ARA-linker-TGFαL3: a novel chimera protein to target breast cancer cells

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Abstract
Targeted cancer therapies based on overexpressed receptors and the fractions containing immunotoxins and bacterial metabolites are one of the well-known methods to overcome the chemotherapy resistance of cancer cells. In this paper, we designed ARA-linker-TGFαL3, using Arazyme, a Serratia proteamaculans metabolite, and a third loop segment of TGFα to target EGFR-expressing breast cancer cells. After cloning in pET28a (+), the expression of recombinant protein was optimized in Escherichia coli strain BL21 (DE3). MDA-MB-468 (EGFR positive) and MDA-MB-453 (EGFR negative) breast cancer cell lines were employed. Also, the chemotherapeutic drug, Taxotere (Docetaxel), was employed to compare cytotoxicity effects. Cell ELISA assessed the binding affinity of recombinant proteins to the receptor, and the cytotoxicity was detected by MTT and lactate dehydrogenase release assays. The interfacing with cancer cell adhesion was evaluated. Furthermore, the induction of apoptosis was examined utilizing flow cytometric analysis, and caspase-3 activity assay. Moreover, RT-PCR was conducted to study the expression of apoptosis (bax, bcl2, and casp3), angiogenesis (vegfr2), and metastasis (mmp2 and mmp9) genes. ARA-linker-TGFαL3 revealed a higher binding affinity, cytotoxicity, and early apoptosis induction in MDA-MB-468 cells compared to the effects of Arazyme while both recombinant proteins showed similar effects on MDA-MB-453. In addition, the Taxotere caused the highest cytotoxicity on cancer cells through induction of late apoptosis. Meanwhile, the expression of angiogenesis and metastasis genes was decreased in both cell lines after treatment with either ARA-linker-TGFαL3 or Arazyme. Our in vitro results indicated the therapeutic effect of ARA-linker-TGFαL3 on breast cancer cells.

Keywords Targeted cancer therapy · Arazyme · Epidermal growth factor receptor · Transforming growth factor alpha · Apoptosis

Introduction
The increasing incidence of breast cancer in males and females in most countries has been reported [1]. In addition to the surgery and radiotherapy, and according to the subtype of breast cancer receptors, a combination of different treatments is administered. These therapy regimes include chemotherapy, endocrine therapy, and drug delivery. Employing nanocarriers, intraductal injection of chemotherapy drugs on the location of tumors, and receptor-based targeting approaches are three strategies of anti-cancer drug delivery [2–4].

Several overexpressing breast cancer cell receptors, namely epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER-2), insulin-like growth factor 1 receptor (IGF-IR), and vascular endothelial growth factor receptor (VEGFR), were utilized to target the exact sites of tumor cells [5–7]. On the other hand, triple-negative breast cancers (TNBC) are resistant to estrogen receptor-α (ER), Her2, and progesterone receptor (PR) targeted therapies [2]. It is therefore necessary to design new anticancer drugs to overcome treatment resistance in cancers [8].
EGFR (HER-1) comprises the transmembrane domain between extracellular ligand binding and intracellular portions [9]. After the ligand-induced receptor dimerization, the cytoplasmic juxtamembrane segment activates the tyrosine kinase domains to phosphorylate the C-terminal tail of the receptor. Subsequently, the signaling pathways responsible for adhesion angiogenesis, cell proliferation, migration, and survival were triggered [10]. Transforming growth factor alpha (TGFα) is a single polypeptide comprising two sets of antiparallel β-sheets and three disulfide loops that pose residues in contact with L1 and L2 domains of EGFR to generate active stable conformation of the receptor [9]. Interestingly, a segment containing the C-terminal third loop of TGFα not only is non-mitogenic but also inhibits cell proliferation in competition with EGF upon binding to the receptor [11]. The use of overexpressed receptors of cancer cells in combination with anti-cancer properties of some bacterial metabolites, including toxins, enzymes, bacteriocins, and biosurfactants has been demonstrated in previous studies [12, 13]. Arazyme, a 51.5 Da metalloprotease is a secondary metabolite secreted by Serratia proteamaculans, which reduces proliferation and metastasis of breast cancer cells [14, 15]. Arazyme possesses hepatoprotective and anti-inflammatory effects and induces CD4+ and CD8+ T lymphocytes as well as macrophages and dendritic cells [16, 17]. In the current study, we designed a fusion protein consisting of Arazyme and the third loop of TGFα to target TNBC breast cancer cell lines and after evaluating the special binding to EGFR, the cytotoxicity of ARA-linker-TGFαL3 was examined by using colorimetric methods and real-time PCR analysis of angiogenesis and apoptosis-related genes.

Materials and methods

**Cell lines and culture condition**

The TNBC breast cancer cell lines, MDA-MB-468 (RRID:CVCL_0419), and MDA-MB-453 (RRID:CVCL_0418) cells were purchased from the Pasteur Institute and Iranian Biological Resource Center (IBRC), respectively. A high level of EGFR expression was revealed in MDA-MB-468 cells. Since there is no detectable EGFR expression in MDA-MB-453, this cell line was utilized as a negative control. The culture media for MDA-MB-468 and MDA-MB-453 were DMEM and RPMI, respectively (both from Bioidea, Iran) [18]. Ten percent fetal bovine serum (Gibco; USA), 100 units/ml penicillin (Bioidea, Iran), and 100 mg/ml streptomycin (Bioidea, Iran) were used as media supplements. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO2.

**The design and codon optimization of chimeric gene**

The amino acid sequence of Arazyme (Accession No. AAX21094) was obtained from the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/). To target EGFR on cancer cell lines, a non-mitogenic binding region of the third loop of TGFα was selected. To increase stability and improve the biological activity of fusion protein, a linker [A(EAAAK)4ALEA(EAAAK)4A] was inserted between the Arazyme and TGFα third loop (Fig. 1a) [19]. The translation of the fusion protein was adapted to the host organism, Escherichia coli BL21, and the vector pET28a (+) was chosen to construct the recombinant ARA-linker-TGFαL3 by Biomatik Company (Ontario, Canada). Also, the recombinant protein of Arazyme was utilized as a control. The design and synthesis of the Arazyme gene construct and its expression optimization were performed as described in the previous study [14].

**Optimization of transformation and expression condition**

The lyophilized plasmid was reconstituted in nuclease-free water to the absolute concentration of 10 ng/μ and the construct was transferred to competent E. coli strain BL21 (DE3). Since the vector, pET28a (+) harbors the kanamycin resistance gene, the transformed bacteria were identified by growing on kanamycin (Solarbio, China) containing (20 mg/ml) Luria–Bertani (LB) agar (HiMedia, India). In addition, the kanamycin resistance colonies were screened by PCR. Then, they were sequenced to verify the correct insertion of the chimeric gene by using colorimetric methods and real-time PCR analysis of angiogenesis and apoptosis-related genes.
Fig. 1 Expression and purification of ARA-linker-TGFαL3 and Arazyme. 

**a** The schematic structure of ARA-linker-TGFαL3. **b** Lane 1, agarose gel electrophoresis of PCR product amplified from fragment encoding ARA-linker-TGFαL3 (1716 bp); Lane M, DNA size marker. SDS-PAGE of Ni–NTA purification of **c** ARA-linker-TGFαL3 (59.3 kDa) and **d** Arazyme (51.5 kDa), which were expressed by inducing with 1 mM IPTG at 37 °C for 4 h; Lane M, protein molecular weight marker, Lane 1, unpurified protein and Lanes 2–4 represent column elution with denaturing wash buffer, native wash buffer and native elution buffer, respectively. **e** Western blot analysis of ARA-linker-TGFαL3 and Arazyme expression; Lane 1, Arazyme; Lane 2, ARA-linker-TGFαL3. **f** The comparison of protease activity between purified and unpurified recombinant proteins, Arazyme and ARA-linker-TGFαL3. The results are shown as means ± SD of three independent experiments. *p < 0.05 represents statistical differences analyzed by paired t-test.
Purification of recombinant proteins

Since only the solid pellet (insoluble inclusion bodies) was resolved by SDS-PAGE analysis, the purification under a hybrid of denaturing and native conditions was performed to remove urea and on-column refolding of recombinant proteins. The procedure described by Invitrogen™ (Ni–NTA Purification System; user manual) has been used with a few modifications as follows: first, the harvested cell pellet was suspended in lysis buffer (7 M urea (Sumchun Chemicals, Korea), 20 mM NaH2PO4, and 500 mM NaCl, pH 7.8). After sonication for 150 s (30 s pulse, 30 s pause, five times at 70% amplitude on ice water) and centrifugation (3000 × g for 15 min), the supernatant was added to the Ni–NTA resin (Qiagen, Germany) which was previously equilibrated by denaturing binding buffer (7 M urea; 20 mM NaH2PO4; 500 mM NaCl; pH 7.8). Subsequently, the column was washed with denaturing binding buffer, denaturing wash buffer (7 M urea 20 mM NaH2PO4; 500 mM NaCl; pH 6), and native wash buffer (20 mM Imidazole, 50 mM NaH2PO4; 500 mM NaCl; pH 8), respectively. At each stage, the supernatant was collected after 30 min with gentle agitation at room temperature and resin was settled by gravity. Ultimately, the chimeric proteins were eluted by the native elution buffer (250 mM imidazole, 50 mM NaH2PO4; 500 mM NaCl; pH 8) and the eluted solution was dialyzed in PBS to remove imidazole. The concentration of purified proteins was determined using a NanoDrop 2000 spectrophotometer system (Thermo Scientific, USA, RRID:SCR_018042).

Western blotting

Western blotting was used to confirm the expression of recombinant proteins. First, SDS-polyacrylamide gel electrophoresis was performed in 10% polyacrylamide gel at 100 V for 1–2 h. Then, the expressed proteins were transferred to the polyvinylidene fluoride (PVDF) membranes (Hi-bond Amersham Biosciences, USA). Subsequently, the membrane was blocked with 5% (w/v) skim milk (Sigma-Aldrich, USA) in Tris-buffered saline containing 0.1% Tween 20 (DNA Biotech Co., Iran) (TBST) at 4 °C for 16 h. Next, the membrane was incubated in a TBST solution containing 5% skim milk and 1:2000 dilution of monoclonal anti-polyHistidine–Peroxidase antibody (Cat# A7058, Sigma-Aldrich, USA, RRID:AB_258326) produced in mouse for 1 h at room temperature. Ultimately, the membrane was washed five times with TBST and stained with 3, 3′-diaminobenzidine substrate (DAB).

Protease activity assay

The protease activity of the recombinant proteins was assessed by the Folin–Ciocalteu method [20]. The purified proteins were added into a solution containing 0.65% w/v casein (Sigma-Aldrich, USA) and 50 mM potassium phosphate buffer (KH2PO4 and K2HPO4 (both from Sigma–Aldrich, USA)), (pH 7.5). After incubation for 15 min at 37 °C, the reaction was stopped by 110 mM trichloroacetic acid (Sigma-Aldrich, USA) solution. Subsequently, the solution was centrifuged at 10,000 rpm for 10 min. The collected supernatant was mixed with 500 mM sodium carbonate solution and Folin–Ciocalteu reagent (both from Sigma-Aldrich, USA) and incubated for 10 min at 37 °C. The optical density of the developed color was read at 660 nm by an ELISA microplate reader (Bio-Rad, Hercules; CA, USA) and the protease activity was defined as unit per mg protein.

Evaluation of the receptor binding by the cell-ELISA method

Elisa based assay was employed to examine the binding ability of ARA-linker-TGFαL3 to EGFR. A 96-well plate was seeded with 8000 MDA-MB-453 and MDA-MB-468 cells. After 24 h of incubation, the cells were washed with PBS. Subsequently, formaldehyde solution (10% w/w in water, Sigma-Aldrich, USA) was utilized for fixation at room temperature for 1 h. Next, the cells were blocked with bovine serum albumin (3% w/v, Merck, Germany). After incubation for 24 h at 37 °C, the cells were washed with PBST (containing 0.1 M PBS and 0.05% Tween 20), the different concentrations of Arazyme and Ara-linker-TGFαL3 (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 μg/ml) were added and incubated for 2 h at 37 °C. Next, the wells were washed three times with PBST. Then, HRP-labeled anti-His antibody (1:2000) was added for 2 h at room temperature. Subsequently, the wells were washed five times with PBST. Then tetramethylbenzidine (TMB) and H2O2 (1.5%) (both from Merck, Germany) were added. Ultimately, the color reaction was prevented with sulfuric acid, 0.1 N (Merck, Germany) and the optical density was read at 450 nm by ELISA microplate reader [21].

Cytotoxicity evaluation of chimera proteins

The effect of ARA-linker-TGFαL3 and Arazyme on the viability of MDA-MB-453 and MDA-MB-468 cells was evaluated by MTT assay [22]. Briefly, the cells at a density of 1 × 10⁴ cells/well were plated on a 96-well plate for 24 h at 37 °C and 5% CO2. Then, different concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 μg/ml) of Arazyme and ARA-linker-TGFαL3 were prepared for each well containing...
serum-free medium for 24 h. Afterward, 5 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (Merck, Germany) was added and incubated for 3 h at 37 °C. After removing the supernatant, 100 µl DMSO (dimethyl sulfoxide) was used to dissolve formazan crystals and the absorbance was read at 570 nm by an ELISA microplate reader. The untreated cell was assumed as a negative control (100% viability) and the concentration of Arazyme and ARA-linker-TGFαL3 that proved to be lethal to 50% (LC50) of treated cells was calculated in µg/ml. Furthermore, the cytotoxicity of the chemotherapeutic drug, Taxotere on MDA-MB-453 and MDA-MB-468 cells was evaluated.

**Apoptosis detection by flow cytometric analysis**

To distinguish apoptosis and necrosis cells, the Annexin/PI flow cytometry assay was performed [23]. MDA-MB-453 and MDA-MB-468 cells were seeded at a density of 5 × 10^5 cells/well in a 6-well plate for 24 h at 37 °C and 5% CO2. Then, the incubated cells were separately treated with 15 and 18.55 µg/ml of Arazyme, ARA-linker-TGFαL3, and Taxotere. After 24 h, the cells were trypsinized and then washed with PBS. Cells were stained with Annexin V-coupled FITC and propidium iodide (BD, San Diego, CA, USA). After 15 min incubation in the dark, the cells were analyzed with FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). According to the manufacturer’s protocol, 500 µl of the lysis buffer was added to cell pellets for 20 min at 4 °C. The dissolved cells were centrifuged and 50 µl of supernatant was mixed with 55.5 µl of the reaction solution (5 mM DEVD-pNA, DTT, and caspase buffer) and incubated for 2 h at 37 °C. The absorption was read at 405 nm by an ELISA microplate reader. Also, the absorption of the different concentrations (0, 10, 20, 30, 40, and 50 µM) of the substrate (pNA) was used to make a standard curve. Finally, caspase activity (µU/ml) was calculated by the following formula:

\[
\text{Caspase activity (µU/ml)} = \frac{\text{µM of pNA} \times \text{Total volume of assay (ml)}}{\text{Volume of sample (ml)} \times \text{Dilution coefficient} \times \text{Time (min)}}
\]

**Gene expression analysis by RT-PCR**

The quantitative expression analysis of some genes responsible for apoptosis (bax, bcl2, and casp3), angiogenesis (vegfr2), and metastasis (mmp2 and mmp9) was performed by SYBR-Green real-time PCR. Briefly, the plated MDA-MB-468 and MDA-MB-453 cells with a density of 1 × 10^6 cells/well were treated with 15 µg/ml of recombinant proteins followed by incubation at 37 °C and 5% CO2 for 24 h. Then, the total RNA was extracted by YTZol reagent (YTA, Iran) and cDNA was synthesized (YTA, Iran) according to the instruction of the manufacturer (M-MLV reverse transcriptase, first-strand buffer [250 mM Tris–HCl (pH 8.3 at 25 °C), 375 mM KCl, 15 mM MgCl2 50 mM DTT], 50 µM Oligo (dT) 18 primer, 40 U/µl RNasein, and 10 mM dNTP). β-Actin gene was used as an endogenous internal control. The PCR reaction mixture included 1 µl cDNA, 0.5 µl of each forward and reverse primers, 6.5 µl of SYBR-Green qPCR Master Mix (2×) (YTA, Tehran, Iran) (100 mM KCl, 5 mM MgCl2, 400 µM dNTPs, 0.1 U/µl Hot Start Taq DNA polymerase, 1 × SYBR Green), and 4.5 µl of nuclease-free water. Each cycle of RT-PCR consisted of denaturation at 95 °C for 2 min, 40 cycles at 95 °C for 5 s, and 60 °C for 40 s, which were conducted on ABI StepOne real-time PCR thermal cycler. The fold change in expression of genes was analyzed by the 2^{−ΔΔC_{t}} method [24].

**Caspase-3 activity determination**

The MDA-MB-468 and MDA-MB-453 cells with a density of 5 × 10^5 cells/well were treated with 15 µg/ml of Arazyme and ARA-linker-TGFαL3 and incubated at 37 °C and 5% CO2 for 24 h. Then, caspase-3 activity assay was done by the colorimetric assay Kiazist kit. Briefly, after trypsinization, the suspension was centrifuged and the supernatant was discarded. According to the manufacturer’s protocol, 500 µl of the lysis buffer was added to cell pellets for 20 min at 4 °C. The dissolved cells were centrifuged and 50 µl of supernatant was mixed with 55.5 µl of the reaction solution (5 mM DEVD-pNa, DTT, and caspase buffer) and incubated for 2 h at 37 °C. The absorption was read at 405 nm by an ELISA microplate reader. Also, the absorption of the different concentrations (0, 10, 20, 30, 40, and 50 µM) of the substrate (pNA) was used to make a standard curve. Finally, caspase activity (µU/ml) was calculated by the following formula:

\[
\text{Caspase activity (µU/ml)} = \frac{\text{µM of pNA} \times \text{Total volume of assay (ml)}}{\text{Volume of sample (ml)} \times \text{Dilution coefficient} \times \text{Time (min)}}
\]

**Lactate dehydrogenase (LDH) release assay**

The cytotoxicity of recombinant proteins was tested through leakage of cytoplasmic lactate dehydrogenase (LDH) to the surrounding culture medium, which occurred upon cell death. Based on the Kiazist kit instruction, Arazyme and ARA-linker-TGFαL3 with a concentration of 15 µg/ml were added to the MDA-MB-468 and MDA-MB-453 cells with a density of 2 × 10^5 cells/well, separately. To prepare positive and negative control cells, 20 µl of Permi Solution (Trition X-100) and sterile water were used, respectively. Then, the suspension was centrifuged (400 × g for 5 min) and 50 µl of supernatants were mixed with 50 µl of a solution, including LDH assay buffer (Resazurin) and co-substrate (NADH) for 30 min at 37 °C in the dark, followed by reading the absorption at 570 nm by the ELISA microplate reader. Background absorption of uncultured wells was subtracted from the absorption of treated and control cells. Finally, the relative cytotoxicity percentage was calculated by the following formula:
Adhesion assay

Arazyme and ARA-linker-TGFαL3 at a concentration of 15 µg/ml were added to the MDA-MB-468 and MDA-MB-453 cell suspension (at a density of 5 × 10^5 cells/well). After 1 h, the treated cells were transferred to a 96-well plate and incubated at 37 °C and 5% CO_2 for 1 h. Then, the unattached cells were removed by washing twice with PBS. After fixation with methanol for 5 min on ice, the attached cells were stained with toluidine blue (1% w/v) in sodium tetraborate (1% w/v) for 5 min followed by washing with PBS. Finally, SDS (1% w/v) was added to each well at 37 °C for 20 min and the absorption of the developed color solution was read at 540 nm. The percentage of adherent cells was calculated and compared to the untreated cells which showed to be 100% adherent [25].

Statistical analysis

All tests were performed in triplicate, and the results were represented as the mean ± SD. Unpaired t-test of independent experiments and one-way ANOVA were performed to analyze data by using GraphPad Prism (8.0.2 for Windows, GraphPad Software, San Diego, California, USA, RRID:SCR_002798) and p-values less than 0.05 were considered significant.

Results

Expression, purification, and confirmation of recombinant proteins

The synthase structure and the length of the araA gene (1482 bp) were verified in our previous study [14]. As shown in Fig. 1b, agarose gel electrophoresis revealed that the length of PCR amplified product of ARA-linker-TGFαL3 was 1716 bp, which is identical to the expected size in the chimeric construct. Meanwhile, the sequence determination of PCR products confirms the construct synthesized by Biomatik Company (data are not shown). The expression of Arazyme and ARA-linker-TGFαL3 in E. coli BL21 harboring cloned vector was optimized at 37 °C for 4 h using 1 mM of IPTG. The concentrations of Arazyme and ARA-linker-TGFαL3 after purification by Ni–NTA affinity chromatography under hybrid conditions were 1.229 and 881 mg/ml, respectively, which were obtained from 1 l of IPTG induced culture (Fig. 1c, d). The western blot analysis confirms that the presence of bands in the 51.5 kDa and 59.3 kDa region on the SDS-PAGE gel is identical to the predicted size of the Arazyme and ARA-linker-TGFαL3, respectively (Fig. 1e).

The activity of purified recombinant proteins was maintained

The measured protease activities of purified Arazyme and ARA-linker-TGFαL3 were 0.87 U/mg and 0.86 U/mg, respectively. In addition, both recombinant proteins revealed higher enzyme activity after purification (p < 0.05, Fig. 1f).

ARA-linker-TGFαL3 shows binding affinity to EGFR

After 24 h, cell culture was treated with different concentrations (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg/ml) of 6His-tagged Arazyme and ARA-linker-TGFαL3 and subsequently, incubated with an HRP-conjugated anti hexahistidine antibody. There is a significant difference between ARA-linker-TGFαL3 and Arazyme in binding to MDA-MB-468 cells (p < 0.05) and in contrast to Arazyme, higher concentrations of ARA-linker-TGFαL3 increased its binding affinity to MDA-MB-468 cells, significantly (p < 0.0001). Furthermore, the binding of different concentrations of both recombinant proteins did not significantly change when added to the MDA-MB-453 cell culture (Fig. 2a, b).

ARA-linker-TGFαL3 inhibits tumor cell growth

After 24 h incubation, the viability of both cell lines was reduced with higher concentrations of Arazyme and ARA-linker-TGFαL3. At concentrations ranging from 0 to 40 µg/ml, the cytotoxicity effect of ARA-linker-TGFαL3 is higher than its effects on MDA-MB-453 cells (p < 0.01), while the cytotoxicity of Arazyme is indifferent between the two cell lines (Fig. 3a, b). Furthermore, both recombinant proteins and Taxotere dose-dependently (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg/ml) decreased cell viability of MDA-MB-468 and MDA-MB-453 cells (p < 0.0001). Meanwhile, in all examined concentrations, when MDA-MB-468 cells were treated with ARA-linker-TGFαL3, the higher cytotoxicity was obtained compared to cells treated with Arazyme (p < 0.0001). Furthermore, the viability of MDA-MB-468 cells was less than 50% when concentrations higher than...
25 µg/ml of ARA-linker-TGFαL3 and Taxotere were separately added to the cell culture (Fig. 3a, c). In addition, Taxotere caused over 50% cell death in MDA-MB-453 cells at lower concentrations compared to Arazyme and ARA-linker-TGFαL3 (Fig. 3d). The calculated IC₅₀ (The half-maximal inhibitory concentration) values for the MDA-MB-468 cells after treatment with ARA-linker-TGFαL3, Arazyme, and Taxotere were 18.66, 64.33, and 18.49 µg/µl, respectively, and for the MDA-MB-453 cells were 30.71, 68.38, and 13.07 µg/µl, respectively.

**ARA-linker-TGFαL3 induces apoptosis in both cell lines**

The cells have been stained with Annexin V and propidium iodide for the evaluation of induced apoptosis. The percentage of early and late apoptotic cells was increased in
a

MDA-MB-468 treated with ARA-linker-TGFαL3

MDA-MB-468 treated with Arazyme

p<0.0001

Cell viability (%)

Concentration (μg/ml)

b

MDA-MB-453 treated with ARA-linker-TGFαL3

MDA-MB-453 treated with Arazyme

p<0.0001

Cell viability (%)

Concentration (μg/ml)

c

MDA-MB-468 treated with Taxotere

p<0.0001

Cell viability (%)

Concentration (μg/ml)

d

MDA-MB-453 treated with Taxotere

p<0.0001

Cell viability (%)

Concentration (μg/ml)
both cell lines after adding 15 (0.25 nM) and 18.66 μg/ml (0.311 nM) of ARA-linker-TGFαL3, Arazyme, and Taxotere compared to the related controls ($p < 0.01$). As shown in Fig. 4a, the percentage of MDA-MB-468 early apoptotic cells increased more than any other cell group after treatment with ARA-linker-TGFαL3 ($p < 0.05$). Furthermore, there was no significant difference between the number of ARA-linker-TGFαL3 and Arazyme treated MDA-MB-453 early apoptotic cells (Fig. 4b). By contrast, the highest percentage of late apoptotic cells of MDA-MB-468 and MDA-MB-453 was obtained after treatment with Taxotere ($p < 0.05$).

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**Fig. 3** The cytotoxic effect of different concentrations of a, b ARA-linker-TGFαL3, Arazyme, and c, d Taxotere on MDA-MB-468 and MDA-MB-453 cell lines. The results are shown as means ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ represents statistical differences analyzed by unpaired $t$-test and one-way ANOVA.

**Fig. 4** Flow cytometry analysis of apoptosis in a MDA-MB-468 and b MDA-MB-453 cells induced by ARA-linker-TGFαL3, Arazyme, and Taxotere in concentrations of 15 μg/ml and 18.66 μg/ml.
ARA-linker-TGFαL3 increased expression of apoptosis genes

The relative expression level of the apoptosis bax gene was elevated by ARA-linker-TGFαL3 and Arazyme in both cell lines. Furthermore, the highest folding expression value of bax and casp-3 genes, as well as the highest bax/bcl2 ratio, was obtained when MDA-MB-468 cells were treated with 15 µg/ml of ARA-linker-TGFαL3 (p < 0.05, Fig. 5a, b). After the addition of ARA-linker-TGFαL3, the expression of the mmp9 in MDA-MB-468 cells was lower than in Arazyme-treated MDA-MB-468 and MDA-MB-453 cells (p < 0.05), while two recombinant proteins similarly caused mmp2 expression reduction. Moreover, the decreased expression level of vegfr2 was detected in ARA-linker-TGFαL3-treated MDA-MB-468 cells as compared to those incubated with Arazyme (Fig. 5c). For all genes evaluated in MDA-MB-453 cells, the effect of both recombinant proteins on the expression level was not significantly different.

ARA-linker-TGFαL3 activated caspase-3

The standard curve was drawn based on different concentrations of activated caspase-3. After adding 15 µg/ml of Arazyme and ARA-linker-TGFαL3 proteins, the level of caspase-3 activity within the treated cells was compared with
each of the above proteins. As shown in Fig. 6a, ARA-linker-TGFαL3 induces caspase-3 activation in MDA-MB-468 more than in MDA-MB-453 cells and has better results compared to Arazyme on both examined cell lines (p < 0.05).

**Increased LDH release indicates cytotoxicity of ARA-linker-TGFαL3**

Following treatment with recombinant proteins, LDH release was determined from both cell lines. At a concentration of 15 µg/ml, ARA-linker-TGFαL3 caused higher cytotoxic effects on MDA-MB-468 cells compared to Arazyme (p < 0.05), while the two recombinant proteins exhibited similar cellular cytotoxicity in MDA-MB-453 cell lines (Fig. 6b).

**ARA-linker-TGFαL3 prevents adherence of tumor cells**

Both recombinant proteins decreased the percentage of attached cells of MDA-MB-468 and MDA-MB-453 cells and the highest decrease resulted after the treatment of MDA-MB-468 cells with 18.66 µg/ml of ARA-linker-TGFαL3, although the differences were not significant (Fig. 6c).
Discussion

The antitumor effects of Arazyme have been well demonstrated due to its cytotoxic effect and induction of antibody production against it. The cross-reaction between Arazyme-specific IgG and matrix metalloprotease MMP8 inhibited metastasis in murine melanoma [14, 25]. The chimeric proteins consisting of TGFαL3 and staphylococcal enterotoxin A and B were examined to eliminate cancer cells [21, 26]. In this manner, we designed ARA-linker-TGFαL3 to concentrate the anti-cancer activity of Arazyme on breast cancer cell lines with overexpression of EGFR. To maintain the true folding of recombinant proteins, the purification has been performed by the hybrid method and the biological activity of the enzyme was confirmed by a protease activity test. To compare ARA-linker-TGFαL3 and Arazyme for specific binding to the receptor, the ELISA test was employed. The efficiency of binding affinity of chimera proteins posing the third loop of TGFα has been revealed in previous studies. Xu et al. examined the affinity of mutant staphylococcal enterotoxin A fused to TGFαL3 (1 ng/ml to 1 µg/ml) for binding to A431 cells as an EGFR-expressing cells which showed the higher binding affinity of fusion protein compared to single enterotoxin A [26]. Yousefi et al. indicated the improved binding ability of the chimera protein comprising of staphylococcal enterotoxin B and TGFαL3 to A431 cells at concentrations ranging between 1 ng/ml and 1 µg/ml [21]. Also, the higher affinity of TGFαL3-SEB (25 to 75 µg/ml) to human colorectal adenocarcinoma (HT-29) was demonstrated [27]. In agreement with previous studies, our data showed a higher affinity of ARA-linker-TGFαL3 to MDA-MB-468, an EGFR positive cell line which may be due to specific binding of TGFαL3 to EGFR.

The cytotoxicity of ARA-linker-TGFαL3 and Arazyme was assessed by MTT assay, based on the detection of mitochondrial activity in living cells [22]. Furthermore, the proportion of the death cells was evaluated by measuring the activity of LDH that was released following membrane damage [28]. Hashimi et al. evaluated the anti-cancer activity of a chimera molecule comprising EGF and Pseudomonas aeruginosa exotoxin A and revealed its cytotoxicity effects on several cancer cells with different expression levels of EGFR. The IC₅₀ values obtained for head and neck cancer cells (HN5) as EGFR positive and breast ductal carcinoma cells (MCF-7) as EGFR negative were 36 and > 10,000 ng/ml, respectively [29].

Yang et al. treated human esophageal cancer cell lines (KYSE-450 and KYSE-150) by employing a fusion protein constructed of an EGFR-targeted antibody and exotoxin A. The calculated IC₅₀ values for KYSE-150 and KYSE-450 were 11.43 and 2.195 nM, respectively [30]. By using MTT assay, Kim et al. reported Arazyme toxicity on human keratinocyte cell line (HaCaT) at concentrations ranging between 10 and 50 µg/ml while human mast cells (HMC-1), human monocytic (THP-1), and human eosinophilic leukemia (EoL-1) cell lines were not affected [31].

Meanwhile, Amjadi et al. revealed the effect of Arazyme (16 to 128 µg/ml) against HT-29 cell viability by employing MTT and LDH assays [14]. The cytotoxicity of Arazyme is related to its metalloprotease activity because the cell viability was not affected by the heat-inactivated Arazyme [25].

In the current study, the results of MTT and LDH assays showed the anti-proliferation activity of both recombinant proteins, but the lower value of IC₅₀ obtained by ARA-linker-TGFαL3 can be due to targeting by its ligand segment. The similar cytotoxicity in concentrations higher than 40 µg/ml may be explained by the limited number of binding sites of the receptor that is saturated at a high ligand concentration [32]. Furthermore, ARA-linker-TGFαL3 and Taxotere showed an almost close IC₅₀ value on MDA-MB-468 cells unlike their different cytotoxic effect on MDA-MB-453 cells, wherein Taxotere shows the most anti-proliferation activity.

Various cellular stresses can lead to one of three forms of cell death, including apoptosis, autophagy, and necrosis. Unlike apoptosis and necrosis, cell death that occurs by autophagy is reversible. Furthermore, necrotic cells suffer from cell membrane disruption, which ultimately leads to inflammatory response while apoptotic cells are noninflammatory and are quickly phagocytosed by macrophages [33]. On the other hand, some anti-cancer therapies can stimulate apoptosis with inflammatory responses due to the release of danger-associated molecules (DAMPs) from cancer cells [34]. The early apoptosis induced in KYSE-450 cells by 10 nM fusion protein comprising EGFR-targeted antibody and exotoxin A was reported after 16 h of incubation while Arazyme (16–128 µg/ml) caused early apoptosis in HT-29 cells after 24 h [14, 30]. In the present study, the targeting ability of ARA-linker-TGFαL3 was shown because compared to the effects of Arazyme, it significantly with a higher percentage caused early apoptosis in MDA-MB-468 cells. On the other hand, the late apoptosis caused by 15 µg/ml TGFαL3 to KYSE-150 was demonstrated due to its cytotoxic effect and induction of anti-apoptosis with inflammatory responses due to the release of danger-associated molecules (DAMPs) from cancer cells [35]. The early apoptosis induced in KYSE-450 cells by 10 nM fusion protein comprising EGFR-targeted antibody and exotoxin A was reported after 16 h of incubation while Arazyme (16–128 µg/ml) caused early apoptosis in HT-29 cells after 24 h [14, 30]. In the present study, the targeting ability of ARA-linker-TGFαL3 was shown because compared to the effects of Arazyme, it significantly with a higher percentage caused early apoptosis in MDA-MB-468 cells. On the other hand, the late apoptosis caused by 15 µg/ml TGFαL3 to KYSE-150 was demonstrated due to its cytotoxic effect and induction of anti-apoptosis with inflammatory responses due to the release of danger-associated molecules (DAMPs) from cancer cells [35].

Downregulation of bcl2, alone or with up-regulation of bax expression, and inhibition of bcl2 related mRNA or protein were used as the targets of anti-cancer therapies [37, 38]. In the current study, both chimera proteins (15 µg/ml) increased bax expression level in MDA-MB-468 cells but not in MDA-MB-453 cells. Subsequently, the elevated
expression ratio of bax/bcl2 can confirm apoptosis detected in MDA-MB-468. Caspase-3 is one of the executioner caspases which is activated after cleavage with initiator caspases-8 and -9 upon ligands binding to death receptors or occurrence of different cellular stresses [39]. Hyatt and Ceresa indicated that only internalized and activated EGFRs can activate caspase-3 in MDA-MB-468 cells [40]. By contrast, Nowsheen et al. showed that EGFR inhibitors block EGFR translocating to nuclear. As a result, DNA repairing capacity was altered and caspase-3 activation was stimulated [41]. On the other hand, Arazyme was displayed to activate intrinsic pathways of apoptosis through activation of caspase-3 and -9 in HT-29 cells [14]. Our findings indicated that Arazyme and ARA-linker-TGFαL3 induced the caspase-dependent apoptosis because of significant activation of caspase-3 and the increased folding expression of its related gene in MDA-MB-468 and MDA-MB-453. Also, the highest obtained folding expression of caspase-3 in ARA-linker-TGFαL3-treated MDA-MB-468 cells may be due to inhibition of EGFR by occupying its binding site, which led to improving the apoptosis induced by Arazyme.

The matrix metalloproteinases, MMP2, and MMP9 are the key biomarkers of tumor development, which elevate tumor invasion and metastasis by degrading extracellular matrices [42]. The anti-adhesion effect of Arazyme was demonstrated in HT-29 cells [14]. Furthermore, Arazyme inhibits the adhesion of B16F10-Nex2 (murine melanoma cell line) through proteolytic cleavage of CD44, a surface protein that is a regulatory molecule for invasion, adhesion, and metastasis in cancer cells [25]. Also, Arazyme reduced the expression of mmp2, and mmp9 in human ovarian cancer (SKOV3) and HT-29 [43]. Based on our study, Arazyme and ARA-linker-TGFαL3 suppressed adhesion of MDA-MB-468 and MDA-MB-453 cells as well as inhibition of the expression of mmp2, while the expression of mmp9 was not affected.

In addition to angiogenesis, VEGFR2 plays role in cell proliferation, survival, and permeability [44]. To target VEGFR2, Safari et al. revealed the sensitivity of human umbilical vein endothelial cell (HUVEC) and Michigan Cancer Foundation-7 (MCF-7) to a fusion protein (10 ng/ml) comprising of anti-VEGFR2 and a truncated form of exotoxin A [45]. The expression of vegfr2 was shown in HT-29 cells and its expression was inhibited by Arazyme [14]. In the current study, ARA-linker-TGFαL3 and Arazyme decreased vegfr2 expression in MDA-MB-453 cells more than what was expected in the MDA-MB-468 cell line, which may be due to differences in vegfr2 expression level between the two cell lines.

In conclusion, the fusion protein ARA-linker-TGFαL3 showed higher anti-cancer activity on EGFR-expressing breast cancer cells when compared with Arazyme while both recombinant proteins possess similar anti-proliferative and anti-invasion effects on low EGFR-expressing cell. Furthermore, the sensitivity of examined TNBC breast cancer cells to ARA-linker-TGFαL3 suggested it as a possible candidate for an anticancer agent against this type of breast cancer cells, although further in vivo studies are required.

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Declarations

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