Original Article

Cytotoxic Effects of a Novel Tagged Apoptin on Breast Cancer Cell Lines

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Abstract

Backgrounds: Apoptin can induce tumor cell-specific apoptosis in a broad range of human tumor cells and is a potential anticancer therapeutic candidate to kill tumor cells.

Materials and Methods: We designed two structures of apoptin fusion protein, SUMO-PTD4-Apoptin, and PTD4-Apoptin. To express these fusion proteins, *E. coli* BL21(DE3) was employed. MTT assay, Flow cytometry, and cell cycle analysis were used to investigate the function of proteins on two breast cancer cell lines (MDA-MB-231 and MCF-7) and MCF 10A cell line (as normal cells).

Results: Expression of the recombinant SUMO-PTD4-Apoptin and PTD4-Apoptin in *E. coli* BL21(DE3) was successful. MTT assay results showed that the IC50 was 6.4 µg/ml for SUMO-PTD4-Apoptin in MDA-MB-231 and was 9.3 after 24 h of treatment in MCF-7. The specific cytotoxicity in both cell lines is significant in comparison with MCF-10A, which is used as a normal cell line (IC50 = 29.4). The IC50 for PTD4-Apoptin was 11.07 µg/ml after 24 h of treatment in MDA-MB-231, while the IC50 of PTD4-Apoptin for MCF7 cells was not significantly different from normal cells. The flow cytometry analysis displayed a significant increment in the apoptosis and late apoptosis number in the MDA-MB-231 cells after treatment with SUMO-PTD4-Apoptin and PTD4-Apoptin protein. PTD4-Apoptin and SUMO-PTD4-Apoptin treatment of MDA-MB-231 cells caused a noteworthy increase in the G0-G1 phase and a reduction in the cell population of S and M/G2.

Conclusion: This study demonstrates that the fusion of PTD4-Apoptin to SUMO-PTD4-Apoptin could provide an effective method to help enhance the expression and solubility of heterologous Apoptin in *E. coli.* BL21 (DE3).

Keywords: Apoptin, apoptosis, breast cancer

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Introduction

Despite tremendous advances and approaches in biomedical research and cancer biology, cancer remains a major health problem. Chemotherapy and radiotherapy, as common cancer treatments, are always associated with many side effects due to damage to healthy cells and tissues. Finding compounds against tumor cells that do not destroy normal cells is always necessary for designing specific cancer treatments.

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Apoptin, a 13.6-kDa serine-threonine-rich protein, was initially derived from the chicken anemia virus (CAV). This virus is one of the smallest chicken viruses that cannot grow in healthy cells. The CAV genome encodes three proteins (VP1, VP2, and VP3), of which VP3 is called Apoptin.[1] Apoptin in many human cancer cell lines makes apoptosis induction. Studies have shown that Apoptin in normal cells cannot activate cell death due to its accumulation in the cytoplasm, while in cancerous cells, Apoptin is located in the nucleus. In addition, its phosphorylation level is higher in cancerous cells than in normal cells.[2] One of the critical points regarding this protein is its ability to destroy tumor cells in which the P53 gene is defective or mutated. As apoptin can induce tumor cell-specific apoptosis in a broad range of human tumor cells, it is a potential anticancer therapeutic candidate for killing tumor cells and cancer therapy.[3]

Considering the important and functional role in destroying all types of tumor cells, the preparation of the recombinant form and the direct application of this protein have been studied to kill cancer cells.

Various fusion tags have been described to help enhance the expression and solubility of heterologous proteins in *E. coli*.[4,5] Among them, the SUMO (small ubiquitin-like modifiers), fused at the N-terminus of proteins, is a more efficient tag for the better expression of some heterologous proteins in *E. coli*.[6] Despite increasing protein expression, with its chaperone property, the SUMO-tag also helps better fold recombinant protein.[7]

The goal of this research project is to explore the role of SUMO fusion on the expression and function of recombinant Apoptin in *E. coli* BL21 (DE3). We also used protein transduction domain 4 (PTD4) before the Apoptin sequence to facilitate the delivery of apoptin across the cellular membrane. Recombinant PTD4-Apoptin protein was expressed in two forms (with and without SUMO-tag) in *E. coli* BL21 (DE3). Finally, the function of two protein forms was investigated while MDA-MB-231 and MCF-7 were selected as the breast cancer cell lines and the MCF-10A acts as the control.

Materials and Methods

Expression of Apoptin and SUMO-Apoptine in E. coli

The transformation phase is performed by transforming the constructed expression vectors, pET28a-SUMO–PTD4-Apoptin and pET28a-PTD4-Apoptin [Figure 1], into *E. coli* BL21 (DE3). After transformation, a single colony of bacteria grown, whereas 50 ml of LB broth (with 30 μg/ml kanamycin) was employed as the medium, the ambient temperature was 37°C, and the shaking speed was 200 RPM to obtain an optical density of OD600 = 0.8. In the next step, the isopropyl-D-1-thiogalactopyranoside (IPTG) (Merck, Germany) has been used to add to a final concentration of 1 mM for inducing the expression of recombinant protein. The PTD4-Apoptin and SUMO–PTD4-Apoptin proteins with the His-tag were exposed to SDS-PAGE using a 12% polyacrylamide gel. To load the samples, they have been mixed with a loading buffer. The gels were stained with Coomassie brilliant blue R-250.

For Western blotting, after isolating the proteins using SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Whatman, UK). The membrane was incubated with 1: 2000-diluted Horseradish Peroxidase (HRP)-labeled anti-His-tag monoclonal antibody (Abcam, UK). To visualize the immunoreactivity, DAB/H_2O_2 substrate solution (Roche, Germany) was employed.

Purification of recombinant PTD4-Apoptin and SUMO–PTD4-Apoptin

The affinity chromatography method using Ni2+-NTA agarose resin (Novagen, USA) has been used to separately purify the recombinant PTD4-Apoptin and SUMO-PTD4-Apoptin. The deposited pellet was lysed on ice using sonication after being suspended in 5 ml of denaturing buffer (6 M Urea, 20 mM NaH2PO4, and 500 mM NaCl, pH 8.0). Also, the supernatant has been loaded on the resin after centrifugation. The centrifugation was performed at a speed of 12000 rpm for 25 minutes. For washing the columns, 10 ml of wash buffer with imidazole's gradient of 10 mM, 20 mM and 30 mM has been used, and the washing process was performed three times. Finally, 1 ml of elution buffer containing 500 mM imidazole[8] has been used to elute the bound target proteins. Also, the protein was dialyzed in phosphate-buffered saline (PBS) at room temperature for two hours.

Cell culture

The required cell lines including human triple-negative breast cancer cell lines, MDA-MB-231 and MCF-7, along with normal breast epithelial cells, MCF-10A, were obtained from Iran Biological Resource Center (IBRC, Tehran, Iran). For culturing the cells, Dulbecco's Modified Eagle Medium (DMEM) has been employed. The medium consisted of 10% FBS and 1% Penicillin/Streptomycin. Also, for culturing MCF-10A cells, the DMEM containing 10% horse serum, epidermal growth factor (EGF) (20 ng/mL), hydrocortisone (0.5 mg/mL), cholera toxin (100 ng/mL), and insulin (10 μg/mL final) with 10% horse serum, 1% penicillin/streptomycin has been used. For incubating the cells, the incubation ambient was adjusted in a humidified atmosphere at 37°C with 5% CO_2.

Effects of PTD4-Apoptin and SUMO–PTD4-Apoptin on cell proliferation and survival

To culture MDA-MB-231, MCF-7, and MCF-10A cells, 96-well plates (3000 cells/well) have been employed. According to the MTT assay, we found 11 concentrations of SUMO–PTD4-Apoptin (0, 2, 5, 8, 10, 15, 20, 25, 30, 60, and 90 μg/ml) and nine concentrations of PTD4-Apoptin (0, 2, 5, 10, 15, 20, 30, 60 and 90 μg/ml). After 24 hours of the cells' incubation, PTD4-Apoptin and SUMO–PTD4-Apoptin in the above concentrations were independently added to the cells, whereas the temperature and CO_2 were 37°C and 5%,

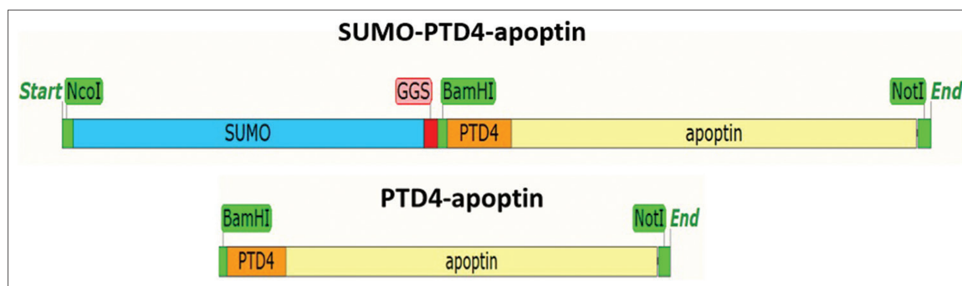

Figure 1: Designed constructs for expression of SUMO–PTD4-Apoptin and PTD4-Apoptin

respectively. Also, the MTT has been correspondingly added to the wells after 24, 48, and 72 hours, and the plates have been incubated for four hours. After discarding the supernatant, a volume of 100 µL of dimethyl sulfoxide (DMSO) was added to each well. To measure the optical density at 570 nm, a BioTek plate reader (USA) has been employed. The experiment was conducted in triplicates, and IC50 of the two forms of apoptin (PTD4-Apoptin and SUMO–PTD4-Apoptin) was determined using MTT assay and statistical analysis.

Apoptosis/Necrosis Assay using Flow cytometry

According to MTT assay results, the suitable concentration of PTD4-Apoptin and SUMO–PTD4-Apoptin was added to cells cultured in six-well plates.

After the incubation of the cells for 24 h, they were trypsinized and collected. In the next step, an AnnexinV-PI kit (Abcam, USA) has been employed to stain the cells. Moreover, a Facscalibur flow cytometer (Becton Dickinson, USA) was used to analyze the cells. Also, the Flowjo software has been employed to analyze the results.

Cell cycle analysis

According to MTT assay results, the suitable concentration of PTD4-Apoptin and SUMO–PTD4-Apoptin was added to cells cultured in six-well plates. After 24 hours of incubation, the cells were trypsinized, collected, and fixed with 70% ethanol and subsequently rehydrated in PBS. The RNase A and Triton X-100 (1 mg/ml) were added to samples and incubated for 40 min. Propidium iodide (PI) (1 µg/ml) was used for staining. The fluorescent intensity was measured using the fluorescent□activated cell sorter instrument (Becton Dickinson FACscan; Becton Dickinson, San Jose, CA) with a 488□nm argon laser. We used Cell Quest (Becton Dickinson) software for data acquisition and Flowjo software to calculate the cells' percentages in the cell cycle phases including G1, S, and G2.

Result

Expression and purification of the recombinant PTD4-Apoptin and SUMO–PTD4-Apoptin

PTD4-Apoptin and SUMO-PTD4-Apoptin, cloned into the pET28a (+) vector, were expressed in *E. coli* BL21 (DE3) and purified by affinity chromatography using the Ni^{2+}-NTA agarose resin. Figure 2 illustrates the results of SDS-PAGE and Western blotting of the purified PTD4-Apoptin (10 kD) and SUMO–PTD4-Apoptin (34 kD). The concentration of PTD4-Apoptin and SUMO–PTD4-Apoptin proteins were 0.05 mg/ml and 0.4 mg/ml, respectively.

In vitro anti-cancer effect of PTD4-Apoptin and SUMO–PTD4-Apoptin proteins

The MTT results and IC50 calculation of the PTD4-Apoptin and SUMO–PTD4-Apoptin proteins revealed in MDA-MB-231, MCF-7 that PTD4-Apoptin and SUMO–PTD4-Apoptin proteins had a significant cytotoxic effect on MDA-MB-231, MCF-7 breast cancer cells and compared with the normal group in a time-24 h and dose-dependent way. The IC50 was 6.4 µg/ml for SUMO–PTD4-Apoptin in MDA-MB-231 and was 9.3 after 24 h of treatment in MCF-7 [Figure 3a]. However, IC50 for PTD4-Apoptin was 11.07 µg/ml after 24 h of treatment in MDA-MB-231; PTD4-Apoptin did not significantly affect the MCF-7 cell line [Figure 3b].

Flow cytometry analysis

The results [Figure 4] showed that the apoptosis and late apoptosis number in the MDA-MB-231 cells increased significantly after treatment with SUMO-PTD4-Apoptin and PTD4-Apoptin protein compared with untreated cells. The necrosis cells also increased not significantly, although the PTD4-Apoptin protein was more effective. The statistical importance of the results has been verified by means of the one-way analysis of variance analysis. Moreover, the obtained apoptosis rate's P values between the treatment and control groups were meaningful (P values ≤ 0.001).

Cell cycle analysis of MDA-MB-231 treated with PTD4-Apoptin and SUMO–PTD4-Apoptin

Extract DNA content and cell cycle analysis of MDA-MB-231 cells were performed 24 h after treatment with PTD4-Apoptin and SUMO-PTD4-Apoptin. The results showed that PTD4-Apoptin and SUMO-PTD4-Apoptin treatment of MDA-MB-231 cells caused a meaningful growth in the G0-G1 phase, indicative of increased pro-apoptotic cells [Figure 5]. They decreased S and M/G2 cell populations, although these changes were more significant for cells treated with SUMO-PTD4-Apoptin.

Discussion

The selective activity of apoptin in tumor cell death induction has caused this protein to be considered in various studies

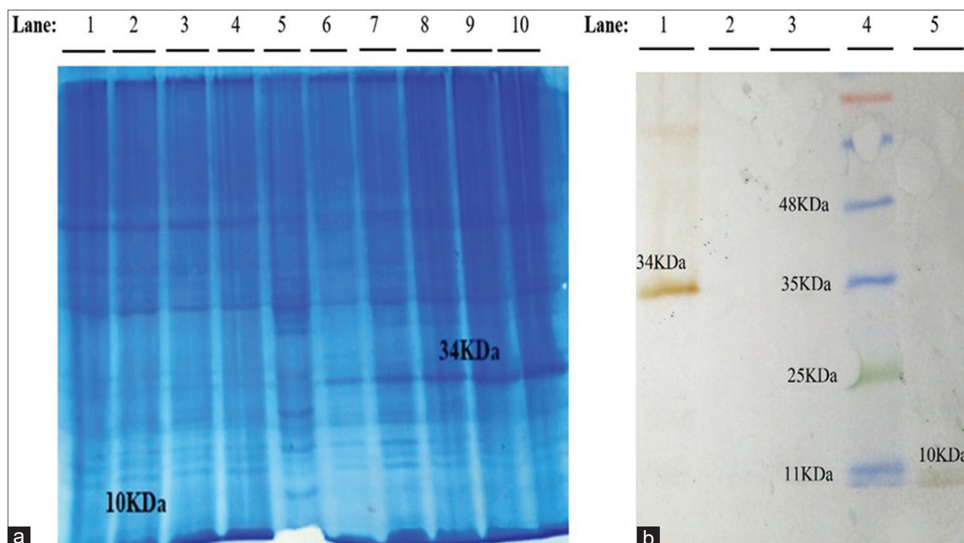

Figure 2: (a) SDS-PAGE analysis of Apoptin and SUMO-Apoptin proteins expression at different times: Lanes (1–4): expressions of apoptin protein at 8, 6, 4, and 2 after induction with one mM IPTG. Lane 5: BL21; Lane 6: t_0; Lanes (7–10): expressions of sumo-apoptin protein at 2, 4, 6, and 8 after induction with one mM IPTG. (b) Western blot analysis of apoptin and sumo-apoptin proteins: Lane 1: SUMO-Apoptin; Lane 2: BL21; Lane 3: t_0; Lane 4: marker; Lane 5: Apoptin

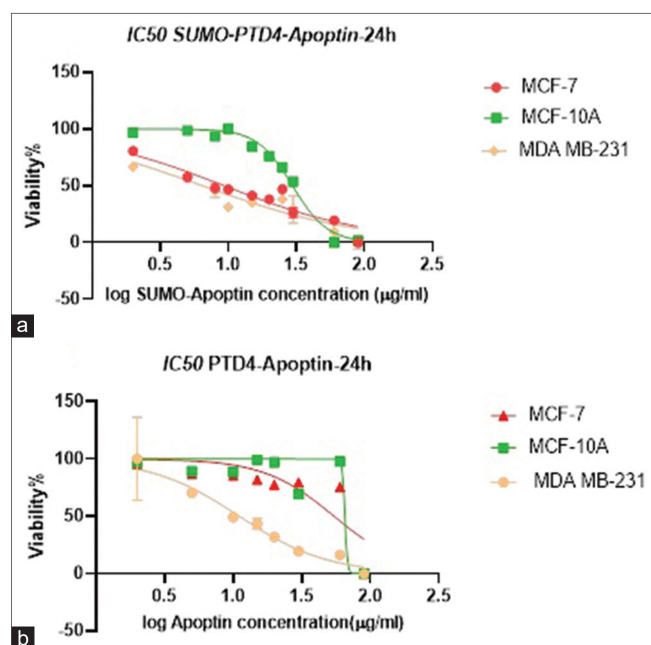

Figure 3: (a) The IC50 for SUMO-Apoptin (a) and Apoptin (b) MDA-MB-231, MCF-7, and MCF-10A

of anticancer therapy.[9-11] It is mentioned that apoptosis induced in tumor cells by apoptin is dependent on p53 and not suppressed via Bcl-2 or BCR-ABL protein.[12] Apoptin can induce arrest in cell cycle G (2)-M and chromatin condensation in cancerous cells.[13] Several studies conducted two apoptin expression and virus expression systems such as plant cells, baculovirus-insect cells, and *E. coli*.[3,14,15] However, *E. coli* is still a noteworthy system in the expression of apoptin compared to the current systems, considering the cost, time, and operation.[16] Several problems have been incidents involving the efficiency of expression and apoptin's protein solubility in *E. coli*.[15] A number of strategies exist to enhance protein expression and improve the solubility of each protein produced in *E. coli*. One of these strategies is producing fusion protein with a suitable tag.[15] Lee and coworkers used GST and His tags separately fused to TAT-apoptin to enhance bacterial expression of TAT-apoptin (trans-acting activator of transcription (TAT) is used as penetrating peptide for protein delivery). They reported that GST causes stronger expression of TAT-apoptin compared to His-tag.[16] A fusion protein named MAEH based on apoptin (MBP-apoptin-EGF^{His}) was designed by Niesler and coworkers. They used maltose-binding protein (MBP) to enhance the solubility of apoptin-EGF^{His}, in which tumor-directed ligand EGF was employed as a targeting agent for apoptin delivery. They reported that MBP fused to apoptin-EGF improved the protein expression and solubility.[17] We tagged apoptin with SUMO-tag in the present study to improve apoptin soluble production. SUMO fusion causes expression and solubility enhancement.[18,19] MDA-MB-231 and MCF-7 are human breast cancer cell lines but have different characteristics. MCF-7 cells are estrogen receptor-positive (ER+) and progesterone receptor-positive (PR+), meaning they can be stimulated to grow by the estrogen and progesterone hormones. MDA-MB-231 cells, however, are triple-negative breast cancer cells, which means they lack expression of estrogen receptor, progesterone receptor, and HER2/neu protein. As a result, MDA-MB-231 cells do not respond to hormonal therapies like tamoxifen or aromatase inhibitors that target ER + breast cancers. MDA-MB-231 cells also tend to be more invasive and metastatic than MCF-7 cells.

Conclusion: In the cytotoxicity study, it was seen that the addition of the SUMO-tag led to a decrease in the IC50 of

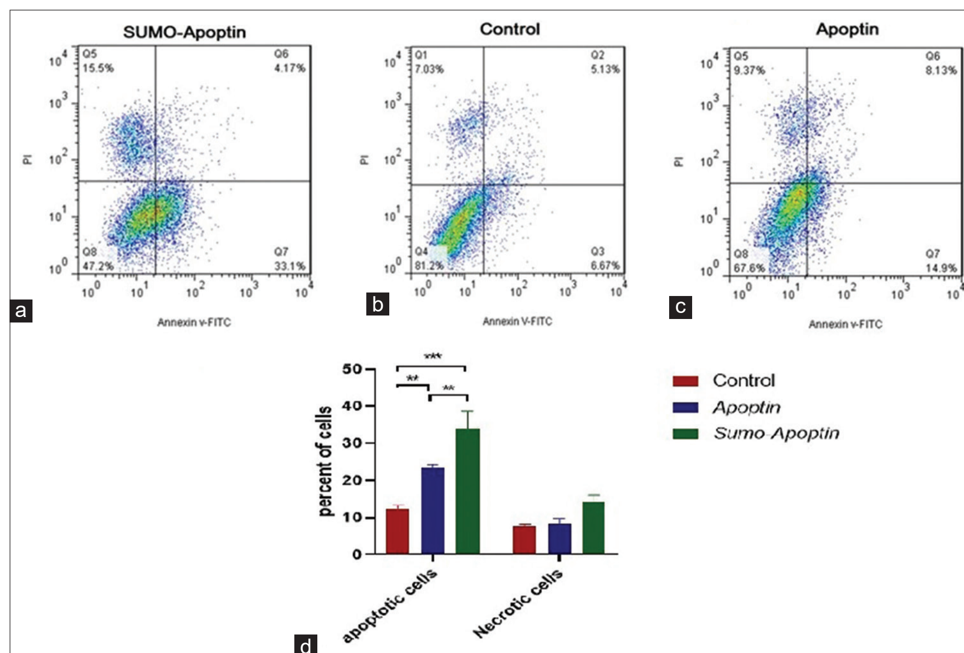

Figure 4: Flow cytometric analysis of MDA-MB-231 cells. (a) cells are treated with SUMO-Apoptin, (b) cells without treatment, (c) cells are treated with Apoptin, and (d) total apoptosis and late apoptosis cell

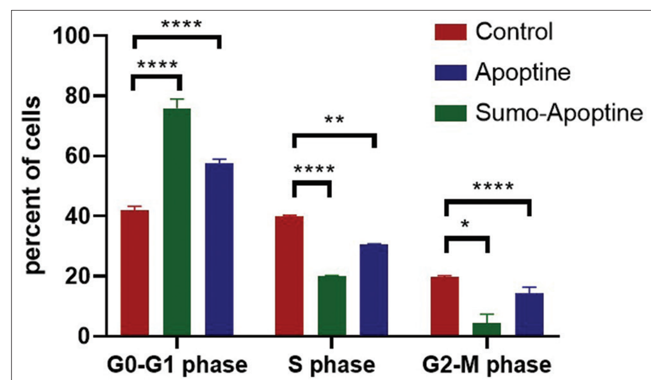

Figure 5: Cell cycle analysis of MDA-MB-231 treated with Apoptin and SUMO-Apoptin

apoptin in both breast cancer cell lines, especially in MDA-MB 231, and thus increased the cytotoxicity of Apoptin. In cell cycle analysis, it can be seen Apoptin can stop the cells in the G0-G1 phase and prevent the cell from entering the S phase and mitosis. This happened with the presence of SUMO-tag in a higher percentage of cells, which confirms the previous data. Also, in the examination of apoptotic cells using Annexin/PI staining, it was seen that SUMO-PTD4-Apoptin, compared to PTD4-Apoptin, leads the cells to apoptosis more significantly. The necrotic cells caused by SUMO-PTD4-PTD4-Apoptin were also more than those caused by Apoptin, but this difference was insignificant.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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