Research in Pharmaceutical Sciences, August 2024; 19(4): 387-396
Received: 25-05-2023
Peer Reviewed: 09-10-2023
Revised: 08-12-2023
Accepted: 21-07-2024
Published: 19-08-2024

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Anticancer and bioactivity effect of the AraA-IL13 fusion protein on the glioblastoma cell line

Rezvan Mehrab[1], Hamid Sedighian[2], Fattah Sotoodehnejadnematalahi[3], Raheleh Halabian[2], and Abbas Ali Imanifooladi[2,*]

[1]*Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.*
[2]*Applied Microbiology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran.*
[3]*Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.*

Abstract

Background and purpose: Glioblastoma (GBM) is an aggressive and malignant brain cancer with the highest mortality and low survival rates. To discover a more specific and efficient treatment for GBM, we synthesized and examined the cytotoxic effect of arazyme-interleukin-13 (*Ara-IL13*) fusion protein on GBM cells.
Experimental approach: At first, the *araA-IL13* chimeric gene in the pET28a (+) vector was designed and synthesized. After transformation into *Escherichia coli* BL21 (DE3), the chimeric gene was verified by colony polymerase chain reaction. Expression optimization and purification of the AraA-IL13 fusion protein was performed and subsequently evaluated by 10% SDS-PAGE. The protein was purified and concentrated using the Amicon® Ultra-15 centrifugal filter unit. The presence of AraA-IL13 was investigated by the western blotting technique. The enzyme was evaluated for proteolytic activity after purification on skim milk agar. The cytotoxic effect of the AraA-IL13 fusion protein was evaluated by MTT assay on U251 and T98G cell lines *in vitro*.
Findings/Results: The chimeric protein had no proteolytic activity on skim milk agar despite high expression. Furthermore, no cytotoxic effect of this fusion protein (up to 400 µg/mL) was observed on the U251 and T98G cell lines.
Conclusion and implications: The lack of proteolytic activity and cytotoxic effect of AraA-IL13 may be due to the disruption of the three-dimensional structure of the protein or the large structure of the arazyme coupled with the ligand and the lack of proper folding of the arazyme to make the active site of the enzyme inaccessible.

Keywords: Arazyme; Cytotoxic effect; Glioblastoma; IL-13; Targeted therapy; Protease.

INTRODUCTION

Due to the uncontrolled proliferation of abnormal cells in cancer, the possibility of metastasis, and cancer recurrence, early diagnosis and effective treatment of cancer are crucial (1). Glioblastoma (GBM) is an aggressive and malignant brain cancer classified as grade IV by the World Health Organization (WHO), with the highest mortality and low survival rate, (2). Standard therapies for GBM include maximum safe resection surgery to protect neurological function but chemotherapy and radiotherapy have limited effectiveness (3,4). Therefore, more specific and efficient GBM therapies are needed to prolong life and offer patients more hope (3).

Immunotoxins (ITs) are a type of targeted protein-based therapeutics. These chimeric proteins consist of two functional domains: a targeting domain for binding to the cancer cell surface and a toxin domain with a lethal function. Numerous studies have recently evaluated the preclinical and clinical efficacy of ITs in cancer therapy, particularly in relapsed or refractory cancers (5).

*Corresponding author: A.A. Imanifooladi
Tel & Fax: +98-2188068924
Email: imanifouladi.a@gmail.com

Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/RPS.RPS_92_23

Some Its, such as moxetumomab pasudotox have been approved by the United States Food and Drug Administration (FDA) for the treatment of drug-resistant hairy cell leukemia. This IT comprises a Fv anti-CD22 monoclonal antibody and *Pseudomonas* exotoxin A (PE38) (6).

Due to their advantages, including the easy production of targeted ITs in microbial hosts, high efficacy, and specific toxicity on tumor cells with low side effects, and other favorable properties (7), ITs hold promise as candidates for advanced cancer therapies (8). Advances in applied immunotherapy techniques for various types of cancer have led to new approaches in treating GBM(9). Molecular studies indicate that vascular endothelial growth factor (VEGF) is overexpressed in GBM. Consequently, bevacizumab, a monoclonal antibody against VEGF-A, is used for recurrent GBM in the USA, Japan, Switzerland, and Australia (9).

In recent years, targeted molecular therapies have been considered an effective cancer treatment. Therefore, studies focus on applying targeted fusion proteins using molecular markers such as monoclonal antibodies, chimeric antigen receptor (CAR) T cells, and fusion protein with a specific ligand to overexpress receptors on tumor cells, potentially leading to novel GBM treatment strategies (3,10,11).

The interleukin-13 α2 receptor (IL13Rα2) is a monomeric receptor with a high affinity for IL13, overexpressed by over 50% of GBM (11). notably, IL13Rα2 is not significantly expressed in normal glial tissue and is thus considered a biomarker of a therapeutic target in GBM treatment (12). Recent studies have used IL13Rα2-redirected chimeric antigen receptor CD8+ T cells (13), IL13Rα2-CAR-T cells (11), and IL-13-immunotoxins (14) to target IL13Rα2 on GBM. IL13-immunotoxins such as cintredekin besudotox (IL13-PE) are chimeric proteins designed for targeting cancers with IL13Rα2 overexpression (15,16).

Arazyme is a secretory proteolytic enzyme with a molecular weight of 51.5 kDa produced by *Serratia proteamaculans* HY-3 (17). This metalloprotease exhibits high proteolytic activity on various substrates such as collagen, albumin, and keratin across a broad range of temperatures and pH levels (18). Arazyme has both proteolytic and non-proteolytic antitumor effects based on CD8 T-cell induction (19). Previous studies have shown that arazyme has anticancer properties on various cancer cell lines, including metastatic melanoma (19), colon, ovarian (20), and breast cancer cell lines (21).

In the present study, Ara-IL13 was used, consisting of a complete form of arazyme as a highly potent targeted toxin with strong antitumor activity and an IL13 part that binds to IL13Rα2 on cancer cells. Since overexpression of IL13Rα2 on GBM enhances survival, proliferation, tumor progression, and metastasis of tumor cells (22), blocking IL13Rα2 with this fusion protein may prevent IL13Rα2-mediated signaling which results in cancer cell death.

Here, we examined the cytotoxic effect of this fusion protein with a specific ligand on GBM cells to discover a more specific and efficient GBM treatment.

MATERIAL AND METHODS

Cell lines and bacterial strains

In this study, we used *Escherichia coli* BL21 (DE3) as a bacterial expression host purchased from Pasteur Institute of Iran (Tehran, Iran). The cell lines used in this research were U251 (human GBM 251 cell line, BN_0012.1.1) as an IL13Rα2-positive cell line, and T98G (human GBM cells, BN_0012.1.45) as an IL13Rα2-negative cell line of GBM. These glioblastoma cell lines were purchased from the Stem Cell Technology Research Center (Tehran, Iran), and were preserved in the Applied Microbiology Research Center at Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences (Tehran, Iran).

Design of araA-IL13 gene construct

In a previous study, we designed a chimeric construct of AraA-IL13, including arazyme (AraA) of *S. proteamaculans* as a functional site and IL13 as a ligand (23). Briefly, after retrieving the sequence of Ara and IL13 from the GeneBank database (with accession numbers AAX21094.1 and X69079.1, respectively), two components were fused with (A(EAAAK)4ALEA(EAAAK)4A)2 as a linker

to obtain two separate functional domains of the fusion protein with or without minimal interference in their native three-dimensional (3D) structure (21,24). Then, the designed fusion protein was optimized by bioinformatics servers and tools (Fig. 1). The design and verification of the *araA-IL13* gene construct was performed in the previous study (23). Finally, the *araA-IL13* chimeric gene in the pET28a (+) vector was synthesized by BioMatik Company (Ontario, Canada).

Transformation and verification of AraA-IL13 chimeric gene

The pET28a (+) vector contains the *AraA-IL13* chimeric gene which was transformed by heat shock transformation into *E. coli* BL21 (DE3) competent cell as a bacterial expression host. First, the positive clones were selected and then recombinant plasmid extraction was performed using a plasmid extraction kit (Gene Transfer Pioneers, GTP, Iran). The chimeric gene was verified by colony polymerase chain reaction (PCR) with universal T7 primers (T7 promoter: 5'-TAATACGACTCACTATAGGG, and T7 terminator: 5'-GCTAGTTATTGCTCAGCGG) and by sequencing. The colony PCR program was performed as follows: initial denaturation at 95 ℃ for 5 min, 35 cycles of denaturation at 95 ℃ for 30 s, primer annealing at 55 ℃ for 30 s, and DNA extension at 72 ℃ for 60 s. Finally, the reaction was carried out at 72 ℃ for 5 min. PCR products were analyzed using 0.8% agarose gel electrophoresis.

Expression optimization and purification of AraA-IL13 fusion protein

Initially, the bacteria carrying the AraA-IL13-pET28a (+) vector were cultured overnight in Luria-Bertani (LB) medium (Himedia, India) containing 20 µg/mL kanamycin (Solarbio, Switzerland). For expression of the AraA-IL13 fusion protein, the culture was grown at 37 ℃ until the optical density at 600 nm reached 0.7-0.9. The expression of AraA-IL13 fusion protein was induced by 1 mM isopropyl β- d-1-thiogalactopyranoside (IPTG; Solarbio, Switzerland), incubated at 37 °C for 16 h. To optimize expression, the fusion protein was induced by several concentrations of IPTG (0.5, 0.6, 0.7, 0.8, 0.9, and 1 mM) incubated at 25, 30, and 37 °C for 4, 6, and 16 h (25). Finally, the expression of the fusion protein was evaluated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

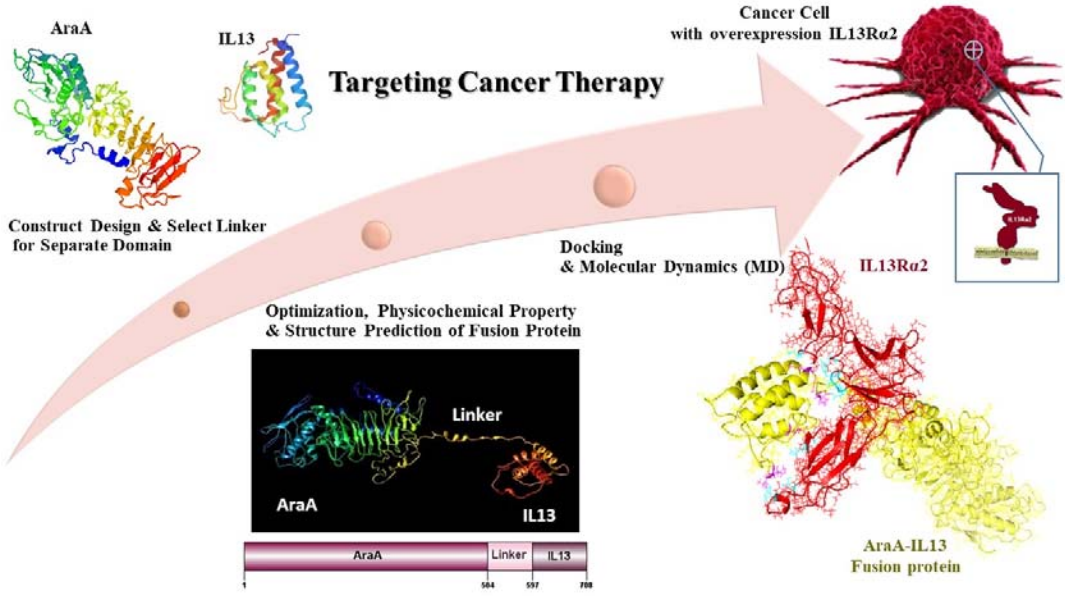

Fig. 1. Designed araA-IL13 gene construct and optimized by bioinformatics server and tools. IL13Rα2, Interleukin 13 alpha 2 receptor.

The purification conditions were set as follows: after suspension centrifugation, the pellet was resuspended in phosphate-buffered saline (PBS) and sonicated six times (sonication time up to 30 s, sonication power of 75 W and 30 s resting between each cycle). The pellet was dissolved step by step in the increasing urea (Samchun, South Korea) concentrations (2, 4, 6, and 8 M). The lysed suspension was then centrifuged at 10,000 rpm, 4 °C for 20 min. The protein was purified and concentrated using the Amicon® ultra-15 centrifugal filter unit 15 mL-30 KDa cutoff (Millipore, USA). This procedure was followed by buffer exchange washing with descending concentrations of urea (8, 6, 4, 2, 1, and 0 M) and PBS. All purified samples were analyzed by SDS-PAGE (10% polyacrylamide gel) (25). The total concentration of purified fusion protein was measured with a NanoDrop 2000 (Thermo Scientific, USA) and Bradford assay using Bradford kit (DNAbiotech, Iran) and bovine serum albumin (BSA; DNAbiotech, Iran) as a standard (26).

Confirmation of AraA-IL13 fusion protein expression by western blotting

The presence of AraA-IL13 was investigated by the western blotting technique. For this purpose, the fusion protein band was transferred from 10% polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane (Amersham, UK, 10600023). The blotted membrane was blocked with 5% skim milk (MirMedia, Iran) in 0.1 mol/L PBS plus 0.05% Tween® 20 by gently shaking at room temperature for 2 h. The membrane was incubated with His-Tag horseradish peroxidase-conjugated antibody (Sigma Aldrich, USA) diluted 1:2000 in tris-buffered saline Tween® 20 (TBS-T) for 1 h at room temperature. After washing five times with TBS-T, antibody binding was detected by staining with 0.5 mg/mL 3,3′-diaminobenzidine (DAB; Alfa Aesar, H54000), an HRP substrate in PBS with 0.1% H_2O_2 (25,27).

Protease assay of AraA-IL13 fusion protein

To screen the protease activity of the AraA-IL13 fusion protein, *E. coli* BL21 (DE3) was cultured on skim milk medium using well diffusion methods. In the presence of protease, a clear zone was created due to casein lysis (casein in milk acts as a substrate). Therefore, 100 µL of the sample was poured into each well-containing skim milk agar. After incubation at 37 °C for 24 h, the diameter of each hydrolysis ring was measured, which was visible as a clear zone. PBS and trypsin (100 µL of each) were spotted on skim milk agar as a negative control and positive control, respectively (28,29).

Cell culture and cytotoxicity assay

U251, the human GBM cell line with overexpression of the IL13Rα2 receptor, was used as the IL13Rα2-positive cell. T98G, a human GBM cell line that does not overexpress the IL13Rα2 receptor was used as IL13Rα2-negative cell line (30,31). Both cell lines were cultured and maintained in DMEM; high glucose (4.5 g/L, Bio-Idea, Iran), supplemented with 10% fetal bovine serum (FBS, Gibco, Germany) and 1% Pen-Strep 100X (Bio-Idea, Iran), and incubated under standard conditions (5% CO_2, 95% humidity) at 37 °C (32,33). After reaching 70-80% confluence, the cells were detached with a trypsin: EDTA (0.05%: 0.02%; w/v) solution (Bio-Idea, Iran) and seeded into new culture flasks T25 (SPL, Spain) containing fresh medium.

To evaluate the cytotoxicity of the AraA-IL13 fusion protein, the MTT assay was performed. The U-251 and T98G cells were seeded in a 96-well plate (JetBiofil, China) at a density of 8×10^3 cells per well and allowed to adhere overnight. The AraA-IL13 fusion protein was added to the wells with a concentration ranging from 25 µg/mL to 400 µg/mL in triplicate. Vincristine (1 mg/mL; Cipla, India) and urea were used as positive controls. The untreated U251 and T98G GBM cells were used as controls to normalize the test set. After 24 and 48 h exposure of cell lines to the fusion protein, the cell viability was measured using the MTT kit (DNAbiotech, Iran). After removing the media, 80 µL of DMEM plus 20 µL of 5 mg/mL of MTT solution were added to each well and incubated at 37 °C for 4 h. Finally, the insoluble formazan crystals produced by living cells were completely dissolved in 100 µL of DMSO.

Then, the absorbance of each well was measured at 570 nm using a microplate reader (Bio-Rad, USA). Values were normalized to untreated controls and analyzed.

Statistical analysis

The data, expressed as mean ± SD, were analyzed using GraphPad® Prism (version 9, USA) through one-way ANOVA followed by Tukey's post-hoc test.

RESULTS

Expression and purification of AraA-IL13 fusion protein

Cloning of the *araA-IL13* gene construct into the pET28a (+) vector was performed by Biomatik Company (Ontario, Canada; Fig. 2).

The gene construct was transformed into *E. coli* BL21 (DE3) and verified by colony PCR (Fig. 3A) and sequencing (data not shown). In the PCR product, a single sharp band was observed on 0.8% agarose gel (Fig. 3A), representing the *araA-IL13* gene construct. The AraA-IL13 fusion protein was expressed in *E. coli* BL21 (DE3) under optimized conditions. Although fusion protein was overexpressed by the induction with 1 mM IPTG and incubation at 37 °C overnight, to prevent the protein aggregation and to increase soluble proteins, fusion protein was expressed with 0.5 mM IPTG and incubated at 25 °C for 4 h (Fig. 3B). The SDS-PAGE results showed the sharp band at ~75 kDa (Fig. 3C, lane 3 before Amicon®). Fusion protein purification with Amicon® 30 KD and buffer exchange with PBS were carried out simultaneously (Fig. 3C, lane 2 after Amicon®). The expression of the purified AraA-IL13 fusion protein was confirmed by western blot (Fig. 3D). As the result showed, the AraA-IL13 fusion protein is more refined after purification using Amicon® 30 kDa (Fig. 3D, lane 5) compared to the fusion protein before purification at ~75 kDa (Fig. 3D, lane 4).

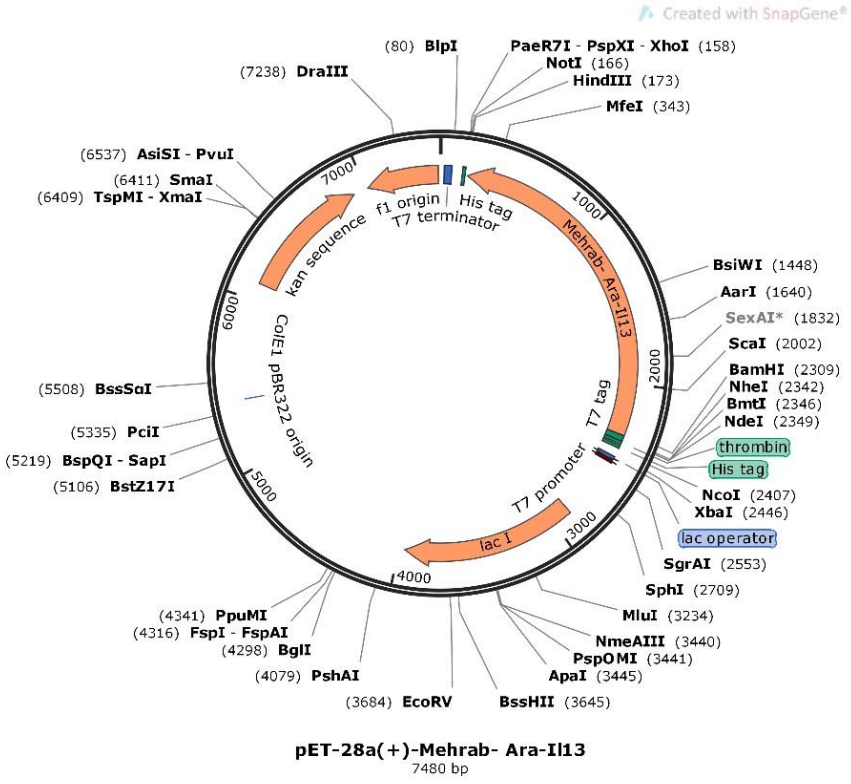

Fig. 2. The *araA-IL13* gene construct with the length of 2142 bp and restriction enzyme site BamHI/HindIII was cloned into the pET28a (+) vector.

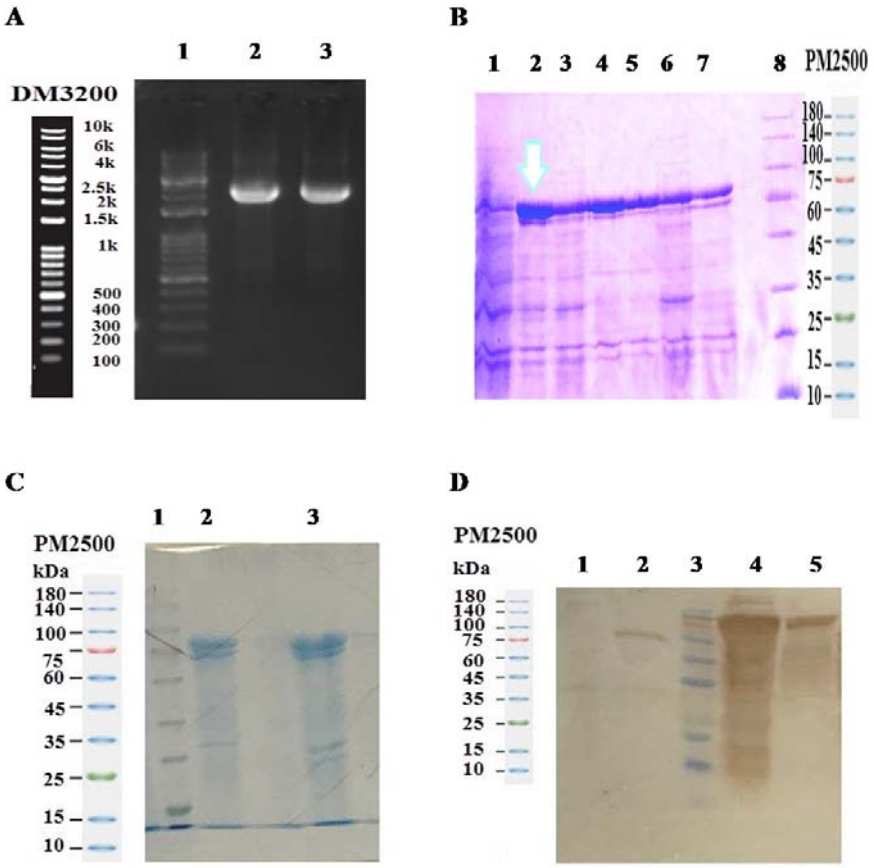

Fig. 3. (A) The result of colony PCR of *araA-IL13* gene on 0.8% agarose gel. Lane 1, ladder DNA (DM3200, SMO Bio, Taiwan); lane 2, *araA-IL13* gene with the size of 2130 bp; lane 3, positive control of colony PCR of gene with the size of 2150 bp. (B) The results of protein expression optimization. Lane 1, negative control; lanes 2 to 7, protein expression by several concentrations of isopropylthio-β-galactoside (1, 0.9, 0.8, 0.7, 0.6, and 0.5 mM, respectively; and lane 8, protein molecular weight marker (PM2500, SMO Bio, Taiwan). (C) The results of protein purification. Lane 1, protein ladder; lanes 2 and 3, the fusion protein after and before purification and buffer replacement with Amicon® 30 kDa, respectively. (D) The results of western blotting. Lane 1, negative control; lane 2, positive control; lane 3, protein ladder marker; lanes 4 and 5, fusion protein before and after purification, respectively with the size of ~75 kDa. PCR, Polymerase chain reaction; IL13, interleukin 13.

Protease activity of AraA-IL13 fusion protein

The protease activity of the fusion protein was evaluated using skim milk assay by the well diffusion method. We did not observe clear zones of proteolysis around the purified fusion protein, indicating the absence of protease activity (Fig. 4A). But the trypsin solution used as a positive control exhibited proteolytic activity by developing a clear zone with 26 mm diameter (Fig. 4B).

The cytotoxic effects of AraA-IL13

The cytotoxic effect of the AraA-IL13 fusion protein was evaluated by MTT assay on U251 and T98G cell lines. No cytotoxic effect of this fusion protein (up to 400 µg/mL) was observed in the U251 and T98G cells after 24 and 48 h incubation (Fig. 5A and B). The half-maximal inhibitory concentration (IC_{50}) values of vincristine as a chemotherapeutic agent in U251 and T98G cell lines were determined to be 90 µg/mL and 150 µg/mL, respectively (Fig. 5C). The significant cytotoxic activity of urea as a positive control was observed against U251 and T98G cells with IC_{50} of 0.201 M and 0.257 M, respectively (Fig. 5D).

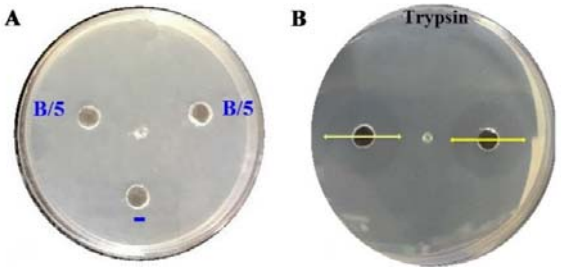

Fig. 4. Detection of protease activity using skim milk assay. (A) AraA-IL13 fusion protein without the clear zone around the well; B/5, fusion protein; -, PBS as negative control. (B) Trypsin with a clear zone of proteolysis around the well.

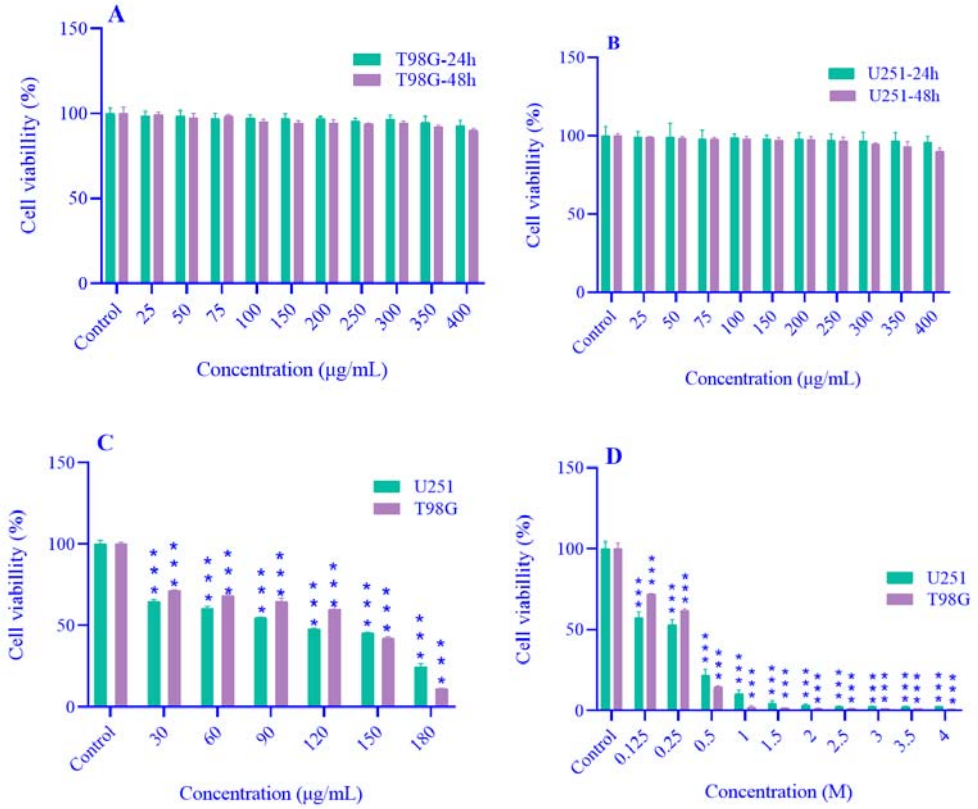

Fig. 5. The results of cell toxicity using MTT assay. (A) The growth inhibition of the T98G cell line; IL13Rα2-negative cells were not observed following treatment with AraA-IL13 fusion protein (25-400 µg/mL) after 24 h and 48 h incubation. (B) The significant growth inhibition of U251 cells; IL13Rα2-positive cells were not seen following treatment with AraA-IL13 fusion protein after 24 h and 48 h of incubation. (C) A concentration-dependent U251 and T98G cell growth inhibition happened when treated with vincristine at different concentrations. (D) The significant growth inhibition of U251 and T98G cells was observed after treatment with urea at different concentrations. Data are presented as mean ± SD, n = 3. $P \leq 0.001$ Indicates significant differences compared to the respective control group. IL13Rα2, Interleukin 13 alpha 2 receptor; Ara-IL13, arazyme-Interleukin-13

DISCUSSION

As GBM is an aggressive malignant brain tumor with high mortality and low cure rates using conventional therapies, novel treatment approaches are urgently needed. Targeted immunotherapy has shown promising results for various cancer treatments in several clinical trials. Recently, the metalloprotease arazyme, known for its proteolysis-dependent activity, has emerged as a strong anti-tumor agent (20). In our previous study, recombinant arazyme from

S. proteomaculans demonstrated a dose-dependent potent cytotoxic effect against human colorectal adenocarcinoma cells (HT29) which could impede cancer cell invasion and adhesion (34). This enzyme also plays a significant role in reducing metastatic gene expression in ovarian cancer cells, which could be considered an approach for treating various types of cancers (20). Thus, in the current study, the effect of arazyme on an aggressive and malignant brain cancer, GBM, was examined. In this case, a novel approach was applied in which a targeted fusion protein with a specific ligand was used. It was hypothesized that to improve the GBM therapy method, the most abundant surface receptor of cancer cells, IL13Rα2, also known as cancer biomarker could be targeted. In addition, the cancer cells using the enzymatic activity of arazyme fused to the ligand of these receptors (IL13) could be treated. Therefore, in the current study, the metalloprotease arazyme was fused to IL13 for binding to IL13Rα2.

To the best of our knowledge, this is the first study of the fusion of full-length arazyme to IL13 using the $(A(EAAAK)_4ALEA(EAAAK)_4A)_2$ linker to create two separate functional domains of the fusion protein so that do not interfere with each other or to minimize their interference compared to their native 3D structure. In a previous study conducted by Ghadaksaz *et al*., the fusion protein of arazyme and the third loop of transforming growth factor alpha (TGFα) was utilized to target TNBC breast cancer cells. Besides, to increase the stability and improve the biological activity of the fusion protein the A(EAAAK)4ALEA(EAAAK)4A linker was employed. Their results showed that the fusion protein had higher binding affinity, cytotoxicity, and apoptosis induction in cancer cells (21).

Based on our results, the recombinant AraA-IL13 as a metalloprotease exhibited the property of inclusion body when overexpressed in the *E. coli* BL21 host due to the presence of disulfide bonds in its structure. Many studies have reported that disulfide bonds are important for the structure and proteolytic activity of metalloprotease (35). Therefore, to prevent protein aggregation and increase the solubility of the recombinant proteins, optimization of the fusion protein expression was performed; the optimized condition was obtained with 0.5 mM IPTG and incubation at 25 °C for 4 h. According to Ghadaksaz *et al*. study, the purification of the fusion protein was performed as a hybrid procedure with denaturation and native conditions, followed by a buffer exchange (washing process) by decreasing urea concentration and increasing pH (8, 6, 4, 2, 1, and 0 M) (21). This procedure was performed to remove urea and refold the recombinant proteins using Amicon® 30 kDa.

Despite using the hybrid method and attempting to remove urea completely, the enzyme was assayed for proteolytic activity (casein lysis) after purification on skim milk agar, and no activity was observed. Subsequently, the cytotoxic effect of AraA-IL13 fusion protein was evaluated by MTT assay on U251 and T98G cell lines. No cytotoxic effect of this fusion protein (up to 400 µg/mL) was observed in the U251 and T98G cell lines.

In a previous study, several constructs were designed and investigated for their anticancer activities. All targeted fusion proteins were cytotoxic to various cancer cell lines. For example, it was shown that the scFv (Herceptin)-PE immunotoxin (IC_{50} 19.98 µM) was more toxic to the HER2-positive breast cancer cell than the scFv (Herceptin)-PE-STXA (IC_{50} 21.25 µM) (36). In another study, the DT389-STXB fusion protein was designed and the results indicated that the DT389-STXB immunotoxin can be used against breast cancer lines with overexpressed GB3 receptors with an average IC_{50} ~18 Mm (25). In the study carried out by Ghadaksaz *et al.,* arazyme was fused to the third loop segment of TGFα (ARA-linker-TGFαL3); The results showed that the IC_{50} value of the constructed fusion protein after the treatment was 18.66 µg/mL in MDA-MB-468 breast cell line with high epidermal growth factor receptor expression level (21). In the present study, no cytotoxic effect of the AraA-IL13 fusion protein could be due to disruption of the 3D structure of the protein; because urea can break the disulfide bonds of sulfhydryl groups and affect the proteolytic activity of metalloprotease (35). These results are consistent with the reports declared by Neto *et al.* They reported that any chaotropic agent like urea can disrupt protein conformation and

hydrophobic interactions (35). Another reason might be the large structure of the enzyme fused to the ligand and the lack of proper folding of the enzyme, therefore the active site of the enzyme is not available.

CONCLUSION

In this study, the AraA-IL13 fusion protein was successfully overexpressed in the *E. coli* BL21 host using 1 mM IPTG induction and incubation at 37 °C overnight. Still, to prevent protein aggregation and increase the solubility of proteins, the fusion protein was expressed using 0.5 mM IPTG incubated at 25 °C for 4 h. The results of SDS-PAGE and western blot confirmed a band of ~75 kDa for our fusion protein, which is consistent with the previous bioinformatics study. Despite the high expression of the AraA-IL13 fusion protein confirmed by western blotting, this chimeric protein had no proteolytic activity. Subsequently, no cytotoxic effect was observed on the U251 and T98G cell lines. The lack of proteolytic activity and cytotoxic effect of AraA-IL13 could be due to the disruption of the protein 3D structure. Moreover, this may be caused by the large structure of the arazyme adjacent to the ligand or owing to the lack of proper folding of this enzyme which makes the active site of the enzyme unavailable. Future studies focusing on the use of IL13Rα2-targeted CAR-T cells to treat GBM are required.

Acknowledgments

This study was a PhD thesis conducted by R. Mehrab and received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors for the research, authorship, and publication of this article.

Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

A.A. Imanifooladi and R. Halabian supervised the project; A.A. Imanifooladi and H. Sedighian conceived the methodology and validation; R. Mehrab performed the experimental parts of the study; R. Mehrab, H. Sedighian, and R. Halabian analyzed the data; R. Mehrab wrote the original draft of the manuscript and provided grammatical revisions to the manuscript with cooperation of F. Sotoodehnejadnematalah. The finalized article was read and approved by all authors.

REFERENCES

1. Yin W, Wang J, Jiang L, Kang YJ. Cancer and stem cells. Exp Biol Med (Maywood). 2021;246(16):1791-1801. DOI: 10.1177/15353702211005390.
2. Abadi B, Ahmadi-Zeidabadi M, Dini L, Vergallo C. Stem cell-based therapy treating glioblastoma multiforme. Hematol Oncol Stem Cell Ther. 2021;14(1):1-15. DOI: 10.1016/j.hemonc.2020.08.001.
3. Chen L, Qin D, Guo X, Wang Q, Li J. Putting proteomics in to immunotherapy for glioblastoma. Front Immunol. 2021;12:593255,1-12. DOI: 10.3389/fimmu.2021.593255.
4. Virtuoso A, Giovannoni R, De Luca C, Gargano F, Cerasuolo M, Maggio N, *et al*. The glioblastoma microenvironment: morphology, metabolism, and molecular signature of glial dynamics to discover metabolic rewiring sequence. Int J Mol Sci. 2021;22(7):3301,1-20. DOI: 10.3390/ijms22073301.
5. Sibuh BZ, Gahtori R, Al-Dayan N, Pant K, Farasati Far B, Malik AA, *et al*. Emerging trends in immunotoxin targeting cancer stem cells. Toxicol In Vitro. 2022; 83:105417. DOI: 10.1016/j.tiv.2022.105417.
6. Dhillon S. Moxetumomab pasudotox: first global approval. Drugs. 2018;78(16):1763-1767. DOI: 10.1007/s40265-018-1000-9.
7. Ataee MH, Mirhosseini SA, Mirnejad R, Rezaie E, Mahmoodzadeh Hosseini H, Amani J. Design of two immunotoxins based rovalpituzumab antibody against DLL3 receptor; a promising potential opportunity. Res Pharm Sci. 2022;17(4):428-444. DOI: 10.4103/1735-5362.350243.
8. Heiat M, Hashemi Yeganeh H, Alavian SM, Rezaie E. Immunotoxins immunotherapy against hepatocellular carcinoma: a promising prospect. Toxins. 2021;13(10):719,1-18. DOI: 10.3390/toxins13100719.
9. Ma R, Taphoorn MJB, Plaha P. Advances in the management of glioblastoma. J Neurol Neurosurg Psychiatry. 2021;92(10):1103-1111. DOI: 10.1136/jnnp-2020-325334.
10. Mikhael E, Kourie HR. Targeting glioblastoma: from dream to reality. Biomark Med. 2021;15(6):385-388. DOI: 10.2217/bmm-2021-0113.
11. Huang B, Li X, Li Y, Zhang J, Zong Z, Zhang H. Current immunotherapies for glioblastoma multiforme. Front Immunol. 2020;11:603911,1-12. DOI: 10.3389/fimmu.2020.603911.
12. Amet N, Lee HF, Shen WC. Insertion of the designed helical linker led to increased expression of tf-based fusion proteins. Pharm Res. 2009;26(3):523-528. DOI: 10.1007/s11095-008-9767-0.
13. Brown CE, Badie B, Barish ME, Weng L, Ostberg JR, Chang WC, *et al*. Bioactivity and safety of

IL13Rα2-redirected chimeric antigen receptor CD8+ T cells in patients with recurrent glioblastoma. Clin Cancer Res. 2015;21(18):4062-4072. DOI: 10.1158/1078-0432.Ccr-15-0428.
14. Kunwar S, Prados MD, Chang SM, Berger MS, Lang FF, Piepmeier JM, *et al*. Direct intracerebral delivery of cintredekin besudotox (IL13-PE38QQR) in recurrent malignant glioma: a report by the cintredekin besudotox intraparenchymal study group. J Clin Oncol. 2007;25(7):837-844. DOI: 10.1200/jco.2006.08.1117.
15. Mazor R, Onda M, Pastan I. Immunogenicity of therapeutic recombinant immunotoxins. Immunol Rev. 2016;270(1):152-164. DOI: 10.1111/imr.12390.
16. Hung AL, Garzon-Muvdi T, Lim M. Biomarkers and immunotherapeutic targets in glioblastoma. World Neurosurg. 2017;102:494-506. DOI: 10.1016/j.wneu.2017.03.011.
17. Kim JH, Lee HN, Bae SK, Shin DH, Ku BH, Park HY, *et al*. Development of a novel denture care agent with highly active enzyme, arazyme. BMC Oral Health. 2021;21(1):365,1-9. DOI: 10.1186/s12903-021-01733-7.
18. Kwak J, Lee K, Shin DH, Maeng JS, Park DS, Oh HW, *et al*. Biochemical and genetic characterization of arazyme, an extracellular metalloprotease produced from *Serratia proteamaculans* HY-3. J Microbiol Biotechnol. 2007;17(5):761-768. PMID: 18051297.
19. Pereira FV, Melo ACL, de Melo FM, Mourao-Sa D, Silva P, Berzaghi R, *et al*. TLR4-mediated immunomodulatory properties of the bacterial metalloprotease arazyme in preclinical tumor models. Oncoimmunology. 2016;5(7):e1178420,1-6. DOI: 10.1080/2162402x.2016.1178420.
20. Amjadi G, Parivar K, Mousavi SF, Imani Fooladi AA. Effect of metalloprotease arazyme on the expression of MMP2 and MMP9 genes in metastasis of colon and ovarian cancer cell lines. Thrita. 2020;8(2):e100004. DOI: 10.5812/thrita.100004.
21. Ghadaksaz A, Imani Fooladi AA, Mahmoodzadeh Hosseini H, Nejad Satari T, Amin M. ARA-linker-TGFαL3: a novel chimera protein to target breast cancer cells. Med Oncol. 2021;38(8):96,1-15. DOI: 10.1007/s12032-021-01546-2.
22. Knudson KM, Hwang S, McCann MS, Joshi BH, Husain SR, Puri RK. Recent advances in IL-13Rα2-directed cancer immunotherapy. Front Immunol. 2022;13,1-10. DOI: 10.3389/fimmu.2022.878365.
23. Mehrab R, Sedighian H, Sotoodehnejadnematalahi F, Halabian R, Imani Fooladi AA. A comparative study of the arazyme-based fusion proteins with various ligands for more effective targeting cancer therapy: an *in-silico* analysis. Res Pharm Sci. 2023;18(2):159-176. DOI: 10.4103/1735-5362.367795.
24. Patel DK, Menon DV, Patel DH, Dave G. Linkers: a synergistic way for the synthesis of chimeric proteins. Protein Expr Purif. 2021;191:106012. DOI: 10.1016/j.pep.2021.106012.
25. Mohseni Z, Sedighian H, Halabian R, Amani J, Behzadi E, Imani Fooladi AA. Potent *in vitro* antitumor activity of B-subunit of Shiga toxin conjugated to the diphtheria toxin against breast cancer. Eur J Pharmacol. 2021;899:174057,1-11. DOI: 10.1016/j.ejphar.2021.174057.
26. Rezaie E, Bidmeshki Pour A, Amani J, Mahmoodzadeh Hosseini H. Bioinformatics predictions, expression, purification and structural analysis of the PE38KDEL-scfv immunotoxin against EPHA2 receptor. Int J Pept Res Ther. 2020;26(2):979-996. DOI: 10.1007/s10989-019-09901-8.
27. Rahmani F, Imani Fooladi AA, Ajoudanifar H, Soleimani NA. *In silico* and experimental methods for designing a potent anticancer arazyme-herceptin fusion protein in HER2-positive breast cancer. J Mol Model. 2023;29(5):160. DOI: 10.1007/s00894-023-05562-z.
28. Araghi A, Hashemi S, Akhavan Sepahi A, Faramarzi M, Amin M. Purification and study of anti-cancer effects of *Serratia marcescens* serralysin. Iran J Microbiol. 2019;11:320-327. DOI: 10.18502/ijm.v11i4.1470.
29. Zhang X, Shuai Y, Tao H, Li C, He L. Novel method for the quantitative analysis of protease activity: the casein plate method and its applications. ACS Omega. 2021;6(5):3675-3680. DOI: 10.1021/acsomega.0c05192.
30. Bhardwaj R, Suzuki A, Leland P, Joshi BH, Puri RK. Identification of a novel role of IL-13Rα2 in human glioblastoma multiforme: interleukin-13 mediates signal transduction through AP-1 pathway. J Transl Med. 2018;16:369,1-13. DOI: 10.1186/s12967-018-1746-6.
31. Sharma P, Roberts C, Herpai D, Fokt ID, Priebe W, Debinski W. Drug conjugates for targeting Eph receptors in glioblastoma. Pharmaceuticals (Basel). 2020;13(4):77,1-17. DOI: 10.3390/ph13040077.
32. Sattiraju A, Solingapuram Sai KK, Xuan A, Pandya DN, Almaguel FG, Wadas TJ, *et al*. IL13RA2 targeted alpha particle therapy against glioblastomas. Oncotarget. 2017;8(26):42997-43007. DOI: 10.18632/oncotarget.17792.
33. Sanaei M, Kavoosi F. The effect of valproic acid on intrinsic, extrinsic, and JAK/STAT pathways in neuroblastoma and glioblastoma cell lines. Res Pharm Sci. 2022;17(4):392-409. DOI: 10.4103/1735-5362.350240.
34. Amjadi G, Parivar K, Fazlollah Mousavi SF, Imani Fooladi AA. Anti-cancer effects of recombinant arazyme from *Serratia proteomaculans*. J Buon. 2020;25(1):531-542. PMID: 32277680.
35. Hamin Neto YAA, de Oliveira LCG, de Oliveira JR, Juliano MA, Juliano L, Arantes EC, *et al*. Analysis of the specificity and biochemical characterization of metalloproteases isolated from *Eupenicillium javanicum* using fluorescence resonance energy transfer peptides. Front Microbiol. 2017;7:2141,1-13. DOI: 10.3389/fmicb.2016.02141.
36. Goleij Z, Mahmoodzadeh Hosseini H, Sedighian H, Behzadi E, Halabian R, Sorouri R, Imani Fooladi AA. Breast cancer targeted/ therapeutic with double and triple fusion Immunotoxins. J Steroid Biochem Mol Biol. 2020;200:105651. DOI: 10.1016/j.jsbmb.2020.105651.