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Research Article

The Cytotoxicity Effect of Recombinant Arazyme on Breast and Ovarian Cancer Cells

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Abstract

Objectives: Investigating new approaches to obtain an effective therapeutic agent for treating life-threatening cancers is critical. The current study aimed to assess the anti-tumor effect of the recombinant arazyme of *Serratia proteamaculans* on ovarian and breast cancer *in vitro*.

Methods: The cytotoxic effects of r-arazyme against MCF-7 and SKOV3 cells were evaluated using MTT and lactate dehydrogenase assays. Potential apoptosis induction by r-arazyme was assessed using the Annexin V/PI kit. The Matrigel invasion test was used to evaluate the ability of r-arazyme to reduce cell invasion. In addition, an adhesion assay was performed. RT-PCR was used to measure the expression of genes involved in angiogenesis, apoptosis, and metastasis.

Results: R-arazyme showed a high cytotoxic effect against MCF-7 and SKOV3 cells in a dose-dependent manner. In addition, r-arazyme has great apoptosis-inducing potential in both cells via the activation of caspase-3 and elevation of the *BAX/BCL-2* ratio. R-arazyme significantly decreased the expression levels of the angiogenesis-related genes *VEGFR-1* and *VEGFR-2* and inhibited both cell adhesion and invasion.

Conclusion: R-arazyme may eventually play an essential role in the development of effective therapies against ovarian and breast cancers, thereby reducing the overall morbidity and death caused by cancer.

Keywords: Apoptosis, breast cancer, ovarian cancer

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Ovarian cancer is the third most prevalent and deadliest gynecological malignancy in the female reproductive system. It presents a significant challenge for healthcare systems worldwide because of its aggressive growth, rapid metastasis, substantial invasion, and poor prognosis.[1] Breast cancer is the most prevalent cancer, and after lung cancer, it is the second leading cause of cancer death.[2]

The shortage of efficient anti-cancer agents and the high incidence, morbidity, and mortality rates of ovarian and breast cancer indicate that designing new and effective therapeutic agents to treat ovarian and breast cancer patients is crucial. Plant- and bacterial-derived products are becoming known as the main sources of new and effective anti-cancer agents to develop for the treatment of metastatic ovarian and breast cancer. In the experimental mod-

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els, a mixture of exogenous proteinases such as trypsin, chymotrypsin, and papain was administered, and tumor growth was effectively inhibited.[3]

The bacterial protease that has a protective effect on cancer cells is arazyme. These zinc metalloproteases belong to the serralysin family. Arazyme with a relative molecular mass of 51.5 kDa was secreted by *Serratia proteamaculans*.[4, 5] A previous study revealed that arazyme, by inducing overexpression of SMP30, inhibition of the TGF-β/Smad pathway, and enhancing antioxidant expression, could protect against acute hepatic damage induced by CCl4.[6] In addition, this enzyme has an anti-inflammatory effect that inhibits inflammation by cleaving cytokines, including IL-6 and MCP-1, and adhesion molecules because it may hydrolyze pro-inflammatory molecules such as bradykinin and histamine.[7, 8] In murine metastatic melanoma, arazyme cleaved tumor matrix metalloprotease 8 (MMP-8) antibodies and the tumor cell surface adhesion receptor CD44. In addition, arazyme effectively inhibits melanoma development.[9]

In this study, various aspects of the *in vitro* anti-cancer effects of recombinant arazyme (r-arazyme) were assessed by evaluating cell viability, adhesion/invasion, and apoptosis pathways in MCF-7 (human breast adenocarcinoma) and SKOV3 (human ovarian cancer) cell lines.

Methods

Bacterial Strain and Cell Lines

The *Escherichia coli* BL21 strain (DE3) and TOP10 were purchased from Novagen Inc. (Madison, WI, USA). MCF-7 and SKOV3 cell lines were purchased from Pasteur Institute (Tehran, Iran).

The Expression of Arazyme Protein

The expression vector pET28a, which harbors a T7 promoter, kanamycin-resistant gene, and 5' six His-tag, was selected for the expression of the arazyme-encoding gene (araA), whose sequence was obtained from the NCBI gene bank (Accession No: AY818193.1). Furthermore, the restriction sites of BamHI and XhoI (Fermentas, Lithonia) were added at the 5' and 3' ends, respectively. The pET28a/araA construct was prepared by Biomatik Corporation (Cambridge, Ont., Canada), and DNA sequencing, restriction digestion, and PCR were used to verify the correct insert of the *araA*. The IPTG concentration (1 mM) used to induce *araA* expression in *E. coli* BL21 (DE3) was optimized in a previous study 10. The size of r-arazyme was approximately 51.5 kDa according to the SDS-PAGE gel.[10] Because the overexpression of the recombinant protein was obtained, the on-column re-solubilization protocol was employed at the same time as the affinity purification of the protein was performed by a Ni-NTA agarose-based procedure, as previously described.[11-15] As previously stated, the pure recombinant protein was verified by Western blotting with a monoclonal anti-polyHistidine–Peroxidase antibody (Cat# A7058, Sigma-Aldrich, USA).[12]

Cell Culture

RPMI-1640 medium (Gibco, Germany) comprising 1% penicillin/streptomycin (100 U/mL penicillin and 100 mg/L streptomycin) and 10% fetal bovine serum (FBS) was used to culture MCF-7 and SKOV3 cells at 37° C in the presence of 5% CO2 for 72 h. All chemicals and cell culture medium were purchased from Gibco (Grand Island, NY, USA).

MTT Assay to Determine Cell Viability

To examine cell viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed for MCF-7 and SKOV3 cell lines, as previously described.[16-18] Briefly, 1 × 105 cells/mL of cells were treated with 4, 8, 16, 32, 64, and, 128 µg/ml of r-arazyme for 24 h. MTT solution (Sigma-Aldrich; USA) was applied to the cells, which was then replaced with dimethyl sulfoxide (DMSO, Sigma-Aldrich). The absorbance was measured at 570 nm. The percentage of cytotoxicity activity was calculated as follows: cytotoxicity activity (%) = [1 − (absorbance of experimental well/ absorbance of negative control well)] × 100.

Lactate Dehydrogenase (LDH) Assay to Assess Cell Cytotoxicity

Briefly, to perform the lactate dehydrogenase (LDH) release assay,[19] MCF-7 and SKOV3 cells were incubated with different concentrations of r-arazyme (16, 32, 64, and, 128 µg/ml) for 24 h. The cells were lysed and mixed with the LDH reaction solution, and the absorbance of the reaction mixture was measured at 490 nm.

Cell Apoptosis Assay by Annexin V/PI Kit Staining

Annexin V/PI staining was used to assess apoptosis. For this purpose, in 6-well tissue culture plates, MCF-7 (2×105 cells/well) and SKOV3 (2×105 cells/well) were treated with concentrations of 16, 32, 64, and, 128 µg/ml of r-arazyme for 24 h. The cell culture supernatant was then separated using a centrifuge (1500 g) for 5 min. The cell pellet was washed twice with PBS and stained with PI (6 mg/ml) and Annexin V (5 mg/ml) solution for 20 min in the darkroom. A FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and FlowJo software version 10 (FlowJo, LLC, USA) were used to examine the cells.

Detection of Caspase-3 and -9 Activity by Colorimetric Assay

Caspase-3 and -9 activities induced by 16, 32, 64, and, 128 µg/ml of r-arazyme were measured using the caspase-3 or caspase -9 colorimetric assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions.[20]

Gene Expression Changes by Real-Time Quantitative PCR

The mRNA expression level of *BAX* and *BCL-2* (apoptosis-regulatory genes) as well as *VEGF-A, VEGFR-1, and VEGFR-2* (angiogenesis genes) were evaluated by quantitative RT-PCR (SYBR Green-based) with specific oligonucleotide primers, as previously described.[21-23] Briefly, the cells were plated at a density of 1 × 106 cells/well and treated with 30 µg/ml r-arazyme, followed by incubation at 37° C in 5% CO2 for 24 h. Total RNA was extracted using YTzol reagent (YTA, Iran), and cDNA was generated using a kit (M-MLV reverse transcriptase, first-strand Buffer (250mM Tris-HCl (pH 8.3 at 25°C), 375 mM KCl, 15 mM MgCl2, 50DTT), 50µM Oligo (dT)18 primer, 50µM Random hexamer primer, 40u/µl RNasein, and 10 mM dNTP). As an endogenous internal control, GAPDH was used. The 2^(-ΔΔCT) method was used to examine the fold change in gene expression.[24]

The Effect of R-arazyme on the Adhesion of Cancer Cells

Adhesion assay was performed according to a previously described.[9] First, r-arazyme-treated (16, 32, 64, and, 128 µg/ml) and untreated cells were fixed with methanol and subsequently stained with 1% toluidine blue in 1% sodium tetraborate and solubilized in SDS 1%. Finally, the absorbance at 540 nm was measured.

Matrigel Assay to Detect Invasion

To detect invasion by the Matrigel assay, cold Matrigel (Basement Membrane Matrix, BD Biosciences, NJ, USA), which was previously diluted with serum-free RPMI-1640 medium, was added to the upper transwell chambers (8-mm pore size, Corning Costar Co., MA, USA). Next, RPMI-1640 medium (supplemented with 10% FBS) was added to the lower chambers. The gel was formed at 37 °C for 30 min. MCF-7 and SKOV3 cells (2×105/mL) were incubated with r-arazyme (16, 32, 64, and, 128 µg/ml) in serum-free RPMI-1640 medium at 37° C and 5% CO2 for 1 h, then added to the upper transwell compartment and incubated at 37 °C and 5% CO2 for 5 h. After eliminating non-invasive cells, paraformaldehyde (3.7%) was employed to fix (for 15 min) the cells underneath the membrane filter and then stained with a 0.1% toluidine blue solution. Next, the filters were incubated with 1% SDS solution (200 mL) at 37 °C for 1 h. The absorbance of this solution was measured at 600 nm on a 96-well ELISA plate.[25]

Statistical Analysis

GraphPad Prism 8.0.2 (GraphPad Software, San Diego, California, USA) was used for statistical analyses. The results were analyzed by one-way analysis of variance and unpaired t-test of independent experiments. All values are presented as the Mean±standard error of the mean (SEM), and a p-value of < 0.05 was considered statistically significant (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns (non-significant)).

Results

R-arazyme Exhibited High Cytotoxic Activity Against MCF-7 and SKOV3 Cells

The cytotoxic effect of r-arazyme against MCF-7 and SKOV3 cells was determined using the MTT assay. Figure 1a and 1b shows that r-arazyme treatment dose-dependently reduced MCF-7 and SKOV3 cell viability (p<0.0001), and at concentrations more than 32 µg/ml, cell viability was reduced by over 50%. The IC50 values of r-arazyme against MCF-7 and SKOV3 were 30.40 and 38.86 µg/ml, respectively.

R-arazyme Induced LDH Releasing from MCF-7 and SKOV3 Cells

The cytotoxicity effect of r-arazyme on MCF-7 and SKOV3 cells was evaluated by using the LDH enzyme release as-

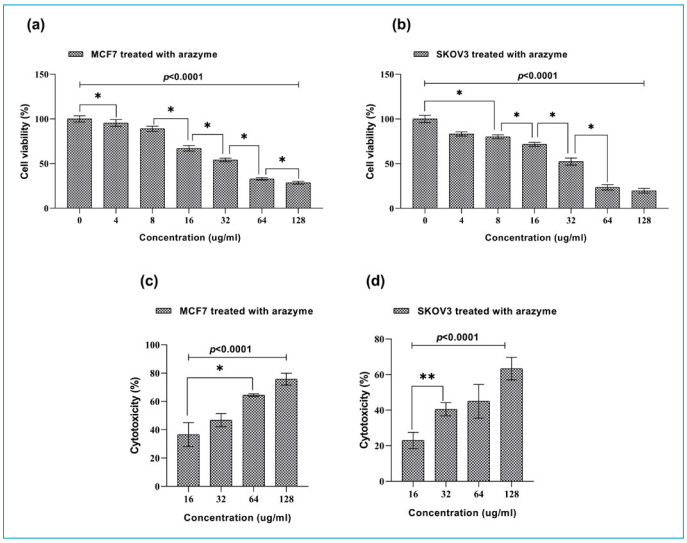

Figure 1. The cytotoxic effects of r-arazyme on MCF-7 and SKOV3 cell lines using MTT **(a and b)** and LDH **(c and d)** tests. The cell lines were incubated with different concentrations of r-arazyme (4-128 µg/ml). Data represent the mean±SEM of three independent experiments. The statistical differences (*p<0.05, **p<0.01) analyzed by unpaired t-test and one-way ANOVA.

say. Similar to the MTT results, the cytotoxicity of r-arazyme was dose-dependently increased following the addition of MCF-7 and SKOV3 cells medium ($p<0.0001$). However, SKOV3 cells were affected by r-arazyme at a concentration lower than that in MCF-7 cells (Fig. 1c and 1d).

R-arazyme Caused Apoptosis of MCF-7 and SKOV3 Cells

To determine whether the cytotoxic effect of r-arazyme against MCF-7 and SKOV3 cells is due to apoptosis or not, Annexin V/PI double staining was performed. As shown in Figure 2, the percentage of viable cells in the r-arazyme-treated groups significantly decreased in a dose-dependent manner ($P< 0.05$). Following treatment, the percentage of early apoptotic MCF-7 cells with 32, 64, and 128 µg/ml of r-arazyme significantly increased from 5.01% in untreated cells to 21.3, 34.6, and 58.7%, respectively ($p<0.05$). After treatment with 16, 32, 64, and 128 µg/ml of r-arazyme, the percentage of late-stage apoptotic MCF-7 cells significantly increased to 10.3, 30.6, 57.5, and 29.6%, respectively. The percentage of early apoptotic SKOV3 cells following treatment with 32, 64, and 128 µg/ml of r-arazyme significantly increased from 3.66% in untreated cells to 14.6, 15.4, and 44.2%, respectively ($p<0.05$). In the presence of 32 and 64 µg/ml of r-arazyme, the percentage of late apoptotic SKOV3 cells was significantly higher than that of early apoptotic cells ($p<0.05$). There was no significant difference between early and late apoptotic SKOV3 cells in the presence of 16 and 128 µg/ml r-arazyme ($p>0.05$).

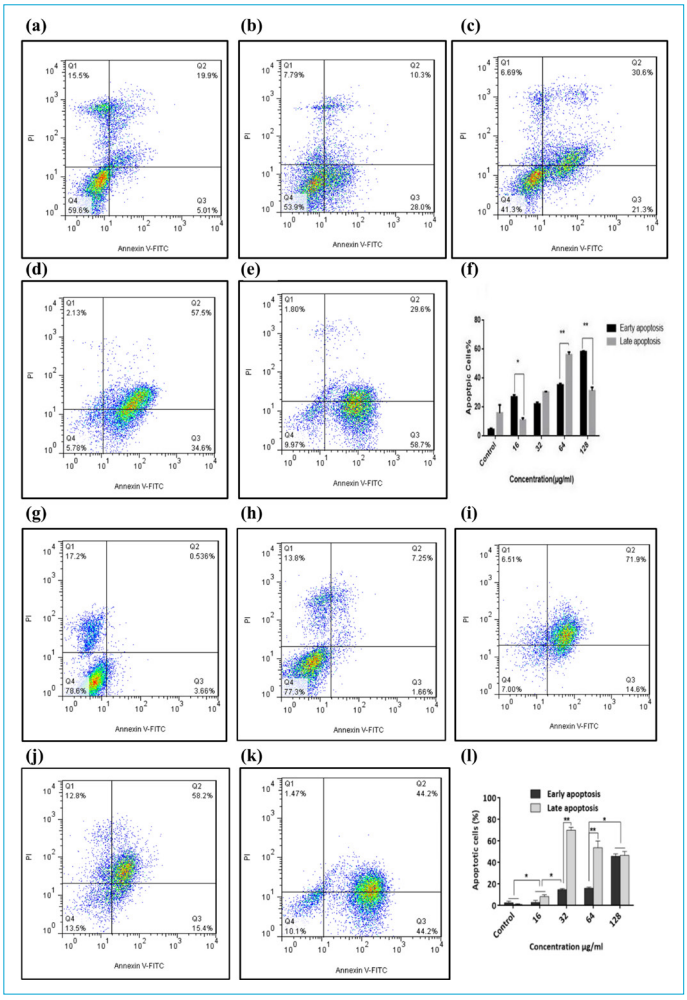

Figure 2. The effect of different concentrations of r-arazyme on MCF-7 (a: control, b: 16, c: 32, d: 64 and e: 128 µg/ml) and SKOV3 (g: control, h: 16, I: 32, j: 64 and k: 128 µg/ml) cells apoptosis using Annexin V/PI staining using flow cytometry. Illustrative figures display the population of viable (Annexin V- PI-), early apoptotic (Annexin V+ PI-), late apoptotic (Annexin V+ PI+), and necrotic (Annexin V- PI+) cells. f and l: The percentage of early and late apoptotic MCF-7 and SKOV3 cells, respectively. Data represent the mean ± SEM of two independent experiments. *p<0.05, **p<0.01.

R-arazyme Increased Caspase Activity

To examine the cell apoptotic pathway induced by r-arazyme, we analyzed caspase-3 and caspase-9 activities in MCF-7 and SKOV3 cells. As shown in Figure 3, the caspase

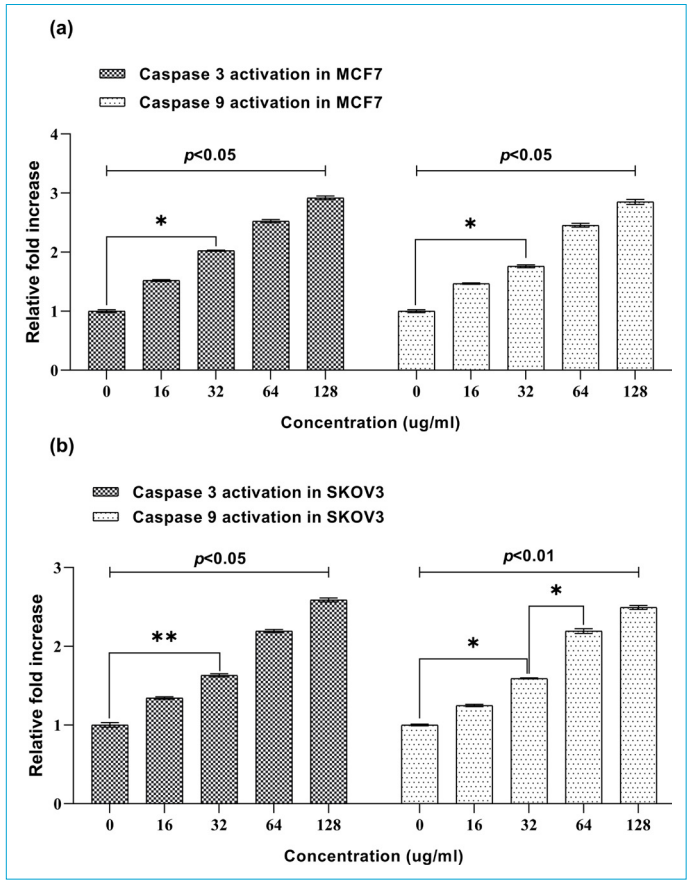

Figure 3. Caspase-3 and –9 activation in MCF-7 **(a)** and SKOV3 **(b)** cells after treatment with r-arazyme. The relative fold caspase-3 and caspase –9 activity were measured in MCF-7 and SKOV3 treated cells with 16-128 µg/ml of r-arazyme. Data represent the mean±SEM of three independent experiments. *p<0.05, **p<0.01.

3 and 9 activity of SKOV3 and MCF-7 cells after treatment with 32 µg/ml of r-arazyme significantly increased compared with the untreated cells ($p<0.05$). Furthermore, at the concentration of 64 µg/ml, caspase-9 activity was significantly increased in the SKOV3 cell line ($p<0.05$).

R-arazyme Decreased Angiogenesis and Apoptosis Genes Expression

Figure 4 depicts the mRNA expression of *VEGF-A, VEGFR-1, and VEGFR-2* in MCF-7 and SKOV3 cell lines using RT-PCR.

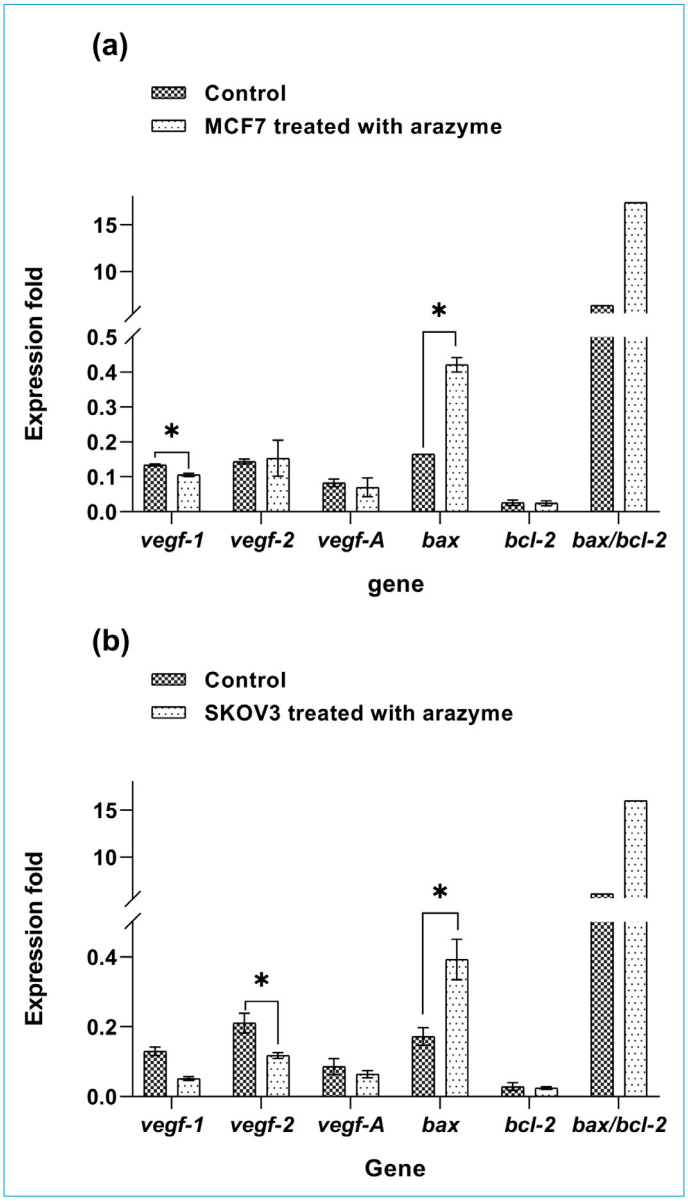

Figure 4. The analysis of apoptosis-related genes in MCF-7 **(a)** and SKOV3 **(b)** cells by Quantitative PCR after treatment with (30 µg/ml) r-arazyme. Relative mRNA levels of BAX, BCL-2, VEGFR-1, VEGFR-2, VEGF-A, and BAX/BCL-2 ratio in treated MCF-7 and SKOV3 cells versus untreated cells are expressed as the mean±SD of at least three independent experiments. *$p<0.05$.

The expression levels of *VEGFR-1* and *VEGFR-2* were significantly reduced in MCF-7 and SKOV3, respectively, compared with the untreated cells ($p<0.05$). Our results indicated that after incubation of MCF-7 and SKOV3 cells with r-arazyme, the *BAX* mRNA expression level was considerably increased ($p<0.05$). Conversely, the expression level of the *BCL-2* gene was remarkably decreased. Taken together, the *BAX*/*BCL-2* ratio of MCF-7 and SKOV3 cells considerably increased.

R-arazyme Reduced Tumor Cell Adhesion and Invasion

As shown in Figure 5, in a dose-dependent manner, r-arazyme may significantly reduce cell adhesion ($p<0.0001$). In addition, the adhesion of MCF-7 (a) and SKOV3 (b) cells in the presence of respectively 32 and 16 µg/ml of r-arazyme significantly decreased compared with the other concentrations and untreated cells ($p<0.05$). In a dose-dependent manner, r-arazyme inhibited MCF-7 (c) and SKOV3 (d) cell invasion to the Matrigel-coated substrate ($p<0.001$). After

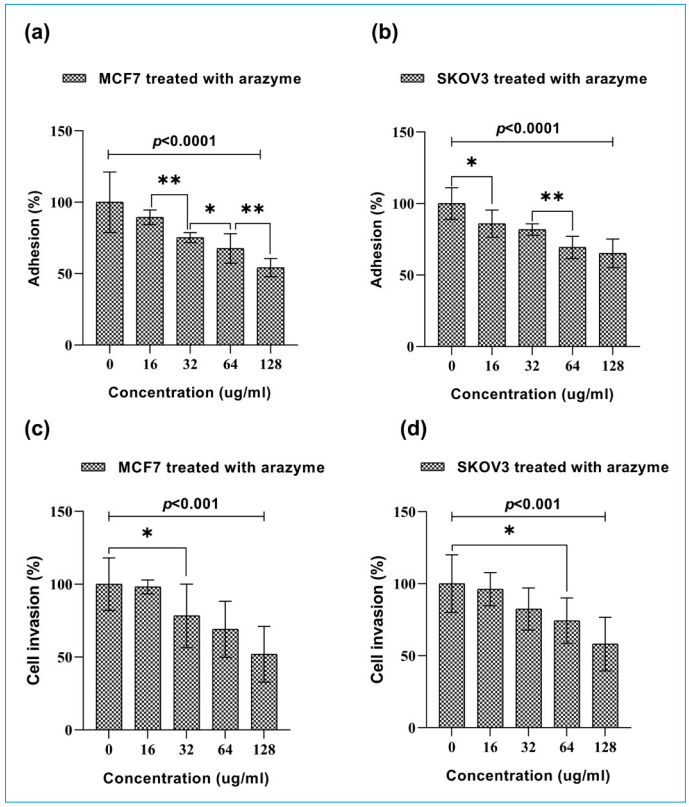

Figure 5. The effect of r-arazyme on MCF-7 and SKOV3 cells adhesion and cell invasion. Different concentrations of r-arazyme (16-128 µg/ml) were added to MCF-7 **(a and c)** and SKOV3 **(b and d)** cells. To detect the adherent cells after 3 hours of incubation, the adherent cells were stained by toluidine blue 1% in sodium tetraborate 1%. To detect the invasion, the migrated cells were stained with a 0.1% toluidine blue solution after 15 min fixation in paraformaldehyde (3.7%). Data represent the mean±SEM of three independent experiments. *$p<0.05$, **$p<0.01$.

exposure of MCF-7 cells to 32 µg/ml of r-arazyme, tumor cell invasion decreased by 78.26% ($p<0.05$). Moreover, incubation of SKOV3 cells with 64 µg/ml of r-arazyme significantly decreased tumor cell invasion by 74.3% compared with untreated cells ($p<0.05$).

Discussion

Bacterial-derived products such as toxins and proteases have been considered therapeutic strategies for treating various human cancers.[9] The metalloprotease arazyme has recently exhibited a significant anti-cancer effect in a mouse metastatic melanoma model by triggering the cleavage of tumor MMP-8 antibodies and tumor cell surface CD44.[9, 22] Arazyme, in addition to its proteolytic action, has been shown to activate macrophages and dendritic cells, as well as enhance pro-inflammatory cytokine production and surface activation markers via TLR4-MyD88-TRIF and MAPK-dependent signaling pathways. It can also boost IFNγ-dependent CD4+ and CD8+ T cell and B lymphocyte responses, which are critical in generating an anti-tumor response.[9] In the current study, the efficacy of r-arazyme as an anti-cancer drug was examined using MCF-7 and SKOV3 cells.

In evaluating the cytotoxic activity of r-arazyme *in vitro* against MCF-7 and SKOV3 cells, we found that r-arazyme has dose-dependent anti-proliferative activity. The MTT test results reveal that r-arazyme has a high potential for suppressing cell growth and killing ovarian and breast cancer cells. Irreversible damage to the cell membrane causes the release of LDH from the cytoplasm of apoptotic and necrotic cells.[26] The results of the LDH release assay indicated that r-arazyme led to potent cytotoxic effects against MCF-7 and SKOV3 in a dose-dependent manner by elevating the LDH level and cell permeability, which increased cell death. The MTT and LDH release assays results indicated that r-arazyme plays a crucial role in inhibiting cell proliferation and inducing human ovarian and breast cancer cell death.

We used an Annexin V/PI flow cytometric assay to evaluate the cell death pathways induced by r-arazyme. Analysis of flow cytometric results showed that r-arazyme treatment increased the number of cells to early and late apoptosis. Unlike necrosis, in apoptosis, dead cells are removed by immune cells such as macrophages, which ultimately does not result in adverse inflammatory responses, no damage to normal cells, or tissue damage but maintains the homeostasis of cells.[27] Cancer cells gain and exhibit resistance to apoptosis to maintain their uncontrolled proliferation; therefore, the potential of r-arazyme to inducing apoptosis can be considered a suitable characteristic to develop a chemotherapeutic agent against cancer in future studies.[28]

Apoptosis is a complex process that is categorized into two mechanisms: caspase-dependent and caspase-independent mechanisms.[29] The two main caspase-dependent pathways, including the extrinsic and intrinsic cascades, are characterized by the presence of caspase-8 or caspase-9, respectively. Otherwise, the activation of caspase-3 was detected in both intrinsic and extrinsic pathways of cell death.[27] In evaluating the molecular mechanism of the apoptosis process, we found that r-arazyme increased the activity of caspase-3 and 9 in a concentration-dependent manner, which confirmed that r-arazyme inhibits the survival of cancerous ovarian and breast cells via both intrinsic and extrinsic apoptosis pathways.

In addition, the apoptotic effects of r-arazyme in MCF-7 and SKOV3 cells were confirmed by the up-regulation of *BAX* and down-regulation of *BCL-2*, which are essential genes in the intrinsic pathway of apoptosis.[30] The anti-tumor effects of many drug components have been demonstrated through the *BAX*/*BCL-2* pathway.[21, 31, 32]

Tumor cell invasion and metastasis are multi-stage dynamic processes that involve cell adhesion, proteolytic degradation, migration, and angiogenesis.[33] Targeted anti-cancer drugs disrupt specific molecules that play a vital role in the growth of tumor cells, survival, migration, and spread of cancer invasion, thereby preventing the progression of tumor cells.[34] Data from the potency assessment of r-arazyme revealed a significant reduction in invaded cells to the Matrigel-coated substrate in a dose-dependent manner, which ultimately inhibited the adhesion and invasion of the treated MCF-7 and SKOV3 cells effectively.

VEGFR-1 and *VEGFR-2* are common anti-angiogenesis target molecules because they are the main intermediaries of angiogenesis in physiological and pathological conditions. Inhibition of angiogenesis is an essential step in the prevention and treatment of cancer because the excess concentration of angiogenesis inhibitors over angiogenesis stimulants can inhibit tumor growth and release it to other organs.[35] R-arazyme effectively inhibited ovarian and breast cancer cell angiogenesis because of a significant reduction in the expression of the angiogenesis-related genes *VEGFR-1* and *VEGFR-2*.

Conclusion

R-arazyme can be used as a suitable option for the treatment of patients with ovarian and breast cancer because it can modulate the activity or expression of regulating proteins during apoptosis, invasion, adhesion, and angiogenesis. According to these findings, a complete evaluation of r-arazyme under controlled clinical conditions seems warranted against different tumor cells. Our results reinforce

the hope that the therapeutic potential of r-arazyme can reduce the high morbidity and mortality of ovarian and breast cancers, although further studies are needed to determine the detailed mechanism of action.

Disclosures

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Authorship Contributions: Concept - A.I.; Design - A.I., H.S.; Supervision - H.S.; Materials - A.I.; Data collection - R.Z., A.G., Z.G., G.A., S.M.; Analysis and/or interpretation - R.Z., A.G., Z.G., G.A., S.M.; Literature search - R.Z., A.G., Z.G., G.A., H.S., S.M.; Writing - R.Z., A.G., Z.G., G.A., S.M.; Critical review - A.G., H.S., AI.

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