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J. Biol. Regul. Homeost. Agents. 2024; 38(7): 5573–5581
https://doi.org/10.23812/j.biol.regul.homeost.agents.20243807.448

Development and Bioactivity Assessment of a Recombinant *Pseudomonas aeruginosa* Azurin-BR2 Peptide Fusion Protein: A Novel Approach to Cancer Immunotherapy

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Submitted: 7 February 2024 Revised: 28 March 2024 Accepted: 8 April 2024 Published: 1 July 2024

Background: Conventional chemotherapeutic drugs have limitations, including non-specific toxicity and drug resistance. Targeted cancer therapy using recombinant fusion proteins may effectively overcome these challenges. Azurin is a bacteriocin with pro-apoptotic activity, while buforin IIb derivative (BR2) is an antimicrobial peptide exhibiting cell-penetrating ability. This study aims to construct a fusion protein combining Azurin and BR2 to explore its potential against breast cancer cell line (MCF-7).

Methods: We designed, expressed, purified, and evaluated a novel recombinant fusion protein named Azurin-BR2, consisting of Azurin and BR2 domains, for targeted cytotoxicity against breast cancer cells. The fusion gene construct was cloned into a pET30a vector and transformed into *E. coli* BL21DE3 for expression. The fusion protein was isolated from inclusion bodies, solubilized, and refolded. The purity and identity of the 19 kDa fusion protein were confirmed by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot analysis. To evaluate its biological activity, the fusion protein was tested using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay on both MCF-7 breast cancer cells and MCF-10F normal cells.

Results: The fusion protein showed significant cytotoxic effects against MCF-7 cells, outperforming Azurin alone. The cytotoxic impact on normal MCF-10F cells was significantly lower. The half-maximal inhibitory concentration (IC_{50}) value for Azurin-BR2 was 52 µg/mL compared to 102 µg/mL for Azurin alone against MCF-7 cells.

Conclusions: The fusion protein Azurin-BR2 demonstrated enhanced anticancer therapeutic efficacy by combining the pro-apoptotic activity of Azurin with the cell-penetrating properties of BR2. This fusion protein holds promise for targeted cancer therapy in clinical applications and warrants further *in vivo* studies.

Keywords: bacteriocin; antimicrobial peptide; recombinant fusion protein; Azurin-BR2; targeted therapy; breast cancer; *Pseudomonas aeruginosa*

Introduction

Cancer ranks among the most prevalent diseases globally, leading to numerous fatalities [1]. Effective cancer treatment requires a careful selection of monotherapies, including radiotherapy, chemotherapy, surgery, and hormonal therapy. However, chemotherapeutic drugs often exhibit non-specific toxicity, causing severe side effects and harm to healthy cells. The efficacy of these monotherapies is constrained by these adverse effects and the emergence of drug resistance, limiting their overall effectiveness. Therefore, there is a need to explore alternative therapeutic approaches that offer more targeted and tolerable cancer treatments [2]. Additionally, the development of drug resistance poses a major limitation, reducing the long-term efficacy of these treatments. Addressing these challenges is crucial for the progress of cancer therapies with improved specificity and reduced resistance [3]. This highlights the need for treatments that selectively target cancer cells, thereby mitigating the potential for unintended tumor development [4].

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

This situation has significantly shifted the focus of cancer research towards the development of targeted drug delivery [5]. This innovative approach seeks to deliver cytotoxic agents to tumor cells, thereby minimizing damage to healthy cells. This precision targeting can be achieved by exploiting the differences in molecular markers, receptors, or pathways between cancer cells and normal cells. Targeted cancer therapy holds the potential to improve therapeutic effectiveness, reduce toxicity levels, and overcome drug resistance [6].

Biologics have great potential in selectively targeting cancer cells, including bacteriocins and antimicrobial peptides. Recombinant fusion proteins are a class of targeted cancer therapeutics that combine different functional domains within a single molecule. These domains can include tumor-specific ligands, cell-penetrating peptides, toxins, cytokines, enzymes, or antibodies. There is growing interest in peptide-based or fusion protein-based anti-cancer agents. The development of a novel Azurin-buforin IIb derivative (BR2) fusion protein aims to improve the specificity and efficacy of cancer treatment, particularly in breast cancer treatment. The use of peptides is a promising strategy for targeting tumors [7]. One key advantage of peptides over small molecules is their heightened specificity and lower toxicity profile [8]. Combining a potential cytotoxic peptide with a tumor-targeting peptide in a chimeric protein represents an effective strategy for targeted cancer therapy with reduced side effects [2,9]. Various types of peptides have exhibited potential in targeted cancer therapy [10], including cell-penetrating cationic peptides with the ability to undergo transmembrane transitions [11]. A cell-penetrating peptide called BR2, a derivative of buforin IIb, is a synthetic antimicrobial peptide that exhibits highly specific cytotoxic activity against cancer cells without affecting normal cells. Thus, this 17-residue peptide can serve as a targeting moiety for the targeted destruction of cancerous cells without showing any toxic effects on normal cells [12]. Azurin (14 kDa) is a *Pseudomonas aeruginosa* periplasmic protein that protects the bacteria from oxidative stress and copper poisoning. Azurin has been shown to have anticancer activity by inducing apoptosis, inhibiting angiogenesis, and modulating immune responses. Azurin has been found to selectively enter cancer cells through the human copper transporter 1 (hCTR1), which is overexpressed in many types of cancers. Azurin is the first bacterial protein capable of forming a complex with p53, allowing cancer cells to undergo apoptosis and degradation [13,14]. The synergistic effects of combining tumor-targeting peptides offer significant potential to cure human cancer more effectively [15,16]. The primary objective of this study was to clone and express the recombinant Azurin-BR2 fusion protein and evaluate its cytotoxicity against MCF-7 cells. We hypothesized that the fusion of Azurin and BR2 would enhance the anticancer efficacy of Azurin by increasing its cellular uptake and delivery.

In the current investigation, we constructed and characterized a novel Azurin-BR2 fusion protein in a pET30a vector. The fusion protein was validated using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blot. The purification process involved a histidine tag Nickel-NTA (Ni-NTA) affinity chromatography scheme. We tested the cytotoxicity of the fusion protein against MCF-7 breast cancer cells and MCF-10F normal cells, and compared it with Azurin alone. The 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay revealed its potent anticancer activity against MCF-7 cells, accompanied by significantly lower toxicity towards non-cancerous MCF-10F cells. The Azurin-BR2 fusion protein could be a strategic approach to ameliorate target precision and cytotoxicity while minimizing the impact on non-cancerous cells.

Materials and Methods

Reagents and Chemical

Gel Extraction Kit Thermo Fisher Scientific (Cat No. K210012, Vilnius, Lithuania), NdeI (Cat No. 11803843, Vilnius, Lithuania), Unstained Protein Marker Thermo Fisher Scientific (Cat No. 26610, Vilnius, Lithuania), Ligation Kit Thermo Fisher Scientific (Cat No. K1422, Vilnius, Lithuania), Taq Polymerase Thermo Fisher Scientific (Cat No. K0171, Vilnius, Lithuania), GeneJET Plasmid Miniprep Kit Thermo Fisher Scientific (Cat No. K0503, Thermo Scientific Vilnius, Lithuania), MTT Thermo Fisher Scientific (Cat No. M6494, Eugene, OR, USA), Tetracycline Sigma Aldrich (Cat No. 87128, Saint Louis, MO, USA), Yeast Extract Fisher Scientific (Cat No. BP9727-2, New Jersey, NJ, USA).

We cultured MCF-7 (Cat # HTB-26, ATCC, Manassas, VA, USA) and MCF-10F (Cat # CRL-10318, ATCC, Manassas, VA, USA) cells in Dulbecco's Modified Eagle Medium (DMEM) and RPMI-1640 from Gibco (Cat No. 12100-061, Grand Island, NY, USA).

Gel Extraction Kit (K210012, Thermo Fisher Scientific, Vilnius, Lithuania), NdeI (Cat No. 11803843, Thermo Fisher Scientific, Vilnius, Lithuania), Unstained Protein Marker (26610, Thermo Fisher Scientific, Vilnius, Lithuania), Ligation Kit (K1422, Thermo Fisher Scientific, Vilnius, Lithuania), Taq Polymerase (K0171, Thermo Fisher Scientific, Vilnius, Lithuania), GeneJET Plasmid Miniprep Kit (K0503, Thermo Fisher Scientific, Vilnius, Lithuania), MTT reagent (M6494,Thermo Fisher Scientific, Eugene, OR, USA), Tetracycline (87128, Sigma Aldrich, Saint Louis, MO, USA), Yeast Extract (BP9727-2, Fisher Scientific, New Jersey, NJ, USA). We cultured MCF-7 and MCF-10F cells in DMEM and RPMI-1640 from.

Fusion Protein Constructs Design

The nucleotide sequence of *Pseudomonas aeruginosa* (*P. aeruginosa*) *Azurin* gene (accession No. M30389) and

BR2 was retrieved from the National Center for Biotechnology Information (NCBI) database. To construct the fusion gene, the cell-penetrating peptide BR2 (RAGLQFPVGRLLRRLLR) was fused with the *Azurin* gene via a rigid linker having eleven residues [P (AP) 5]. To facilitate the purification process, a 6X-Histidine tag was placed at the fusion construct N-terminal. The 3D structure of the designed fusion protein construct was predicted through the Swiss Model online server (https://swissmodel.expasy.org) to ensure that each domain has the freedom to act. Finally, the fusion gene construct was synthesized from GeneScript (Piscataway, NJ, USA) and used for further expression studies.

Cloning of the Azurin-BR2 Gene

The *Azurin-BR2* gene, synthesized and cloned into pUC 57 with *Ndel* at 5′ end and *Xho1* at 3′ end, was subcloned into a pET30 vector under the control of a T7 promoter for protein expression. Confirmation of cloning involved PCR amplification and subsequent digestion with the specific *Nde1* and *Xho1* restriction enzymes. The recombinant construct was then transformed into BL21DE3 *E. coli* strains using the $CaCl_2$ method.

Expression Studies of Azurin-BR2 Fusion Construct

Shake flask fermentation was used for the expression studies of the *Azurin* gene and the *Azurin-BR2* fusion gene. Positive transformants were cultured for 24 h at 37 °C on LB medium supplemented with the necessary antibiotics. Following the overnight incubation, the 2.5 L baffled shake flasks were inoculated with 500 mL of M9 medium, appropriate antibiotics, and salts. The flasks were then placed in a shaking incubator set at 37 °C. After 18 h of continuous growth, the culture was induced with IPTG at an OD600 to reach a final concentration of 1 mM. The cell culture was then centrifuged, and the harvested biomass was stored at –20 °C for use in subsequent experiments.

Cell Disruption

To isolate inclusion bodies from the collected cell biomass, suspended cells were mechanically disrupted using a French press cell disrupter system at high pressure. The harvested cells were lysed by passing through the system at 2.5 kbar. Following this, the cell debris and insoluble proteins were removed by centrifugation at high speed. The supernatant was discarded, and the collected inclusion bodies (IBs) were further washed with a washing buffer.

Solubilization and Refolding

To recover the bioactive protein, the separated IBs were resuspended in chaotropes and a high-denaturant buffer containing 6 M guanidine hydrochloride. The resuspended IBs were then incubated with continuous stirring for 30 min at room temperature. Subsequently, the sample mixture underwent centrifugation at 8000 ×g for 20 min at 4 °C to eliminate cell debris and other insoluble components. The final concentration of the isolated protein was determined using the Bradford assay [17]. The refolding of the solubilized protein involved a dilution process. The solubilized protein was added drop by drop into a freshly prepared refolding buffer with gentle stirring. The reaction was allowed to proceed at 20 °C for 24 h in a shaking incubator with continuous stirring.

Azurin-BR2 Fusion Protein Purification

The purification process of the Azurin-BR2 fusion protein involved several steps. Initially, the refolded protein underwent desalting to eliminate salts, including L-arginine, and was concentrated using a Millipore flow system equipped with a nitrocellulose membrane cartridge (0.45 µm) and an optical filter with a 10 kDa cutoff. Subsequently, the desalted protein was subjected to Ni-NTA affinity chromatography. The protein purification process involved loading the protein onto a column that had been pre-packed with nickel Sepharose and pre-equilibrated with buffer A [pH 7.4, containing sodium phosphate (20 mmol), Tween 20 (0.01%), and sodium chloride (0.5 M)]. The column was washed successively with the buffers A and B [sodium phosphate (20 mmol) pH 7.4, Tween 20 (0.01%), sodium chloride (0.5 M), and imidazole (0.01 M)]. Finally, an elution buffer was employed to elute the purified proteins from the column, thereby completing the Ni-NTA affinity chromatography-based purification process.

SDS-PAGE and Western Blot

The purity of Azurin-BR2 fusion was confirmed through 12% SDS-PAGE using the Hoffer gel apparatus (SE260-10A-75, GE Healthcare, Piscataway, NJ, USA) following the Laemmli method [18]. A 1:1 ratio of protein to sample buffer was used to prepare the protein sample. The samples were boiled for 10 min and run for 100 min at a constant voltage of 110 V on a 12% SDS gel. The target protein bands on the gel were observed by staining the gel with Coomassie Blue. For analysis by Western blot, the separated protein bands on the SDS gel were subsequently transferred via a semi-dry blotting procedure onto a nitrocellulose membrane using Towbin buffer in a semiphor Western blot system by Hoffer [19]. Subsequently, the membrane was blocked with a blocking buffer at room temperature overnight. The diluted (1:1000) mouse anti-6X histidine tag primary antibody (Cat No. sc-8036, Santa Cruz Biotechnology, Texas, CA, USA) was then added and incubated for 1 h. Following this, the membrane was washed with washing buffer and finally incubated with the goat anti-mouse HRP-conjugated secondary antibody at a 1:10,000 dilution (Cat No. sc-2031, Santa Cruz Biotechnology, Texas, CA, USA) to detect the 6X histidine tag at the N-terminal region of the Azurin-BR2 fusion protein.

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

Cytotoxicity Assay

To assess the cytotoxic effects of Azurin-BR2 and Azurin proteins on MCF-7 and MCF-10F cells, an MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was conducted. The cell lines (MCF-7 and MCF-10F) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) (Cat No. HTB-22 & CRL-10318). The passage number of both cell lines was 6 at the time of the cytotoxicity assay. The cell lines were routinely tested for mycoplasma contamination using the MycoAlert™ Plus Mycoplasma Detection Kits from Lonza (Cat. No. LT07-701, Basel, Switzerland). Each test included a positive control containing mycoplasma and a negative control containing no mycoplasma. The results were expressed as the ratio of positive control to test sample luminescence (PCT). A PCT value of less than 0.9 indicated a negative result. A PCT value of more than 1.2 indicated a positive result. The cell line was tested before MTT assay and showed negative results for mycoplasma contamination. The cell line was also verified for its identity and origin using the FTA Sample Collection Kit for Human Cell Authentication Service (Cat. No. 135-XV, ATCC, Manassas, VA) following the provider's protocol. The cell lines were maintained and handled under strict aseptic conditions to prevent any potential contamination. The cell culture media, supplements, and reagents were sterilized through filtration and stored at appropriate temperatures. Furthermore, all cell culture flasks, plates, tubes, and pipettes were sterile and disposable.

MCF-7 and MCF-10F cells were cultured in DMEM supplemented with streptomycin, penicillin, and 10% FBS in a 5% carbon dioxide incubator at 37 °C. After 72 h of culture to achieve desired cell densities, the cells were treated with varying concentrations (10–140 µM) of Azurin and Azurin-BR2 fusion protein in triplicate, with untreated MCF-7 and MCF-10F cells serving as controls. Following a 24-hour incubation in a 5% carbon dioxide incubator at 37 °C, MTT reagent (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. The formazan crystals were dissolved in 100 µL dimethyl sulfoxide, and the absorbance of the plate was measured at 570 nm using an ELISA plate reader (SpectraMax Molecular Devices, San Jose, CA, USA). This assay provided valuable insights into the proteins' impact on cell viability and their potential cytotoxic effects on the tested cell lines. The formula for determining cell viability is as follows:

$$\text{Percentage of cell viability (\%)} = \frac{(\text{Test wavelength 570 nm} - \text{ reference wavelength 630 nm})}{\text{Control 570 nm} - 630\text{ nm}} \times 100$$

Statistical Analysis

GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. The mean and standard deviation (SD) of the results were calculated using descriptive statistics after conducting a triplicate experiment. Using one-way Analysis of Variance (ANOVA) ($p \leq 0.05$), the cytotoxicity of Azurin and Azurin-BR2 against breast cancer and normal breast epithelial cell lines was investigated. Post-hoc comparisons were conducted using Tukey's honestly significant difference test for one-way ANOVA.

Results

Fusion Proteins Construct Design

The mature full-length fragment of 450 bp fragment of the *Azurin* gene was obtained from the NCBI database and fused with the cell-penetrating peptide BR2 using a rigid linker [P (AP) 5]. The target fused gene construct was amplified with gene-specific primers and sequence verified, showing 99% homology. Protein 3D structure prediction was conducted for both Azurin and the recombinant Azurin-BR2 fusion construct using the Swiss Model web service (https://swissmodel.expasy.org), representing the successful fusion of the target gene (Fig. 1A,B). The positive fusion construct clones, after digestion with Nde1 and Xho1, were subcloned into pET30 vectors and transformed into *E.coli* BL21DE3 strain for expression.

IB isolation, Solubilization, and Refolding of Azurin and Azurin-BR2 Fusion Protein

The *E.coli* system was used to express Azurin and the Azurin-BR2 fusion protein [20]. To achieve high-density culture expression of Azurin and the Azurin-BR2 fusion protein, shake flask fermentation was performed as previously described [21]. Generally, proteins expressed in prokaryotic systems may not fold correctly into their native conformation, leading to the formation of insoluble inclusion bodies (IBs) within the cellular compartment [17]. Therefore, a critical focus lies in the recovery of active proteins from these aggregates (IBs). The main strategy applied in this study to recover active proteins involved the isolation of inclusion bodies, their solubilization, and subsequent proper refolding. To isolate IBs from the harvested cell culture containing Azurin and Azurin-BR2, cell biomasses were lysed using a constant cell disrupter system (Constant Systems Ltd., Daventry, UK) at varying pressures (0.5 kb, 1 kb, 1.5 kb, and 2 kb). A significant increase in the recovery of IBs was observed when the cells were lysed at a pressure of 1.5 kb.

To maximize the recovery of the target protein, a 6 M guanidine hydrochloride solution, previously optimized [22], was used to solubilize the inclusion bodies of both Azurin and Azurin-BR2 fusion proteins. The most efficient solubilization of the proteins was achieved using the above-mentioned buffer. Proper refolding of solubilized proteins into their native conformations is required to obtain a high level of biologically active protein recovery, as the process yield is directly influenced by the effectiveness of refolding.

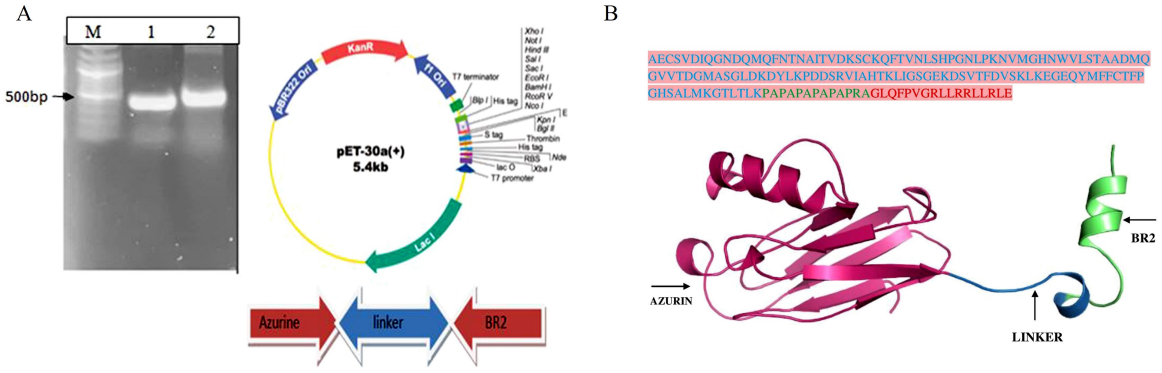

Fig. 1. Construct design of the fusion protein. (A) Schematic representation of amplification and cloning of Azurin and Azurin-BR2 fusion protein in a pET30 expression vector. (B) Amino acid sequence and 3D protein structure of Azurin and buforin IIb derivative (BR2) containing a linker [P (AP) 5)] between Azurin and BR2.

Table 1. Refolding and purification process yield.

Construct	Inclusion bodies (a) (g/L)	Total protein (b) (mg/L)	Refolded and Diafilteration (c) (mg/L)	Purification (d) (mg/L)	Overall yield (e) (%)
Azurin	3.5 (±0.2)	450 (±5.0)	315 (±5.0)	100 (±1.0)	45 (±1.0)
Azurin-Br2	3.2 (±0.1)	420 (±5.0)	295 (±5.0)	85 (±1.0)	35 (±1.0)

(a) Inclusion body of Azurin and Azurin-BR2 calculated after cell lysis. (b) Total protein concentration of Azurin and Azurin-BR2 measured by Bradford assay after solubilization of inclusion bodies. (c) Protein quantification after refolding. (d) Purified Azurin and Azurin-BR2. (e) Overall yield.

The refolding of solubilized Azurin and Azurin-BR2 fusion proteins was carried out using a simple dilution method, and the overall yield is summarized in Table 1 [14].

Purification of Azurin and Azurin-BR2 Fusion Protein

Azurin and Azurin-BR2 fusion proteins were efficiently purified using diafiltration and Ni-NTA affinity chromatography. Before the purification process, the refolded protein was diafiltered to remove particulates and concentrate the proteins up to 25% by passing through a 10 kDa (0.45 µm) cut-off nitrocellulose membrane cartridge. Through Ni-NTA affinity chromatography, the purity of the recombinant Azurin and Azurin-BR2 fusion proteins exceeded 98%, as demonstrated by the product yield on SDS–PAGE (Fig. 2A,B). The identification of the purified proteins was confirmed by their reactivity to monoclonal anti-6X His tag antibody in Western blot analysis (Fig. 2C).

Biological Activity Study

The cytotoxicity of Azurin and Azurin-BR2 fusion proteins was evaluated on MCF-7 and MCF-10F cells. The cells were treated with various concentrations of Azurin and Azurin-BR2 fusion proteins (10–140 µM) for 24 h. Following cell viability measurement using the MTT assay, it was observed that both Azurin and Azurin-BR2 fusion proteins inhibited the viability of MCF-7 cells in a dose-dependent manner. Fig. 3A,B show the cytotoxic effect after 24 h. The results indicated a significant decrease in MCF-7 cell survival with Azurin-BR2 at 30, 50, and 60 µM (60%, 40%, and 20%) compared to Azurin at 60, 80, and 120 µM (80%, 60%, and 40%). Azurin-BR2 with an half-maximal inhibitory concentration (IC_{50}) of 52 µM indicated higher cytotoxicity than Azurin with an IC_{50} of 102 µM. The apoptotic impact of Azurin and Azurin-BR2 fusion proteins was significantly less in normal cells. To determine whether both compounds possess equivalent cytotoxicity in normal cells, the MCF-10F normal memory epithelial cell line was examined. In cytotoxicity assays, it is common to test a range of concentrations to determine the dose-dependent effects of potential drug candidates. The highest concentration is often included to assess the maximum tolerable dose before normal cell viability is compromised. The highest concentration of 130 µg/mL was used for the MCF-10F normal cell line to demonstrate the safety margin of Azurin and Azurin-BR2. Only 13.7% and 21.4% of the MCF-10F cells were nonviable after 24 h of treatment with 130 µg/mL of Azurin and Azurin-BR2, respectively. Even at this high concentration, both proteins exhibited minimal cytotoxicity towards normal cells. The results suggest a potentially advantageous therapeutic window. This helps to validate the selectivity of the fusion protein, which is a crucial factor in targeted cancer therapy.

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

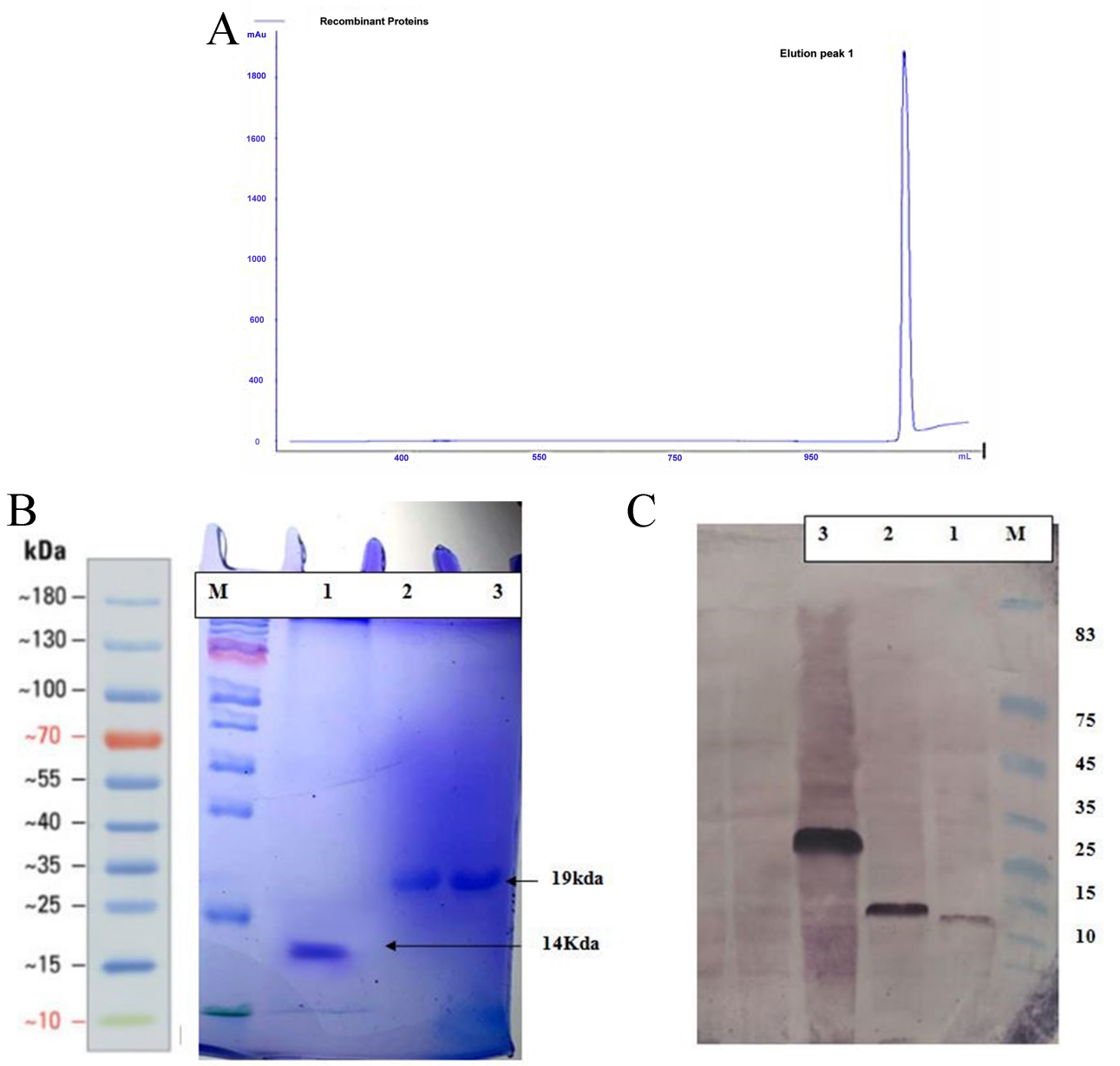

Fig. 2. SDS-PAGE and Western blot analysis of affinity chromatography purified fusion protein Azurin-BR2. (A) Affinity exchange chromatography was used to purify the fusion protein. Peak fractions were pooled and characterized by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). (B) Lane M: prestained marker (26617). Lane 1: Azurin. Lanes 2–3: Azurin-BR2 (Western blot). (C) Lane M: prestained ladder (26617), Lanes 1–2: Azurin. Lane 3: Azurin-BR2 fusion protein.

Discussion

The fusion of a tumor-targeting peptide with recombinant interleukin demonstrates elevated anti-cancer potential due to their combined anti-tumor synergistic effects. The targeted application of these therapeutic fusion proteins is currently generating significant interest, with recombinant DNA technology transforming them into novel biomolecules with multifunctional capabilities within the biopharmaceutical field [15,23]. The fusion protein results in a single polypeptide and has the benefits of both target genes. The dimeric forms of this fusion protein, due to their synergistic effects, exhibit a 10 to 20-fold increase in therapeutic potential compared to the monomeric form of the protein [24]. The fusion of a divalent antibody and toxin results in a significant increase in cytotoxicity. Combining a Fab fragment with two exotoxin A molecules from *Pseudomonas* resulted in a 40-fold increase in activity compared to using only one toxin molecule [25]. Bioactive compounds and effective anticancer therapeutics are most likely to originate from bacterial peptides and proteins [26]. Azurin (14 kDa, 128 amino acids), an essential bacteriocin produced by *Pseudomonas aeruginosa*, plays a vital role in

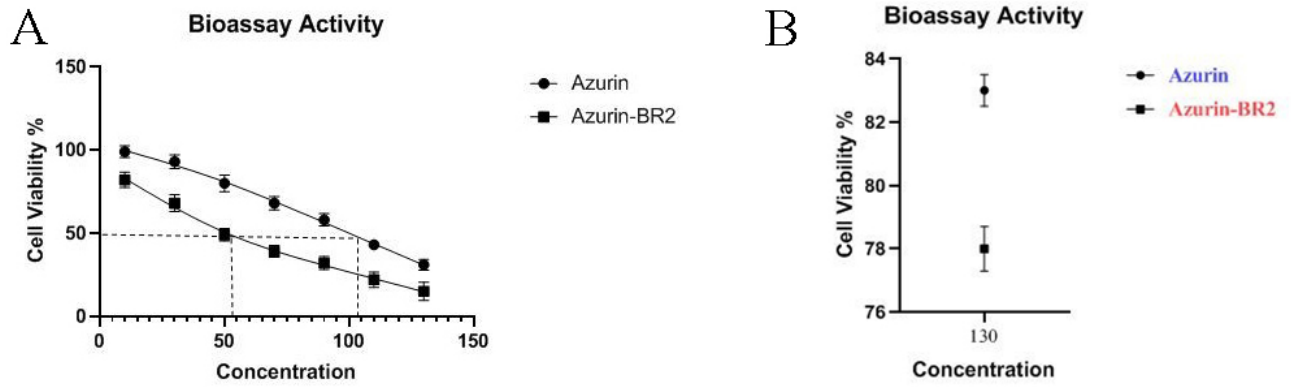

Fig. 3. Cytotoxicity assessment assay against the breast cancer cell line MCF-7 and the normal breast epithelial cell line MCF-10F. The experiment was performed in triplicates. The cytotoxic values at all concentrations were significant ($p < 0.05$) *vs* the control group. (A) Biological activity of the Azurin and Azurin-BR2 protein against breast cancer MCF-7 cells. (B) Biological activity of Azurin and Azurin-BR2 fusion against normal memory epithelial MCF-10F cells.

prompting apoptosis and cytotoxicity [27]. After penetrating tumor cells, Azuring may integrate into the cytosol and nuclear material, thereby stabilizing the p53 protein, which is capable of destroying the tumor cell and facilitating an increase in its level within the cell to inhibit the development of cancer [28]. The most important approach for reducing side effects caused by the use of conventional chemotherapeutic agents is targeted delivery to malignant growth cells and tissues [29]. BR2, a derivative of the anticancer peptide buforinIIb, has been found to be highly effective in delivering therapeutic proteins to the target cells.

BuforinIIb is renowned for its capability to penetrate cancerous cells through gangliosides, facilitated by electrostatic interactions that are present on the surface of tumor cells [30]. Recently, a range of studies have been conducted to evaluate the synthesis and expression of Azurin and peptide fusion proteins [31,32]. The production of fusion proteins, achieved by combining two or more protein domains, leads to the creation of unique functional combinations that exhibit enhanced bioactivity. This approach finds wide application in various biotechnological and biopharmaceutical fields [33]. In a previous study, the fusion protein DT386-BR2 was expressed, and purified, and resulted in significantly enhanced cytotoxic effects against HeLa and MCF-7 cell lines, while showing no toxicity towards normal cell lines [34]. Similarly, in another investigation, recombinant IL-24 was fused with BR2 and the expressed IL24-BR2 fusion protein showed higher cytotoxicity with IC_{50} of 2 µM against MCF-7 cancer cells compared to wild type IL24 [35]. According to the previous studies on the design, expression and purification of recombinant fusion proteins with BR2 and their effects in field of therapeutics, we decided to clone and express *P. aeruginosa* azurin and BR2 peptide as a single recombinant fusion protein. The expressions of the fusion protein and peptide in *E. coli* have been confirmed, but different items like expression vector, host strain, growth medium, and temperature must be optimized for efficiency and sufficient quantity production of the recombinant protein [36]. The fusion protein was designed and expressed at high levels by cloning it into a pET30 vector under the control of the T7 inducible promoter and transforming it into the *E. coli* BL21DE3 expression host strain. High-level expression of the Azurin-BR2 fusion protein was achieved in the *E. coli* BL21DE3 strain. Additionally, we used a carboxyl-terminal Histidine tag for purifying fusion proteins using a Ni-NTA affinity column and identifying them through Western blot. Several studies reported the robust affinity of the resin-nickel agarose system for fusion proteins tagged with six histidine residues, enabling selective purification [25,37,38]. For instance, IL-24 and IL24-BR2 fusion proteins were purified using Ni-NTA affinity chromatography. Similarly, the purification of recombinant Arginine deiminase-Azurin protein was achieved through Ni-NTA chromatography, with the purity of the product confirmed via SDS-PAGE and Western blot analysis. We achieved a high-yield purification of the recombinant protein azurin-BR2 using Nickel Sepharose Fast Flow chromatography. Azurin, an essential bacteriocin found in *Pseudomonas aeruginosa*, belongs to a promising group of bioactive compounds and potent anticancer drugs. It plays a vital role in promoting apoptosis and cytotoxicity [39]. Zaborina *et al*. [40] first identified Azurin as a promising anticancer drug, demonstrating its cytotoxic activity against the murine macrophage cell line J774. Subsequently, study has shown the role of Azurin in inducing apoptosis and significant cytotoxicity in human cancer cell lines such as osteosarcoma, melanoma, and breast cancer [41]. Mohamed *et al*. [41] conducted a study on the cytotoxic properties of recombinant Azurin on various cancer cell lines. In the present study, we assessed the cytotoxic effects of both Azurin and Azurin-BR2 fusion protein specifically against the MCF-7 cell line. The MTT assay revealed that the Azurin-BR2 fusion protein exhibited substantial toxicity at 50 µg/mL, surpassing the cyto-

Journal of
BIOLOGICAL REGULATORS
and Homeostatic Agents

toxic effect of Azurin at 102 µg/mL. This significant activity underscores the potential of the Azurin-BR2 fusion protein as a promising candidate for anti-tumor drug development, particularly targeting the MCF-7 cell line. A previous study was conducted on the cytalyse fusion protein, which consists of a *Pseudomonas* exotoxin and a single-chain variable fragment (scFv) designed to target the CD38 receptor. This receptor is overexpressed in multiple myeloma cells. Cytalyse is currently undergoing phase 1 clinical trials for the treatment of relapsed or refractory multiple myeloma. However, further research is needed to fully evaluate their safety and efficacy in humans and to identify the most promising candidates for future development.

In the current study, there are several limitations along with the promising results. The *in vitro* setting fails to fully replicate the complex *in vivo* microenvironment. Consequently, the observed efficacy and selectivity of the Azurin-BR2 fusion protein require further validation in cancer animal models to assess the pharmacokinetics, biodistribution, efficiency, and safety profile of the fusion protein. Moreover, expression systems in prokaryotes such as *E. coli* are cost-effective for protein synthesis. However, the expressed protein needs refolding, potentially affecting the function of the protein. Furthermore, the possible immunogenicity of the fusion protein remains unexplored. Exploring fusion proteins with additional tumor-targeting ligands may also provide valuable insights into potential synergistic effects. In summary, the innovative approach of the Azurin-BR2 fusion protein signifies a leap forward in the field of cancer therapeutics. The translation of this novel strategy from bench to bedside to treat cancer is associated with addressing these limitations and leveraging the prospects.

Conclusions

The present study expressed and purified recombinant Azurin-BR2 protein in *Escherichia coli* (BL21DE3). Notably, the fusion protein demonstrated significant activity against MCF-7 cells compared to Azurin alone, indicating its potential as an anti-cancer drug candidate. Importantly, both Azurin and Azurin-BR2 fusion protein showed considerably low cytotoxicity towards the normal epithelial cell line MCF-10F, suggesting a level of selectivity for cancer cells. The limitations of the study, including the *in vitro* nature of the experiments and the need for further *in vivo* studies, are acknowledged. The proposed novel strategy has the potential to advance the development of next-generation cancer therapeutics.

Availability of Data and Materials

All experimental data included in this study can be obtained by contacting the corresponding author if needed.

Author Contributions

Methodology, formal analysis, validation: HS, HB, MZS, MYA. Investigation resources, data curation: MA, MS, TN, ST, UH, OS and original draft preparation: HB, OS. All authors read and approved the final manuscript. All authors contributed to important editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

The authors extend their appreciation to University Higher Education funds for funding this research work under research support program for Central Lab at King Khalid University through the project number CL/CA/7.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

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