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# Design and Cytotoxicity Evaluation of a Cancer-targeting Immunotoxin Based on a Camelid Nanobody-PE Fusion Protein

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## ABSTRACT

**Background:** Developing effective targeted treatment approaches to overcome drug resistance remains a crucial goal in cancer research. Immunotoxins have dual functionality in cancer detection and targeted therapy.

**Objective:** This study aimed to engineer a recombinant chimeric fusion protein by combining a nanobody-targeting domain with an exotoxin effector domain. The chimeric protein was designed to bind surface-expressed GRP78 on cancer cells, facilitating internalization and inducing apoptosis to inhibit proliferation and survival.

**Methods:** Using a flexible linker, we designed two constructs linking VHH nanobody domains to *Pseudomonas* exotoxin (PE) domains II, III, and Ib. These constructs were then optimized for expression in *E. coli* BL21 (DE3) using the pET28a vector. Following the expression of the recombinant proteins, we purified them and tested their binding capability, cytotoxicity, and ability to induce apoptosis in breast cancer cell lines MDA-MB-231 and MCF-7, as well as in control cell lines HEK-293 and MDA-MB-468. The binding affinity was measured using a cell-based ELISA, internalization was assessed through Western blotting, cytotoxicity was evaluated by an MTT assay, and apoptosis was determined using flow cytometry with an Annexin V kit.

**Results:** The immunotoxin specifically bound to cancer cells expressing csGRP78. The results of the cytotoxicity test showed that the cytotoxic effect of two constructs, I and II, depended on concentration and time. With an increase in both components, the effect of recombinant proteins also increased. Both constructs were able to penetrate and induce apoptosis in csGRP78+ cells.

**Conclusion:** These immunotoxin structures showed therapeutic potential against GRP78-expressing cancers, making them suitable candidates for targeted therapy pending *in vivo* studies.

**Keywords:** Breast Cancer, Exotoxin, GRP78, Nanobody, Recombinant Protein

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## INTRODUCTION

Breast cancer is the most common form of cancer diagnosed in women, accounting for nearly a third of all cancer cases identified among females (1). Cancer continues to be one of the most significant challenges in medicine despite important medical and therapeutic advances (2). Breast cancer treatments are determined based on the progression of the disease, which includes surgery, chemotherapy, radiation therapy, and immunotherapy (3). In recent years, a deeper understanding of the pathogenesis of breast cancer has led to new interventions, including targeted treatments and cancer immunotherapy (4). Monoclonal antibodies (mAbs) and their variants can precisely target cancer cells, making them a promising part of targeted therapies to develop effective cancer treatments with fewer side effects (1, 5).

The role of Glucose-Regulated Protein 78 (GRP78) in survival, proliferation, angiogenesis, metastasis, poor prognosis, and resistance to chemotherapy makes it an attractive target for targeted therapy (6-8). GRP78 functions principally as a chaperone in the Endoplasmic Reticulum (ER) lumen, facilitating proper protein folding and assembly. In stressed cells, GRP78 translocates to the cell surface (csGRP78), interacts with various ligands, and triggers intracellular signaling cascades (9). The expression of GRP78 on the surface of breast cancer cells increases as the disease progresses (10). Due to its cancer-specific expression pattern, csGRP78 has potential as a targeting antigen for antibody-based therapies (9).

Monoclonal antibodies are highly complex biomolecules. Synthetic conjugates, particularly foreign immunoglobulins such as murine, chimeric, and humanized IgG, may cause overstimulation of the immune system (1). In addition to the challenge of developing a strong immune response, resistance to treatment remains a major obstacle (11). Identifying heavy-chain antibodies in camels, which contain only a single variable domain called VHH, led to the characterization of these domains as nanobodies (Nbs) or single-domain antibodies. Due to their beneficial properties, Nbs were explored as a potential substitute targeting module (1, 12). Nbs are the smallest known functional antibody fragments, with high stability, solubility, specificity, and affinity, and can be easily produced in bacteria (12, 13). The significant thing is that, without an Fc region, Nbs cannot directly initiate Fc-mediated immune responses. Extensive research has explored utilizing Nbs as smart drug delivery systems to selectively protect healthy tissues while eliminating cancer cells (14). Nbs have various applications in cancer therapy through conjugation with other treatment modalities such as nanoparticles, viral vectors, or imaging agents (15, 16). Most antibodies that bind to targets are non-cytotoxic. Current research is focused on optimizing recombinant single-chain antibodies as targeted cancer therapeutics (17). A breakthrough in in reducing the size of antibodies, in the form of nanobodies, has enabled improved penetration into solid tumors. This advancement allows researchers to conjugate Nbs with other toxic proteins (9). This strategy combines antibody specificity with toxin-induced cytotoxicity, optimizing the overall therapeutic impact (18-20).

*Pseudomonas* exotoxin A (PE) acts as a bacterial ADP-ribosyltransferase to irreversibly inhibit translation within targeted cells. Truncated PE38, in particular, is commonly used as the cytotoxic cargo in immunotoxins due to its proven efficacy in preclinical and clinical studies (21, 22). Resistance to mutations that do not affect immunotoxin activity, improved stability, and markedly reduced immunogenicity make PE a favorable candidate for cancer therapy applications (23, 24).

using an appropriate linker domain is crucial for maintaining the proper folding and functions of the targeting and cytotoxic domains within fusion proteins.

Immunotoxins frequently use a linker such as (G4S)3 to spatially separate domains and enhance folding stability thereby facilitating the expression of recombinant proteins (25). (G4S)3 linker creates the required space and flexibility between the nanobody and the toxin. This can enhance both the binding strength and the overall effectiveness of the toxin. Studies have shown that the (G4S)3 linker helps maintain the structure and function of both components of the fusion protein, ultimately improving its therapeutic potential (15). On the other hand, directly attaching the toxin to the nanobody without a linker might reduce the size of the protein., However, it could also interfere with the proper folding and function of the domains due to physical constraints. Therefore, a linker is generally more effective and reliable for maintaining the therapeutic efficacy of nanobody-toxin fusion proteins.

Many researchers are exploring the use of naobodies for targeted treatments due to their unique properties. In this regard, Xu et al. evaluated the antitumor effects of a nanobody-based combination drug on pancreatic cancer cells expressing TROP2. This conjugated Nb was able to eradicate pancreatic cancer cells (26). In another study, Li et al. reported positive results on the anticancer effects by targeting CD147 using a Doxorubicin-conjugated nanobody (27). A new nanobody against GRP78 named V80 was introduced by Aghamollaei et al. They reported specific binding of this Nb to cancer cell lines, such as HepG2 and A549, which exhibit high expression of the GRP78 protein (28).

In this study, we combined a targeting domain (V80) with a potent bacterial toxin agent for the first time. We then evaluated the effects of two new immunotoxin compounds (V80-PE38KDEL) on breast cancer cells. Considering the effect of GRP78 expression on cancer patients' poor prognosis and the development of drug resistance, we aim to overcome these fundamental challenges by developing this new immunotoxin.

## MATERIAL AND METHODS

*Design and Construction of Chimeric Genes*

In the present study, exotoxin A was used with two different sequence combinations. The initial sequence from the 5' to the 3' side was as follows: 5'-Ia-II-Ib-III-3'. After removing the Ia part on the 5' side, the nanobody gene sequence was inserted. The first optimized exotoxin sequence was cloned and utilized in the pET28a vector based on the research by Rezaie et al. (18). The final composition after homogenization was 5'-VHH-II-Ib-III-3' (Fig. 1A). The second sequence from the 5' to 3' side was 5'-III-Ib-II-Ia-3'. The sequence orientation of the second exotoxin is the reverse of the sequence of the first exotoxin. This optimized sequence was used based on the research by Kashtvarz et al. (23). The nanobody sequence was also obtained in the form of cDNA from Aghamollaei's research (28) and replaced domain Ia after sequencing and amplification by PCR. Its final composition was 5'-III-Ib -II-VHH -3' (Fig. 1B).

*Isolation and Purification of Chimeric Recombinant Proteins*

The pET28a vector containing the chimera gene was transformed into the host strain *E. coli* BL21 (DE3). After 16h of IPTG induction, the recombinant proteins were isolated . Bacterial pellets were washed with phosphate-buffered saline (PBS) and then resuspended in lysis buffer (50 mM Tris−HCl containing 5 mM DTT, 1 mM EDTA). The bacterial cells were lysed using sonication (5×30 s pulses at 200W). Supernatants were discarded after centrifugation at 6000 ×g for 30 min, and pellets were washed with washing buffer (1 M urea in 50 mM Tris−HCl, pH=8.0, containing 5 mM EDTA and 1 mM DTT) two times, followed by centrifugation at 11,000 ×g. The urea buffer consisting of 0.01 M Tris/base, 0.1 M NaH2PO4, and 8 M urea with a pH=8 was used toextract proteins from the washed inclusion bodies. After overnight incubation, the samples were centrifuged for 20 min.

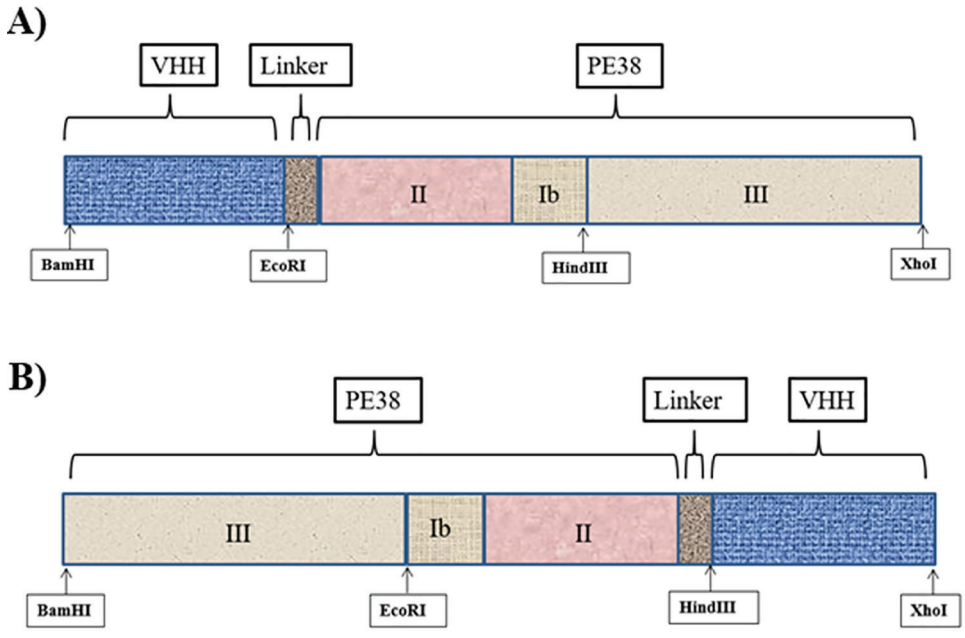


**Fig. 1.** Components and their positions in constructions I and II. (A) construction I: The linker used has a flexible sequence (GGGGS). The first nanobody (VHH) is on the N-terminal side and PE38 is on the C-terminal side. (B) construction II: The linker used has the sequence TCTGCTAGCGGCGGTCCAGAA. The second nanobody (VHH) is located on the C-terminal side and PE38 is on the N-terminal side. According to the gene map of the pET28a vector, HisTaq is located at the amine end of both constructs.

The supernatants were then analyzed by SDS-PAGE. Subsequently, the supernatants were applied to equilibrate Ni-NTA columns. Proteins were eluted with elution buffer (50 mM NaH2PO4, 300 mM imidazole, and 300 mM NaCl at pH=8) (17). Salts, imidazole and denaturing agents were removed by dialysis of the purified protein samples. To perform the dialysis process, dialysis bags with a pore size of 2.4 nm (molecular weight between 12 and 14 kDa) were boiled in distilled water for 10 minutes. Protein samples were then poured into these membranes and left for 14 hours. Subsequently, dialysis bags containing recombinant proteins were placed in PBS buffer at 25°C for 14 hours on a stirrer. This step is necessary to facilitate the refolding of recombinant proteins. Finally, the recombinant proteins were electrophoresed on an SDS-PAGE gel after dialysis.

*Cancer Cell Culture*

The breast cancer cell lines MDA-MB-231 (ATCC: CRM-HTB-26), MCF-7 (ATCC: CRL-3435), and MDA-MB-468 (ATCC: HTB-132) were utilized in this study. MDA-MB-468, which does not express surface GRP78 (28), served as a negative control. HEK-293 (ATCC: CRL-1573) cells known as human embryonic kidney cellswere also included as a standard cell line. All cell lines were obtained from the Pasteur Institute of Iran cell bank and were cultured at 37°C in a humidified, 5% $CO_2$ incubator in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

*Analysis of the Binding Potential Recombinant Molecules to Cell Surface Receptors*

After purification, the antigen binding affinity of the fusion proteins was assessed using a cell-based enzyme-linked immunosorbent assay (ELISA). MDA-MB-231, MCF-7, HEK-293, and MDA-MB-468 cells were separately seeded at a density of $5\times10^3$ cells per well in 96-well plates. Following a 24-hour incubation period, the culture medium was discarded, and the cells were washed with PBS. The cells were then fixed and blocked using 10% paraformaldehyde and 3% bovine serum albumin (BSA). Subsequently, the cells were treated for 1h at 37°C with various

concentrations (1, 3, 6, 12.5, 25, 50 and 100 µg/ml) of purified proteins. The wells were washed in PBS-T five times, then incubated with horseradish peroxidase (HRP)-conjugated anti-histidine antibody (1:2000) for 2h at room temperature. Orthophenol diamine (OPD) solution containing 1.5% hydrogen peroxide was added to the wells. Color development was stopped by adding sulfuric acid stop solution, and optical density was measured at 492nm using an ELISA plate reader (29). This allowed evaluation of the binding affinity of the fusion proteins to cell surface receptors using a quantitative and label-based ELISA technique.

*Internalization Assay*

The internalization of constructs I and II into MDA-MB-231 and MDA-MB-468 cells was investigated. For this purpose, the cells were cultured in 6-well plates ($3\times10^5$ cells) at 37°C and 5% CO2. After 24 hours of incubation, the cell culture medium was replaced with culture medium containing 50 µg/ml of recombinant proteins. The treated cells were then incubated for 2 hours at 37°C. Susequently, the culture medium containing the recombinant proteins that did not enter the cell was removed. The cells were washed with PBS, separated from the bottom of the dish by trypsin and collected. Next, the cells were lysed using a lysing buffer (50 mM Tris-HCl, pH=7.4 and 2% SDS) to break down their membrane structure. The cell contents were then separated using the SDS-PAGE method and an anti-His Tag antibody was used to identify constructs I and II that entered the cell.

*MTT Assay for Recombinant Immunotoxin Cytotoxicity*

MDA-MB-231, MCF-7, HEK-293, and MDA-MB-468 cells were cultured separately in 96-well plates at a density of $1\times10^4$ cells/well in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were then incubated at 37°C in 95% air and 5% $CO_2$ humidified atmosphere. For 48h, the cells were treated with varying concentrations of recombinant protein (1, 3, 6, 12.5, 25, 50, and 100 µg/ml). Each experiment was performed in triplicate. Afterwards, the medium was replaced with fresh medium containing 10 mg/ml MTT, and the cells were incubated at 37°C for 4h. Formazan crystals were dissolved in 150µl isopropanol and absorbance was measured at 570nm using a microplate reader to determine cell viability (30).

*Immunotoxin Apoptosis Assay by Flow Cytometry*

The apoptotic effects of the immunotoxin on MDA-MB-231, MCF-7, HEK-293, and MDA-MB-468 cells were evaluated using an Annexin V kit. Cells were seeded at a density of $1\times10^5$ cells/well in 12-well plates and incubated at 37°C with 5% CO2. Cells were treated with 12.5 and 25 µg/ml of recombinant proteins for 48h. Susequently, the culture media was removed, and the treated cells were trypsinized at 37°C for 5 min. The collected cells were then resuspended in 1 ml of Annexin V binding buffer containing 5 µl of Annexin V-FITC and propidium iodide. After incubating at room temperature in the dark for 30 minutes apoptosis was analyzed by flow cytometry (31).

*Statistical Analysis*

Experiments were done in three replicates, and the results were reported as mean ± standard deviation. Differences between group means were analyzed using one- and two-way ANOVA. A p-value below 0.05 was considered statistically significant.

## RESULTS

*Amplification of Nanobody Sequence Using PCR*

Two PCR reactions were performed using two different primer pairs on the vector pET28a carrying the nanobody sequence against GRP78. Both reactions produced a 453bp product. The PCR results were visualized on a 1% agarose gel electrophoresis as shown in Fig. 2A.

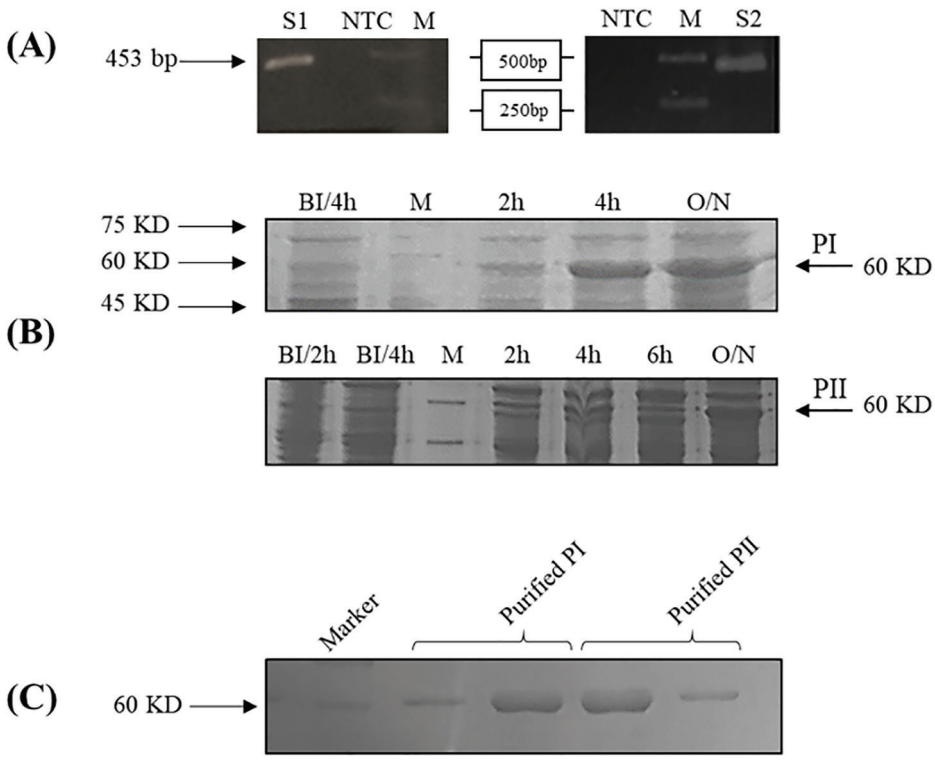


**Fig. 2.** (A) The electrophoresis image of the optimization of PCR conditions for the first and, second primer pair for the nanobody gene. (NTC: negative control, M: DNA molecular weight indicator (GeneRuler TM 1Kb DNA Ladder), S: PCR product, The size of the PCR product for two constructs I and II is approximately 450 kDa. (B) The pattern of total proteins of BL21(D3) bacteria during the induction process for Products I and II. BI: 2 or 4 hours before induction with IPTG, M: protein marker, 2h: At the second hour of induction, 4h: at the 4th hour of induction, 6h: at the 6th hour of induction, O/N: 14 hours after induction with IPTG at a concentration of 1 mM. (C) Results of electrophoresis of purified recombinant proteins with Ni-NTA column and dialysis with the [PM2600] ExcelBand™ 3-color High Range Protein Marker.

Two linearized vectors containing PE toxin subunit sequences in opposite directions were then ligated to GRP78 in two separate reactions.

*Recombinant Protein Expression in Bacterial Host and Purification*

SDS-PAGE analysis of the expressed gene PE38-VHH (PE38-Nb) in BL21 bacteria-induced with 1 mM IPTG revealed that the approximate size of the chimeric protein in both constructs is around 60 kDa (Fig. 2B). Figure 2C shows the SDS-PAGE results after removing salts, imidazole, and denaturing agents.

*Cell ELISA Binding of Recombinant Proteins*

The ELISA method was used to evaluate the binding of recombinant immunotoxins to csGRP78. The results showed that recombinant immunotoxins I and II were able to bind to MCF-7 and MDA-MB-231 cells. However, no binding was observed with HEK-293 and MDA-MB-468 (negative control cell) (Fig. 3A).

*Western Blotting for Recombinant Protein*

The recombinant protein expressed in the pET28a vector contains a sequence of six histidine amino acids. Therfore, the immunoblot technique using the Anti-His Tag antibody was able to identify the recombinant protein (Fig. 3B).

*In vitro Cytotoxic Effects of Recombinant Proteins*

The MTT assay was conducted using various concentrations of chimeric protein

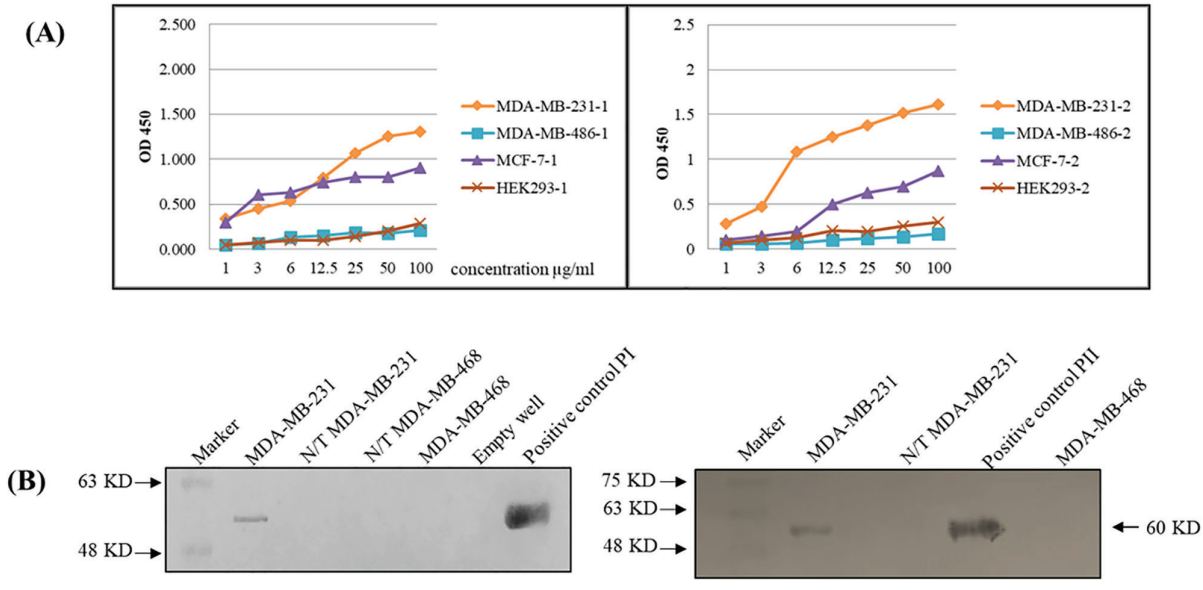


**Fig. 3.** (A) The ability to bind immunotoxins I and II to MDA-MB-231, MCF-7, HEK-293 and MDA-MB-468 cell lines. The MDA-MB-468 cell line served as the negative control. This assay was conducted at 7 different concentrations (1, 3, 6, 12.5, 25, 50 and 100 µg/ml). (B) Internalization of immunotoxins (PI & PII) into MDA-MB-231 and MDA-MB-468 was confirmed through western blotting using an Anti His Tag antibody and a Prestained Protein Ladder (Sinaclon SL7011).

(1, 3, 6, 12.5, 25, 50 and 100 µg/ml) on four cell lines: MCF-7, MDA-MB-231, HEK-293 and MDA-MB-468 (used as a negative control due to the lack of surface GRP78) (28). HEK-293 cell line served as a normal cell and a negative control for csGRP78 (32). The results showedindicated that increasing the concentration of immunotoxins I and II resulted in a stronger lethal effect on MDA-MB-231 and MCF-7 cells. Immunotoxins do not affect the MDA-MB-468 cell line, which cannot express the GRP78 protein. They also do not affect HEK-293 cells that do not have csGRP78. As a result, these immunotoxins can target and destroy cancer cells expressing the GRP78 protein. The cytotoxic effects of immunotoxins I and II on MDA-MB-231, MCF-7, HEK-293, and MDA-MB-468 cell lines at 24, 48, and 72h are depicted in Figure 4.

*Apoptosis Analysis*

The amount of early and late apoptosis induced by the immunotoxin was determined in four cell lines, MDA-MB-231, MCF-7, HEK-293, and MDA-MB-468 (used as a negative control). This was done using the Annexin V kit with the flow cytometry technique. The cell lines were treated with 12.5 and 25 µg/mL of the proteins and then incubated at 37°C for 24h. Following incubation, flow cytometry analysis was performed. A significant increase was observed in the population of both early and late apoptotic cells in MDA-MB-231 and MCF-7 compared to the controls. The apoptotic cell numbers observed in MDA-MB-231 cells treated with immunotoxin at 12.5 and 25 µg/ml concentrations in construct I was 27% and 64%, respectively. In construct II, the numbers were 25% and 60%. Additionally, the apoptotic cell numbers observed in MCF-7 cells were 20% and 45% in construct I and 18% and 25% in construct II, respectively (Fig. 5). Statistically, no changes were observed in the amount of apoptosis and necrosis in MDA-MB-468 cells as a negative control and HEK-293 cells as a normal cells after 24h of incubation at 37°C with increasing concentrations of immunotoxins (33).

Furthermore, increasing the concentrations of the chimeric proteins in treated cells resulted in significant shifts in the ratio of live to apoptotic cells for MDA-MB-231 and MCF-7 cells in a dose-dependent manner. However, for MDA-MB-468 and HEK-

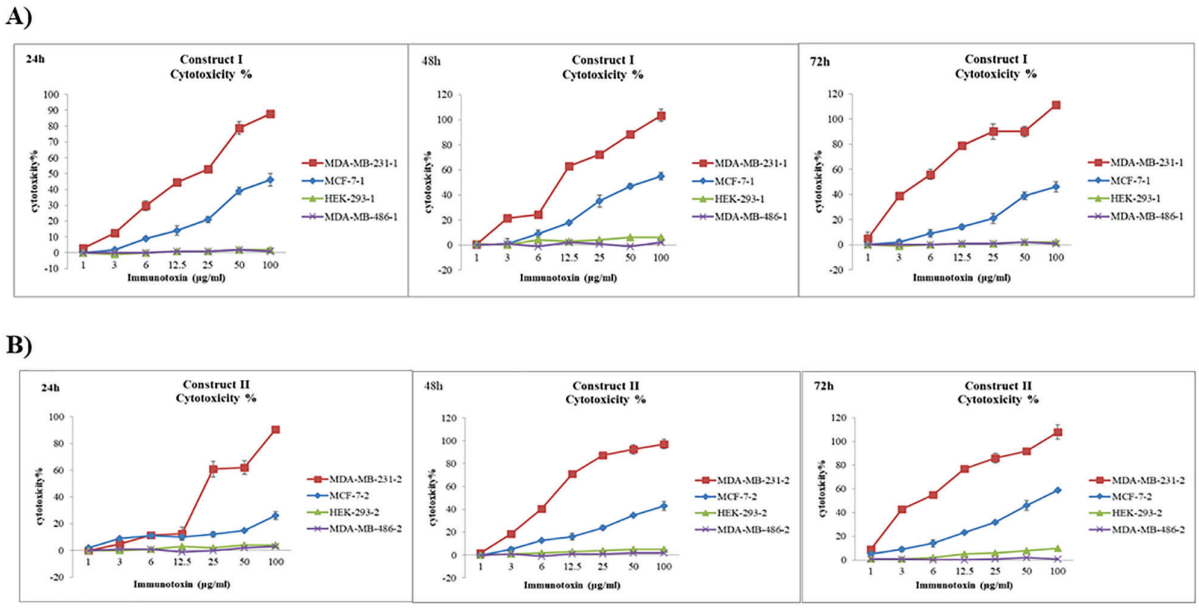


**Fig. 4.** Cytotoxicity results of immunotoxins I (A) and, II (B) on MDA-MB-231, MCF-7, HEK-293, and MDA-MB-468 cell lines at 24, 48, and 72h with varying concentrations of chimeric protein (1, 3, 6, 12.5, 25, 50 and 100 µg/ml). The cytotoxicity was determined using the MTT assay. The inhibition of cell-growth occured in a dose-dependent manner in MDA-MB-231 and MCF-7 cell lines. The results showed that increasing the concentration of immunotoxins I and II enhances the lethal effect on MDA-MB-231 and MCF-7 cells. The data is presented as mean ± SD. Significance levels are; *P<0.05; **P<0.01; **** P<0.0001.

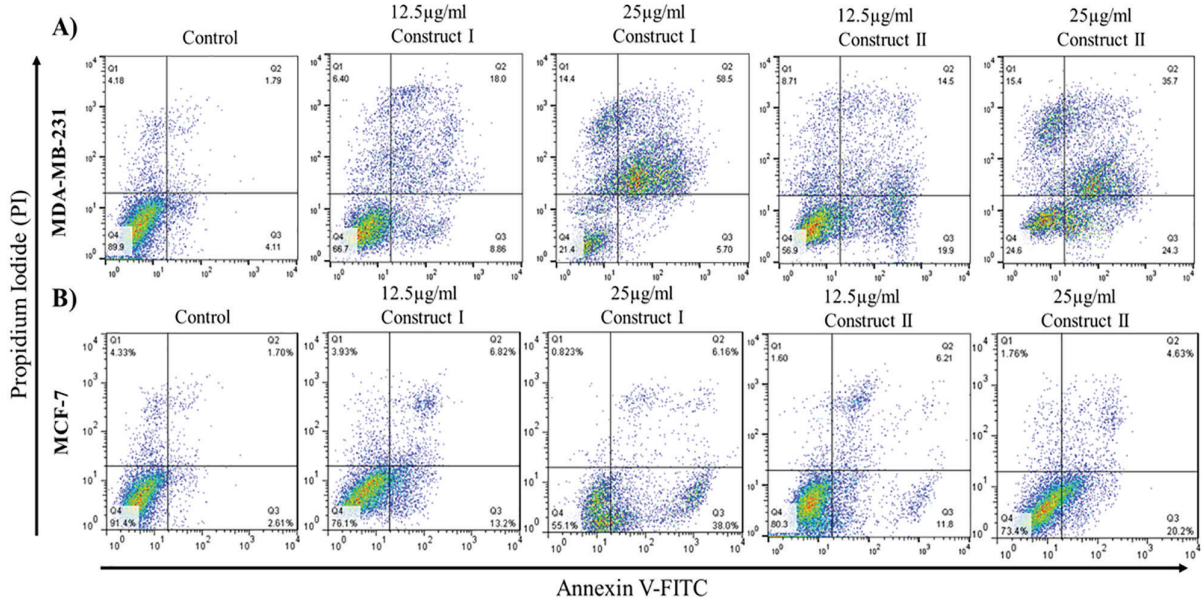


**Fig. 5.** Apoptosis and necrosis for (A) MDA-MB-231, and (B) MCF-7 cell lines, 24h after incubation with immunotoxins I and II at 37 °C and 5% $CO_2$. As the concentration of immunotoxins increased from 12.5 to 25 µg/ml, a significant increase in the number of apoptotic cells was observed.

293 cells (33), there was no noticable dose-dependent increase in apoptotic cell numbers compared to controls. These findings suggest that the engineered proteins were able to selectively induce apoptosis in a concentration-dependent manner in the breast cancer cell lines MDA-MB-231 and MCF-7, but not in the non-cancerous HEK-293 cells or the GRP78-negative MDA-MB-468 cells (Fig. 6).

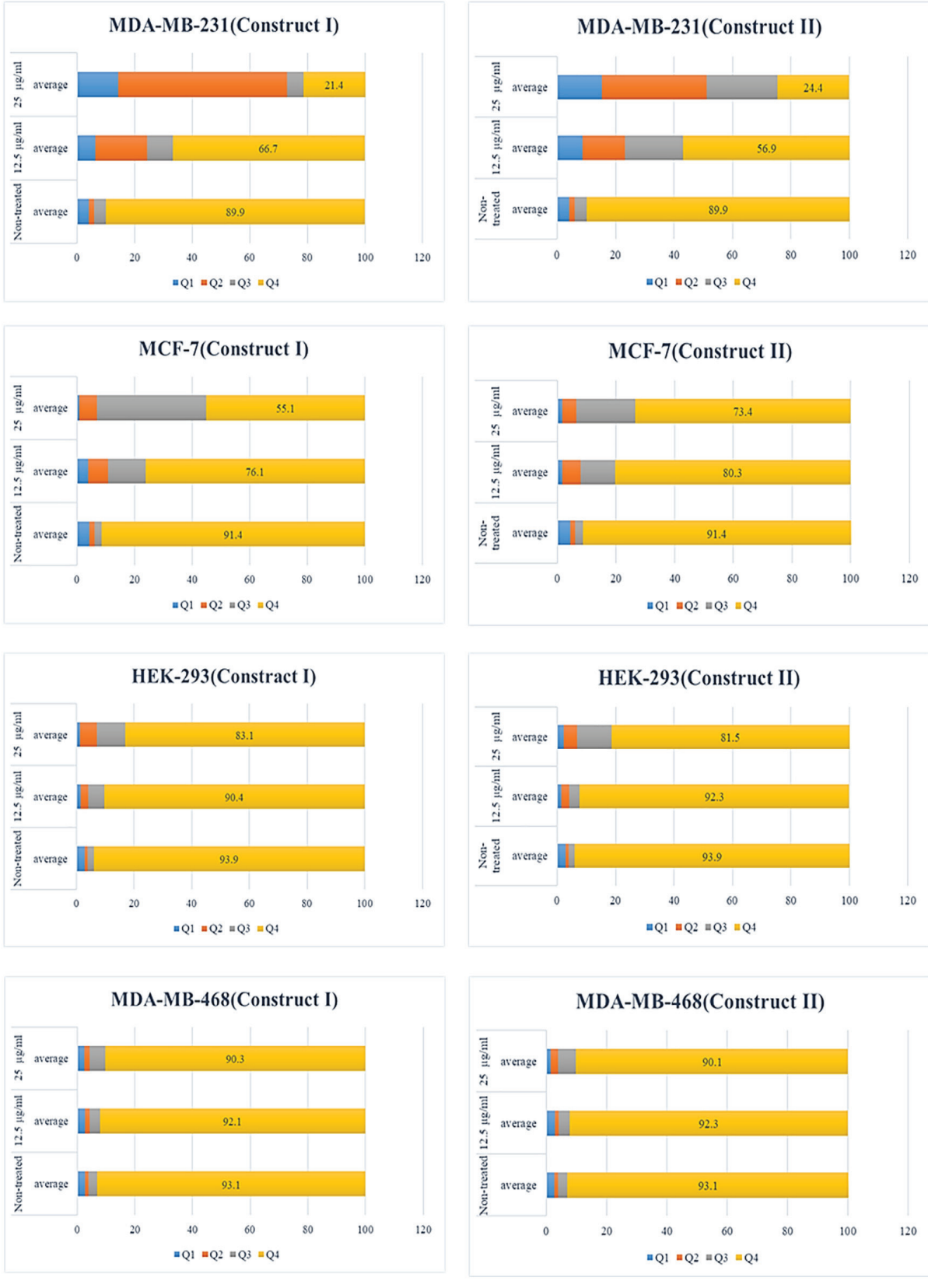


**Fig. 6.** Charts of apoptosis and necrosis for (A) MDA-MB-231, (B) MCF-7, (C) HEK-293, and (D) MDA-MB-468 cells, 24h after incubation with immunotoxins I and II at 37°C and 5% CO2. As the concentration of immunotoxins increased from 12.5 to 25 µg/ml, a significant change in the number of apoptotic cells was observed.

## DISCUSSION

Immunotoxins with their dual function in cancer detection and targeted therapy, offer a promising pathway for developing effective treatment approaches to address drug resistance in cancer research. In this study, a recombinant protein, PE38KDEL-Nb, was designed by combining nanobody molecules, an exotoxin, and novel chimeric proteins expressed in prokaryotic hosts. The cytotoxic effects of these engineered proteins were subsequently evaluated on breast cancer cell lines. Recent studies have highlighted the potential of nanobody-based immunotoxins in cancer therapy. Nanobody-drug conjugates have shown significant tumor growth inhibition and favorable biodistribution in preclinical models of HER2-positive breast cancer (34). These findings csupport our results, denonestrating that V80-PE38KDEL constructs specifically bind to csGRP78-positive breast cancer cells and effectively induce apoptosis.

A key component of immunotoxins is toxin selection, which significantly impacts specificity and targeted delivery to cancer cells (35). The choice of antigen also determines specificity. In this work, the GRP78 antigen was selected due to its favorable properties for the objective. Cell surface GRP78 is a cancer-associated antigen highly expressed in malignant cells. Additionally, it is involved in cell surveillance pathways that regulate motility, proliferation, and resistance to apoptosis (28, 36). Compared to standard cell lines, GRP78 protein levels are significantly higher in the breast cancer cell lines, MCF-7 and MDA-MB-231, making it a relevant targeting antigen for this application (37). The high reactivity of csGRP78 to nanobody V80 allows for specific entry into the cancer cells. The affinity of V80 to csGRP78 was determined to be $2.1\times10^{-7}$ M (28). The nanobodies were designed to interact with a protein that is highly expressed on the cancer cell surface. Once bound, the nanobodies are internalized by the cancer cells and release their toxin cargo (38). In this study, we constructed a recombinant PE38KDEL-Nb to enhance cytotoxicity. This construct consist of PE toxin in two different orientations, bound to the V80 nanobody to facilitate the delivery of PE38 into target cells. Our study support previous research highlighting the potential of nanobody-based immunotoxins for targeted cancer therapy. For example, Rezaie et al. (2020) developed an immunotoxin combining PE38KDEL with an scFv targeting the EPHA2 receptor, demonstrating significant cytotoxic effects in breast cancer cell lines (15). Similarly, Keshtvarz et al. (2021) developed the STX2a-PE15-P4A8 chimeric protein, which showed high specificity and efficacy against cancer cells (39). In our study, the V80-PE38KDEL constructs specifically bound to csGRP78-positive breast cancer cells and efficiently induced apoptosis. This aligns with the advantages of nanobodies, like V80, known for their stability and high affinity (28). Moreover, our results support the dose-dependent cytotoxicity seen in earlier studies, emphasizing the need for optimal toxin concentrations to maximize therapeutic outcomes (40, 41). These comparisons validate our approach and suggest that nanobody-based immunotoxins hold great promise for targeted cancer therapy.

According to the cell ELISA results, both immunotoxins bind to cancerous cells expressing csGRP78. The binding to csGRP78-positive cells was notable at a concentration of at least 3 µg/ ml. No binding of the immunotoxin was observed in MDA-MB-468 a triple-negative breast cancer (TNBC) cell line (28, 42, 43) and HEK-293. This is likely due to the fact that no significant accumulation of GRP78 as an unfolded protein response marker has been reported in these cell lines.

The cytotoxicity of PE38KDEL-Nb was analyzed using MTT assays. PE38KDEL-Nb was efficiently transported into csGRP78-positive cell lines and demonstrated significant cytotoxicity, clearly revealing target-specific cell killing. This supports its

potential as an immunotherapeutic. MDA-MB-231 cells were highly susceptible to the recombinant immunotoxins, while MCF-7 cells showed a weaker response, possibly due to lower csGRP78 surface levels (44). Despite similar cytotoxicity values between cell lines, csGRP78 expression, and internalization rates varied markedly (Fig. 4). Therefore, both levels of csGRP78 and internalization kinetics determine the cytotoxic activity of PE38KDEL-Nb. The produced immunotoxin exhibited highly specific binding to csGRP78-positive cell lines. The cytotoxicity of constructs I and II depended on concentration and time in a dose-dependent manner. previous research has shown that increasing the incubation time and toxin concentration enhances toxin uptake per cell and reduces viability (45).

In this study, flow cytometry demonstrated the levels of early and late apoptosis in the cancerous cell lines treated with csGRP78. At a concentration of 12.5 µg/ml, structure I induced approximately 27% apoptosis in MDA-MB-231 and 20% in MCF-7 cell lines. Additionally, necrosis levels were 6% and 4% in the respective cell lines. At a concentration of 25 µg/ml of construct I, apoptosis levels in the MDA-MB-231 cell line increased to 65%, with necrosis at 14%. In MCF-7 apoptosis level was at 45% and necrosis at 0.8%. Apoptosis induced by construct II at a concentration of 12.5 µg/ml were estimated to be 35%% in MDA-MB-231 and 18%, inMCF-7 cell lines while necrosis rates were 8.7% and 1.6%, respectively. At a concentration of 25 µg/ml apoptosis rate rose to 60% in MDA-MB-231 and 25% in MCF-7 cell lines with necrosis rate of 15% and 1.7% , respectively.

The ELISA and MTT results indicate thst the apoptosis rate would be lower in MCF-7 cells due to their reduced csGRP78 protein expression. Both immunotoxin designs could potentially bind to csGRP78-positive breast cancer cells, internalize, and eliminate them via apoptosis. This *in vitro* study concluded that the recombinant immunotoxins demonstrate therapeutic potential against csGRP78-positive breast cancer. Flow cytometry analyses revealed that there was a high capacity for cancer cell penetration and induction of apoptosis . The different reactions to PE38KDEL-Nb among cell lines were directly related to their csGRP78 expression levels. The amount of Nbs-PE internalized into the cell lines was proportional to the density of surface antigens. Therefore, cells with higher surface antigens internalized more immunotoxins, leading to greater cytotoxicity at a given dose compared to cells with lower surface antigens (36). This data support the targeted functionality of the immunotoxin against csGRP78-positive breast cancer cells *in vitro.*

Bacterial toxins such as PE in immunotoxins induce cancer cell death by irreversibly modifying and inactivating eukaryotic elongation factor 2 (eEF2), disrupting the cellular protein synthesis machinery. The intracellular transport of the immunotoxin complex to the cytosol is crucial for anticancer efficacy, as it enables the toxin to reach its cytoplasmic target. Specifically, the targeting moiety of the immunotoxin first interacts with the cancer cell surface receptor, leading to the endocytic internalization of the bound complex. The trafficking and processing of the immunotoxin depends on the toxin and the target antigen, ultimately leading to the translocation of the toxin's enzymatically active domain into the cytosol. This allows for cytotoxic disruption of essential protein translation pathways within the targeted cancer cell. The immunotoxin's dual properties of specific cell surface receptor recognition and effective cytosolic delivery of the toxin component underlie its mechanism of action as a tumor-directed anticancer agent (40, 46).

## CONCLUSION

In conclusion, the immunotoxin structures developed in this study demonstrate promising potential as effective agents against GRP78-

expressing cancer cell lines in a preclinical context, making them suitable candidates for targeted therapy. The results indicate the designed immunotoxins, which specifically interact with GRP78 proteins to induce apoptosis, show promise as a therapeutic approach. Further research is warranted by utilizing *in vivo* animal models and intratumoral administration methods to explore the clinical translational potential of these immunotoxin structures. Areas of interest could include addressing drug resistance mechanisms and improving outcomes in cancer treatment. Additional *in vivo* studies using appropriate disease-relevant models would help confirm these proof-of-concept findings and propel the immunotoxins closer to clinical applications; positive outcomes would justify further development.

## AUTHORS' CONTRIBUTION

JA conceived the original idea. JA and MK designed the experiments; MK performed experiments and collected data; MK, JA, and HA discussed the results and strategy; JA and MMF supervised, directed, and managed the study; All authors approved the final version to be published.

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## ETHICAL APPROVAL

"This article does not contain any studies with human participants or animals performed by the authors."

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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