptin)-PE Inhibited Cell Growth in Breast Cancer Cell Lines.
A, SKBR-3 and MCF-7 cells were treated with different concentrations of scFv(Herceptin)-PE-STXA. B, cells were treated with different concentrations of scFv (Herceptin)-PE for 48 h.
The amount of viable cells was determined by MTT assay. The inhibition of cell-growth happened in a dose-dependent manner in SKBR-3 cell line. But chimeric proteins do not inhibit cell growth in MCF-7 cells.
The data represent mean ± SD. Statistical differences between treated and untreated control groups were analyzed by ANOVA. Significance levels are; *P < 0.05; **P < 0.01; **** P < 0.0001.

for scFv(Herceptin)-PE-STXA and scFv(Herceptin)-PE chimeric genes, respectivly (Figs. 2A and 3 A). Then, two constructs were transferred into BL21 (DE3) expression system. After protein expression, the optimized concentration of IPTG were 0.8 mM for scFv(Herceptin)-PE-STXA and 1 mM for scFv(Herceptin)-PE (Figs. 2B and 3 B).

3.2. Purification and confirmation

Following the induction of expression, two fusion proteins were purified using Ni-NTA agarose resin (Figs. 2C and 3 C). SDS-PAGE analysis revealed scFv(Herceptin)-PE-STX as a major band (~66 kDa) in the purified fractions. The molecular mass of recombinant scFv (Herceptin)-PE protein was estimated approximately 70 kDa. These proteins were confirmed by western blot analysis (Figs. 2D and 3 D).

3.3. In vitro cytotoxic effects of recombinant proteins

The cytotoxic effect of recombinant proteins was evaluated by the MTT assay using SKBR-3 and MCF-7 cells. After 48 h of incubation with scFv(Herceptin)-PE-STX recombinant protein, the viability of SKBR-3 cells were decreased in a dose-dependent manner to approximately 15 %, 27.3 %, 36 %, 46 % and 54 % at the concentrations of 10, 25, 50, 75 and 100 µg/ml, respectively ($p < 0.001$) (Fig. 4A). Similarly, scFv (Herceptin)-PE protein reduced the proliferation of SKBR-3 cells to 22.7 %, 33.3 %, 40 %, 52 % and 60 % at the concentrations of 10, 25, 50, 75 and 100 µg/ml, respectively ($p < 0.001$) (Fig. 4B). No cytotoxicity effect of these two chimeric proteins was observed in the MCF-7 cells compared to SKBR-3 cells (except at the concentrations of 75 and 100 µg/ml). Moreover, these treatments were performed on HEK-293 cells and there was no significant difference between treated and untreated cells. The IC50 (median inhibition concentration) values of scFv(Herceptin)-PE-STX and scFv(Herceptin)-PE were determined to be 21.25 and 19.98 µM, respectivly. Comparing the results of two chimeric proteins showed that the scFv(Herceptin)-PE chimeric protein is more effective and have more cytotoxicity for SKBR-3 cells.

3.4. Cell ELISA binding of chimeric proteins

Cell ELISA was used to examine the binding affinity of scFv (Herceptin)-PE-STX and scFv(Herceptin)-PE proteins. For this purpose, SKBR-3 and MCF-7 cells were cultured in 96-well plates. After 24 h, the culture medium was replaced with different concentrations of 6His-tagged chimeric proteins (1, 10, 25, 50, 75 µg/ml). These fusion proteins bound to SKBR-3 HER2-expressing cells with a high affinity and the binding affinity of these cells were 30 %, 45 %, 80 % and 90 % for the scFv(Herceptin)-PE chimeric protein at the concentrations of 10, 25, 50 and 75 µg/ml, respectively ($p < 0.001$) whereas, the binding affinity of scFv(Herceptin)-PE fusion protein to MCF-7 cells was low (Fig. 5A). The specific binding of the scFv(Herceptin)-PE-STXA protein to HER2/neu-positive cells at the concentrations of 1, 10, 25, 50, 75 µg/ml scFv (Herceptin)-PE-STX protein was 25 %, 45 %, 80 % and 90 %, respectively (Fig. 5B). Two chimeric proteins in the same concentration had the same binding affinity to the receptors.

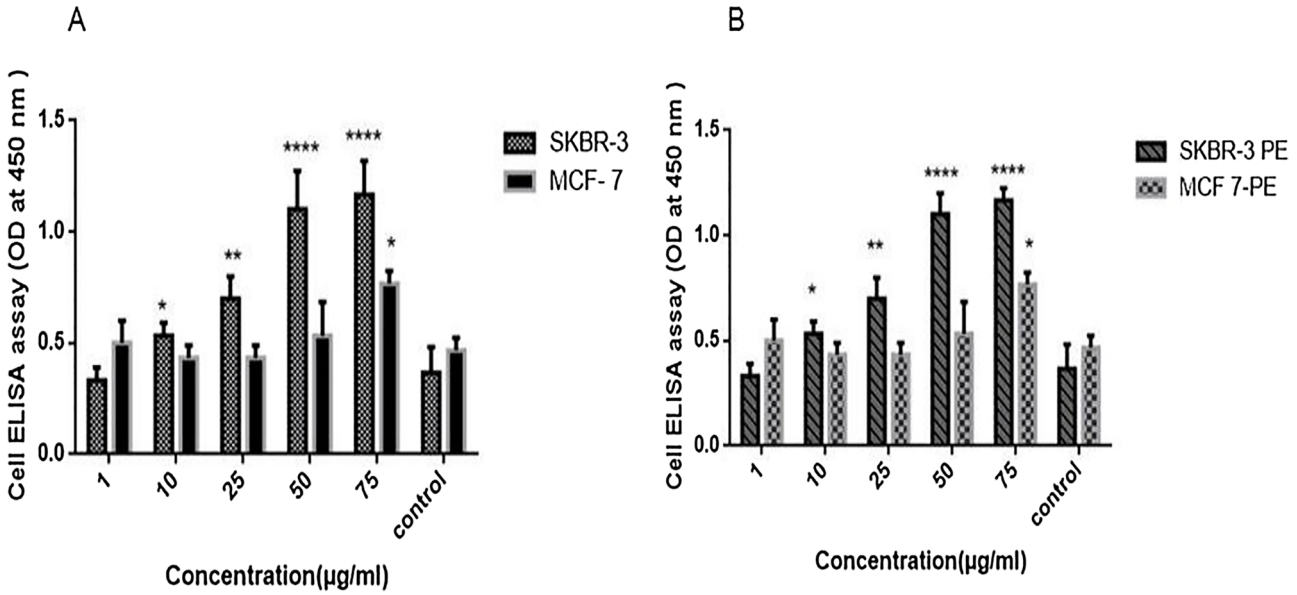

Fig. 5. Binding of Fusion Proteins to HER2 by Cell ELISA.
A, SKBR-3 and MCF-7 cells were treated with different concentrations of scFv(Herceptin)-PE-STXA. B, cells were treated with different concentrations of scFv (Herceptin)-PE. Cells were seeded in a 96-well plate. After fixation with 10 % neutral formaldehyde at room temperature for 1 h, various concentrations (1 − 75 µg/mL) of fusion proteins were added and the binding was evaluated using cell ELISA. The results indicated that scFv(Herceptin)-PE-STXA and scFv(Herceptin)-PE fusion proteins bind to HER2-expressing cells (SKBR-3) with a significant affinity ($P < 0.0001$) whereas, these chimeric proteins bind to MCF-7 with low affinity, the binding affinity was compared to the PBS as the negative control. The data represent mean ± SD. Statistical differences between treated and untreated control groups were analyzed by one-way ANOVA. Significance levels are $^{*}P < 0.05$; $^{**}P < 0.01$;**** ($P < 0.0001$).

3.5. Internalization assay

The ability of the chimeric proteins to be internalized by cells were assessed in HER2-overexpressing SKBR-3 cells and inHER2–negative MCF-7 cells. After exposure of cells to the chimeric proteins, the cells were collected and lysed by RIPA buffer, then, the lysates were analyzed by SDS/PAGE and western blot. As shown in Fig. 6, this chimeric protein was uptaked by SKBR-3 cells at the concentrations of 25 and 50 µg/ml. The internalization was increased dose-dependently but in MCF-7 cells, the internalization rate was low.

3.6. Induction of apoptosis

Annexin V and PI double staining method was used to assess the apoptosis induced by chimeric proteins on SKBR-3 cells. According to the manufacturer's instruction, Annexin V+/PI- cells were recognized as the early apoptotic cells. In addition, Annexin V+/PI + cells were considered as late apoptotic cells and Annexin V-/PI- cells were considered as controls. MCF-7 and SKBR-3 cells were treated with 10, 25 and 50 µg/ml of chimeric proteins and then incubated at 37 °C for 48 h followed by flow cytometry analysis.

A considerable increase was observed in the number of early and late apoptotic cells of SKBR-3 cells. The amount of apoptotic cells observed in SKBR-3 cells treated with scFv(Herceptin)–PE-STXA at the concentrations of 10, 25 and 50 µg/ml were 20.8 %, 38.9 % and 46.3 %, respectively (Fig. 7A) and when SKBR-3 cells treated with scFv(Herceptin) – PE at the same concentrations, the amount of apoptotic cells were 62.8 %, 89.7 % and 88.3 %, respectively (Fig. 7C). In addition, the increase in the concentrations of chimeric proteins in the treated cells, resulted in remarkable alteration of live to apoptotic cells in a dose-dependent manner in SKBR-3 but, in MCF-7cells significant elevation in the amount of apoptotic cells was not detected, except in high concentration of ITs (Fig. 7B and D). The results showed that in the same concentrations, the scFv(Herceptin)-PE immunotoxin induced more apoptosis effect compared to scFv(Herceptin)–PE-STXA immunotoxin.

4. Discussion

Today, a wide range of research is performed in this topic area to improve and to optimize the recombinant single-chain antibodies for targeted therapy [23]. In addition, decreasing the size and thus, improving the infiltration into solid tumors by recombinant methods, have permitted the investigators to attach antibodies to other proteins such as toxins. Immunotoxins are protein-based therapeutics consisting of at least two efficient domains, one part permits IT to bind to the target cells and another part eradicates the cells after internalization [24,25].

In recent years, research on ITs has become very popular. ITs are extremely potent cytotoxic proteins [26]. Primarily, these constructs were generated by chemically conjugating an antibody to a protein toxin [27]. By the improvement of the recombinant DNA technology, antibody fragments have been used for delivering various toxins to HER-positive cancer cells [28].

In this study, we used cytotoxic form of PE in recombinant immunotoxins which is a 38 kDa truncated form, namely PE38. It is resistant to the mutations without affecting the activity of IT. This property is associated with improved stability of PE and remarkably

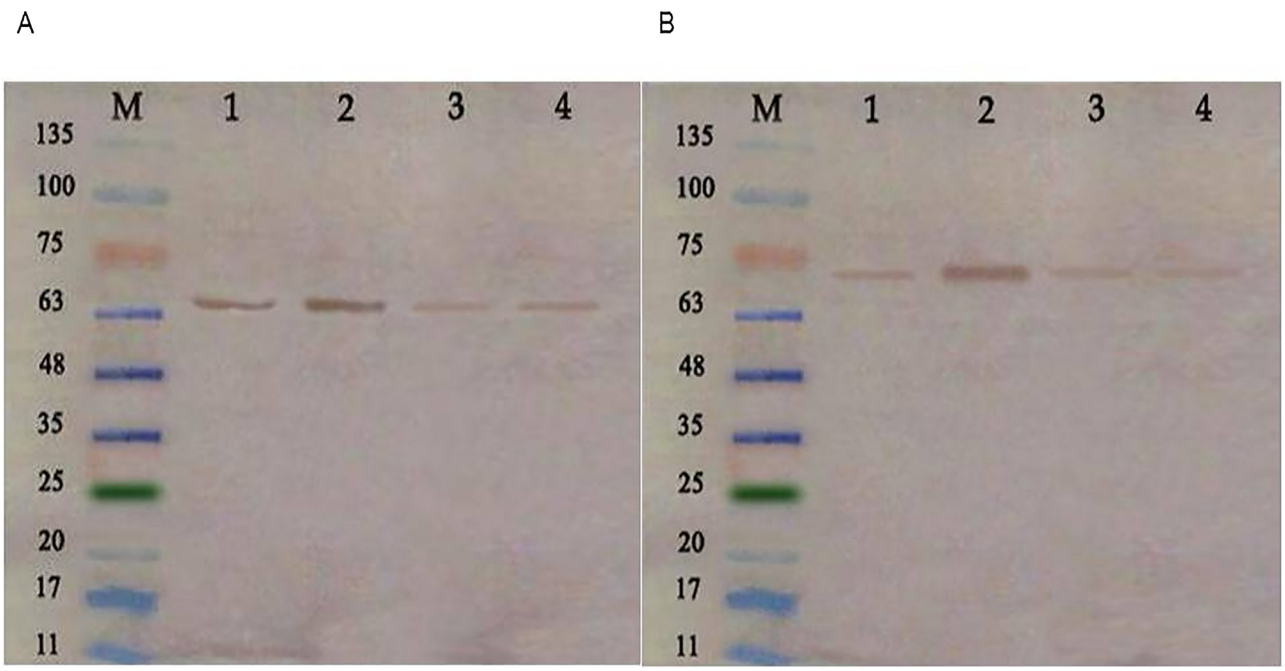

Fig. 6. Evaluation of recombinant protein internalization by western blotting.
Anti-His-tag antibody identified scFv(Herceptin)-PE-STXA and scFv(Herceptin)-PE recombinant proteins in SKBR-3 and MCF-7 cell lysates. A, M: Protein size marker. 1: Lysate of SKBR-3 cells treated with 50 μg/ml scFv(Herceptin)-PE-STXA (1 h) 2: Lysate of SKBR-3 of cells treated with 50 μg/ml scFv(Herceptin)-PE-STXA (2 h). 3: Lysate of MCF-7 cells treated with 50 μg/ml scFv(Herceptin)-PE-STXA (1 h). 4: Lysate of MCF-7 cells treated with 50 μg/ml scFv(Herceptin)-PE-STXA (2 h).
B, M; Protein size marker. 1: Lysate of SKBR-3 cells treated with 50 μg/ml of scFv(Herceptin)-PE(1 h) 2: Lysate of SKBR-3 of cells treated with 50 μg/ml of scFv (Herceptin)-PE(2 h). 3: Lysate of MCF-7 cells treated with 50 μg/ml of scFv(Herceptin)-PE (1 h). 4: Lysate of MCF-7 cells treated with 50 μg/ml of scFv(Herceptin)-PE (2 h).

reduces immunogenicity of IT; In addition, makes it an appropriate candidate for cancer therapy [40,41]. Applying a proper linker is a key factor to keep the proper folding and functions of the

binding and toxic moiety of the fusion proteins [29] therefore other features of these immunotoxins are the use of ASGGPE and (G4S)3 linkers to separate different segments and alsoincreasing folding stability of IT which facilitates the expression of recombinant proteins [35,42].

Recently, the new chimeric proteins expressed in prokaryotic hosts were purified and subsequently the cytotoxic effect of these proteins were evaluated in breast cancer cell lines.

Here, we used SKBR-3 and MCF-7 as HER2-positive and -negative cell line, respectively.

According to Yucao, the MCF-7 cells are not completely negative for HER2 receptor and they rarely express it but, in some other studies MCF-7 cells are considered negative for HER2 receptor [18,30]. Therefore, at high concentrations the immunotoxin can bind to MCF-7 and then enter the cell and cause cell death; this event does not take place at low concentrations of immunotoxin.

We carried out the cytotoxic effect of recombinant immunotoxins by MTT assay. The cytotoxic action of recombinant immunotoxins has obviously revealed the target-specific cell killing. Here, we demonstrated that SKBR-3 cells have high susceptibility to recombinant immunotoxins whereas MCF-7 cells are not reactive at low concentration but at higher concentrations have low cytotoxicity.

The total number of HER2 internalized into these cell lines is proportional to the antigen density at the cell surface. Thus, the more HER2 located on a cell surface, the more internalization of immunotoxins happens. Therefore, the cytotoxicity of a certain dose of immunotoxins in the cells with higher surface antigens increases in comparison to the cells with lower surface antigens [31]. The rate of the construct internalization may be mainly related to HER2/neu receptor recovery and the amount of antigens absorbed, which seems not to be affected by the affinity of immunotoxin binding. In addition, intracellular transfer and distribution of the toxin associated with ribosomal subunits may be the important factors that can explain the immunotoxin sensitivity [32–35]. As it has been mentioned before, the essential part of the ITs is the toxin, hence, the selection of antigen is another significant element that should be considered in planning the exact time of conjugation, since this event determines the selectivity and the specific transfer of the toxin to the target cancer cells [36]. The best antigen for this purpose must specifically be expressed on the cancer cells, but such antigens have not yet been recognized. The HER2 antigen has been selected for this study as it has two prominent features required for this objective: 1. It is a cancer-related antigen that is mainly present in breast cancer and 2: It is involved in cellular surveillance systems such as, motility, proliferation and resistance to apoptosis. HER2 is well-known as a surface marker of 20–25 % of breast cancers [37]. After internalization, the performance of Herceptin-HER2 complexes differ and is related to the HER2 expression levels, particularly, in the cells that overexpress HER2 and Herceptin is efficiently recycled. However, amino portion of antibody-HER2 complexes is degraded by the lysosomal pathway. Contrarily, in the cells with low HER2, the recycling of Herceptin cannot be identified and antibody-HER2 complexes enter the lysosomal pathway [38]. It should be considered that, for assessing the efficacy of ITs, the internalization is of great importance. Transportation of the cytotoxic domain of an IT to the cytosol of target cell is dependent on a series of events. It has been

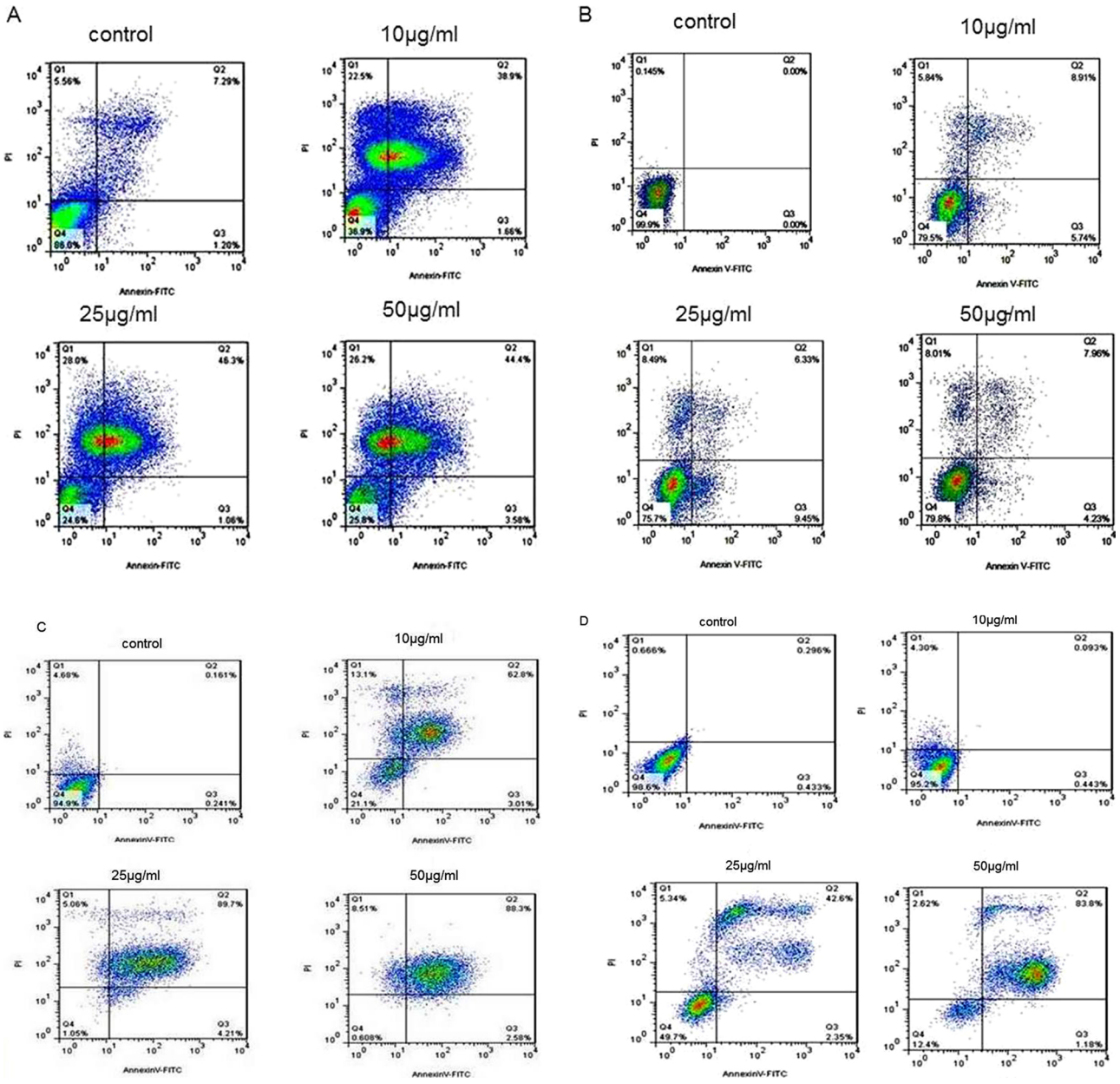

Fig. 7. scFv(Herceptin)-PE-STXA induced apoptosis in breast cancer cell lines.
SKBR-3 (A) and MCF-7 (B) cells were treated with different concentrations of scFv(Herceptin)-PE-STXA for 48. SKBR-3 (C) and MCF-7 (D) cells were treated with different concentrations of scFv(Herceptin)-PE for 48 h. Apoptosis was evaluated by flow cytometric analysis using annexin V and PI staining as explained in materials and methods. Cells that are Annexin V-FITC (+)/ PI(-) are apoptotic. The cell population with Annexin V-FITC (+)/PI (+) has been described as necrotic or advanced apoptotic and Annexin V-FITC (-)/ PI (+) may be bare nuclei, cells in late necrosis or cellular debris. The scFv(Herceptin)-PE-STXA increased the number of early apoptotic cells (AnnexinV positive cells) and late apoptotic cells (AnnexinV and PI positive cells) and necrotic cells in a dose-dependant manner in SKBR-3 cell line; But in MCF-7 cells the number of early apoptotic cells and late apoptotic cells increased. scFv(Herceptin)-PE increased the number of early apoptotic cells (AnnexinV positive cells) and late apoptotic cells (AnnexinV and PI positive cells) in a dose-dependant manner in SKBR-3 cell line; But in MCF-7 cells, apoptotic and necrotic cells were observed.

shown that by increasing the incubation time and toxin concentration, the amount of the toxin uptake per cell increases and in contrary, the cell viability reduces [39].

As ITs recruit various cell-specific binding moieties to increase their entry into the cells, the evaluation of the efficacy of different trafficking pathways is complicated.

The bacterial toxins that are used in ITs eradicate cells by disturbing cellular protein synthesis. Intracellular transport of immunotoxins to the cytosol is necessary for antitumor activity. Subsequently, the IT targeting moiety binds to the cancer cell surface receptor and stimulates the endocytic uptake of this complex. Trafficking and processing of IT is toxin- and target-specific, however, it ultimately leads to the translocation of the enzymatically active portion of the toxin to the cytosol [40].The bacterial toxins such as PE and diphtheria toxin (DT) irreversibly change and inactivate eukaryotic elongation factor 2 (eEF2), an important element of the cellular protein synthesis machinery. Considerable advances have been made for understanding the mechanism of action of anticancer IT therapy using PE and PE-based ITs. Generally, the toxin binds to its cell-surface receptor and the IT binds to the specific target molecule by its targeting moiety and the following events are common in both PE and the PE-based ITs [41]. The toxin/IT enters by receptor-mediated endocytosis into clathrin-coated pits, then,

the furin protease cleaves the translocated subunit in the endosome and then generates two separated domains. Subsequently, the catalytic subunit, along with the translocation subunit, moves in the retrograde direction to the Golgi system and the catalytic- translocation subunit migrates to the ER, but, the transport mechanism of the subunit from the ER to the cytosol is still unknown [42].

Finally, the catalytic subunit, ADP ribosylate elongation factor 2 lead to inhibition of protein synthesis. Shiga toxin can exert its effects on eukaryotic cells by one of three known mechanisms: Firstly, it can inhibit the synthesis of cytoplasmic protein by inactivation of ribosomes that result in cell death [43]. Secondly, Shiga toxin generates depurinated 28S rRNA in ribosomes that begins a unique signal-transduction response known as the 'ribotoxic stress response' (RSR) that causes activation of cytokines, chemokines or other factors that lead to several different events including apoptosis of the affected cell [44]. In this study, we used PEII- in the structure of scFv(Herceptin)-PE-STXA immunotoxin for translocation and delivery of STX into the target cells to facilitate cytotoxicity induced by STX. Li Zhang,et al. used HER2-targeting recombinant protein with truncated *pseudomonas* exotoxin A translocation domain that efficiently kills breast cancer cells. The second domain of *Pseudomonas* exotoxin A (PEII) has been recruited to simplify the translocation of extracellular contents into the cytoplasm and to transfer heterologous molecules into target cells. They also use the PEII fragment in combination with an active granzyme B (ImmunoGrB) to kill HER2-positive tumor cells. They created four ImmunoGrB fusion proteins consisting of different PEII deletions and examined their ability to kill HER2-positive cells. Their data indicated that while a whole deletion of PEII in ImmunoGrB resulted in an inability to kill cancer cells, ImmunoGrBs containing PEII could powerfully kill HER2-positive SKBR-3 cells [14].

In brief, the scFv(Herceptin)-PE-STXA immunotoxin is both specific and effective against HER2-expressing breast cancer cells. In this investigation, the results presented here show that two chimeric proteins inhibit cell proliferation in HER2-positive breast cancer cell line. The scFv(Herceptin)-PE protein was more toxic to the SKBR-3 cell line than scFv(Herceptin)-PE-STXA immunotoxin and the scFv(Herceptin)-PE-STXA protein induces apoptosis in SKBR-3 whereas, induction of apoptosis in the MCF-7 cell was low.

5. Conclusions

In the current study, two ITs were constructed by using Herceptin antibody against HER2 for recognizing cell surface receptors and PE and STX toxin were recruited to eradicate cancer cells.

The scFv(Herceptin)-PEIT was more toxic to theSKBR-3 cell line, that overexpressed the antigen HER2, than to the scFv(Herceptin)-PE-STXA immunotoxin. Furthermore, the binding of these ITs and subsequent internalization of these immunotoxins were intense in SKBR-3 cell line compared to MCF-7cell line. These data indicated that binding and cytotoxicity of these ITs were receptor-dependent, because apoptosis could not be induced in the MCF-7cells that did not overexpress the HER2 antigen.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgment

This paper was extracted from the thesis.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jsbmb.2020.105651.

References

[1] M. Tan, D. Yu, Molecular mechanisms of erbB2-mediated breast cancer chemoresistance, Breast Cancer Chemosensitivity (2007) 119–129.
[2] S.K. Pal, M. Pegram, HER2 targeted therapy in breast cancer… beyond Herceptin, Rev. Endocr. Metab. Disord. 8 (3) (2007) 269–277.
[3] S. Choudhary, M. Mathew, R.S. Verma, Therapeutic potential of anticancer immunotoxins, Drug Discov. Today 16 (11) (2011) 495–503.
[4] J. Madhumathi, R.S. Verma, Therapeutic targets and recent advances in protein immunotoxins, Curr. Opin. Microbiol. 15 (3) (2012) 300–309.
[5] S. Kunwar, S.M. Chang, M.D. Prados, M.S. Berger, J.H. Sampson, D. Croteau, et al., Safety of intraparenchymal convection-enhanced delivery of cintredekin besudotox in early-phase studies, Neurosurg. Focus 20 (4) (2006) E15.
[6] A. Antignani, D. Fitz Gerald, Immunotoxins: the role of the toxin, Toxins 5 (8) (2013) 1486–1502.
[7] V.S. Allured, R.J. Collier, S.F. Carroll, D.B. McKay, Structure of exotoxin A of Pseudomonas aeruginosa at 3.0-Angstrom resolution, Proc. Natl. Acad. Sci. 83 (5) (1986) 1320–1324.
[8] J.E. Weldon, L. Xiang, O. Chertov, I. Margulies, R.J. Kreitman, D.J. FitzGerald, et al., A protease-resistant immunotoxin against CD22 with greatly increased activity against CLL and diminished animal toxicity, Blood 113 (16) (2009) 3792–3800.
[9] J. Hwang, D.J. Fitzgerald, S. Adhya, I. Pastan, Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in E. coli, Cell 48 (1) (1987) 129–136.
[10] I. Pastan, R. Hassan, D.J. FitzGerald, R.J. Kreitman, Immunotoxin treatment of cancer, Annu. Rev. Med. 58 (2007) 221–237.
[11] A.R. Melton-Celsa, Shiga toxin (Stx) classification, structure, and function, Microbiol. Spectr. 2 (2) (2014).
[12] M.H. Roudkenar, S. Bouzari, Y. Kuwahara, A.M. Roushandeh, M. Oloomi, M.J.W.Jo G.W. Fukumoto, Recombinant hybrid protein, Shiga toxin and granulocyte macrophage colony stimulating factor effectively induce apoptosis of colon cancer cells, World J. Gastroenterol. 12 (15) (2006) 2341.
[13] B. Hotz, M.V. Backer, J.M. Backer, H.-J. Buhr, H.G.J.N. Hotz, Specific targeting of tumor endothelial cells by a shiga-like toxin-vascular endothelial growth factor fusion protein as a novel treatment strategy for pancreatic cancer, Neoplasia 12 (10) (2010) 797.
[14] L. Zhang, J. Zhao, T. Wang, Jia L.-T. Yu C-J, Y.-Y. Duan, et al., HER2-targeting recombinant protein with truncated Pseudomonas exotoxin: a translocation domain efficiently kills breast cancer cells, Cancer Biol. Ther. 7 (8) (2008) 1226–1231.
[15] X. Li, P. Stuckert, I. Bosch, J.D. Marks, Marasco WAJCgt, Single-chain antibody-mediated gene delivery into ErbB2-positive human breast cancer cells, Cancer Gene Ther. 8 (8) (2001) 555.
[16] M. Mahboobi, H. Sedighian, M.H. CH, B. Bambai, S.E. Soofian, J. Amani, Applying bioinformatic tools for modeling and modifying type II E. coli L-asparginase to present a better therapeutic agent/drug for acute lymphoblastic leukemia, Int. J. Cancer Manag. 10 (3) (2017).
[17] Z. Goleij, H.M. Hosseini, M. Amin, J. Amani, Elham Behzadi, A.A.I. Fooladi, In silico evaluation of two targeted chimeric proteins based on bacterial toxins for breast cancer therapy, Int J Cancer Manag. 12 (2019) e83315.
[18] Y. Cao, J.D. Marks, Q. Huang, S.I. Rudnick, C. Xiong, W.N. Hittelman, et al., Single-chain antibody-based immunotoxins targeting Her2/neu: design optimization and impact of affinity on antitumor efficacy and off-target toxicity, Mol. Cancer Ther. 11 (1) (2012) 143–153.
[19] H.M. Hosseini, Soleimanirad J. Fooladi AAI, M.R. Nourani, S. Davaran, M. Mahdavi, Staphylococcal entorotoxin B anchored exosome induces apoptosis in negative esterogen receptor breast cancer cells, Tumor Biol. 35 (4) (2014) 3699–3707.
[20] F. Yousefi, S.F. Mousavi, S.D. Siadat, M.M. Aslani, J. Amani, H.S. Rad, et al., Preparation and in vitro evaluation of antitumor activity of tgfαl3-seb as a ligand-targeted superantigen, Technol. Cancer Res. Treat. 15 (2) (2016) 215–226.
[21] P. Saffarian, S.N. Peerayeh, J. Amani, F. Ebrahimi, H. Sedighian, R. Halabian, et al., TAT-BoNT/A (1–448), a novel fusion protein as a therapeutic agent: analysis of transcutaneous delivery and enzyme activity, Appl. Microbiol. Biotechnol. 100 (6) (2016) 2785–2795.
[22] F. Maleki, N. Sadeghifard, H.M. Hosseini, S. Bakhtiyari, Z. Goleij, E. Behzadi, et al., Growth-inhibitory effects of TGFαL3-SEB chimeric protein on colon cancer cell line, Biomed. Pharmacother. 110 (2019) 190–196.
[23] M.G. Rosenblum, L.H. Cheung, Y. Liu, Marks JW 3rd, Design, expression, purification, and characterization, in vitro and in vivo, of an antimelanoma single-chain Fv antibody fused to the toxin gelonin, Cancer Res. 63 (14) (2003) 3995–4002.
[24] N. Becker, I.J.A. Benhar, Antibody-based Immunotoxins for the Treatment of Cancer, (2012) 1(1):39-69.
[25] E. Rezaie, A.B. Pour, J. Amani, H.M. Hosseini, Bioinformatics predictions, expression, purification and structural analysis of the PE38KDEL-scfv immunotoxin against EPHA2 receptor, Int. J. Pept. Res. Ther. (2019) 1–18.
[26] K.K. Jain, Use of bacteria as anticancer agents. expert opinion on biological therapy, Expert Opin. Biol. Ther. 1 (2) (2001) 291–300.
[27] G.C. Rodriguez, M.P. Boente, A. Berchuck, R.S. Whitaker, K.C. O'Briant, F. Xu, et al., The effect of antibodies and immunotoxins reactive with HER-2/neu on growth of ovarian and breast cancer cell lines, Am. J. Obstet. Gynecol. 168 (1) (1993) 228–232.
[28] M. Jain, G. Venkatraman, S.K. Batra, Optimization of radioimmunotherapy of solid tumors: biological impediments and their modulation, Clin. Cancer Res. 13 (5) (2007) 1374–1382.

[29] E. Rezaie, M. Mohammadi, A. Sakhteman, P. Bemani, S. Ahrari, Application of molecular dynamics simulations to design a dual-purpose oligopeptide linker sequence for fusion proteins, J. Mol. Model. 24 (11) (2018) 313.
[30] Y. Cao, J.D. Marks, J.W. Marks, L.H. Cheung, S. Kim, Rosenblum MGJCr, Construction and characterization of novel, recombinant immunotoxins targeting the Her2/neu oncogene product: in vitro and in vivo studies, Cancer Res. 69 (23) (2009) 8987–8995.
[31] X.-Y. Zhao, H.-L. Liu, B. Liu, J. Willuda, G. Siemeister, M. Mahmoudi, et al., Tomoregulin internalization confers selective cytotoxicity of immunotoxins on prostate cancer cells, Transl. Oncol. 1 (2) (2008) 102–109.
[32] R. Mandler, C. Wu, E.A. Sausville, A.J. Roettinger, D.J. Newman, D.K. Ho, et al., Immunoconjugates of geldanamycin and anti-HER2 monoclonal antibodies: antiproliferative activity on human breast carcinoma cell lines, J. Natl. Cancer Inst. Monographs 92 (19) (2000) 1573–1581.
[33] L.L. Tortorella, N.H. Pipalia, S. Mukherjee, I. Pastan, D. Fitzgerald, Maxfield FRJPo, Efficiency of immunotoxin cytotoxicity is modulated by the intracellular itinerary, PLoS One 7 (10) (2012) e47320.
[34] T.K. Bera, J. Viner, E. Brinkmann, Pastan IJCr, Pharmacokinetics and antitumor activity of a bivalent disulfide-stabilized Fv immunotoxin with improved antigen binding to erbB2, Cancer Res. 59 (16) (1999) 4018–4022.
[35] X. Du, R. Beers, D.J. FitzGerald, Pastan IJCr, Differential cellular internalization of anti-CD19 and-CD22 immunotoxins results in different cytotoxic activity, Cancer Res. 68 (15) (2008) 6300–6305.
[36] M.K. Tur, M. Huhn, T. Thepen, M. Stöcker, R. Krohn, S. Vogel, et al., Recombinant CD64-specific single chain immunotoxin exhibits specific cytotoxicity against acute myeloid leukemia cells, Cancer Res. 63 (23) (2003) 8414–8419.
[37] N. Jiang, J.J. Lin, J. Wang, B.N. Zhang, A. Li, Z.Y. Chen, et al., Novel treatment strategies for patients with HER2-positive breast cancer who do not benefit from current targeted therapy drugs, Exp. Ther. Med. 16 (3) (2018) 2183–2192.
[38] The level of HER2 expression is a predictor of antibody-HER2 trafficking behavior in cancer cells, in: S. Ram, D. Kim, R.J. Ober, E.S. Ward (Eds.), MAbs, Taylor & Francis, 2014.
[39] C.M. Pirie, B.J. Hackel, M.G. Rosenblum, K.D. Wittrup, Convergent potency of internalized gelonin immunotoxins across varied cell lines, antigens, and targeting moieties, J. Biol. Chem. 286 (6) (2011) 4165–4172.
[40] M. Keshtvarz, J. Salimian, M. Yaseri, S.Z. Bathaie, E. Rezaie, A. Aliramezani, et al., Bioinformatic prediction and experimental validation of a PE38-based recombinant immunotoxin targeting the Fn14 receptor in cancer cells, Immunotherapy 9 (5) (2017) 387–400.
[41] L.L. Tortorella, N.H. Pipalia, S. Mukherjee, I. Pastan, D. Fitzgerald, F.R. Maxfield, Efficiency of immunotoxin cytotoxicity is modulated by the intracellular itinerary, PLoS One 7 (10) (2012) e47320.
[42] H.K. Gan, M. van den Bent, A.B. Lassman, D.A. Reardon, Amjnrco Scott, Antibody–drug conjugates in glioblastoma therapy: the right drugs to the right cells, Nat. Rev. Clin. Oncol. 14 (11) (2017) 695.
[43] T. Obrig, P. Del Vecchio, J. Brown, T. Moran, B. Rowland, T. Judge, et al., Direct cytotoxic action of Shiga toxin on human vascular endothelial cells, Infect. Immun. 56 (9) (1988) 2373–2378.
[44] D.M. Jandhyala, A. Ahluwalia, T. Obrig, Thorpe CMJCm, ZAK: a MAP3Kinase that transduces Shiga toxin-and ricin-induced proinflammatory cytokine expression, Cell. Microbiol. 10 (7) (2008) 1468–1477.