Original Article

Antiproliferative Effects of Recombinant Apoptin on Lung and Breast Cancer Cell Lines

Masoud Ezami Razliqi, MSc[1]; Gholamreaza Olad, PhD[2*]; Rouhollah Dorostkar, PhD[1]; Sahar Heydari, PhD[2]; Hadi Esmaeili Gouvarchin Ghaleh, PhD[1]

[1]Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2]Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

Abstract
Background: Selective therapy has always been the main challenge in cancer treatments. Various non-replicative oncolytic viral systems have revealed the safety and efficacy of using viruses and these products. The aim of this paper is to examine the impact of recombinant apoptin on the proliferation of lung cancer and breast cancer cell lines.
Methods: The present study consisted of two steps of expression of recombinant apoptin and its anti-proliferative effects on normal and cancer cells. In the first step, following bioinformatics and optimizing apoptin gene sequencing and synthesis, it was expressed using vector PET28a and E. coli BL21 (DE3). The expressed recombinant apoptin was confirmed by analytical SDS-PAGE and then purified using Ni affinity chromatography. In the second step, the antiproliferative effects of recombinant apoptin on lung cancer, breast cancer and primary cell lines were determined using MTT assay.
Results: According to the results of SDS-PAGE gel assay, recombinant apoptin was visible in the 14 kDa band. Also, the MTT assay results indicated that the antiproliferative effects of recombinant apoptin in cancer cell lines was different compared with the primary cell line, and followed a dose-dependent manner in both cell lines. The highest cytotoxicity (lowest cell viability) groups were 0.2 mg/mL in lung cancer (0.32 ± 0.015) ($P<0.001$), and in breast cancer (0.33 ± 0.031) ($P<0.001$) and 0.032 mg/mL in primary cells (0.17 ± 0.004) ($P<0.01$), as compared to the control groups.
Conclusion: Our results confirmed that recombinant apoptin can induce antiproliferative effects in lung cancer and breast cancer cell lines, but not in normal monkey kidney cell line Vero; thus, it can be introduced as a promising novel specific antitumor agent after further evaluation in clinical trials.
Keywords: Breast cancer, Chicken anemia virus, Lung cancer, Oncolytic virotherapy, Recombinant apoptin
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Introduction

Cancer refers to the abnormal growth, proliferation, and irregularity in the cell cycle with probable extension to body cells in an unnatural manner (metastasis). The onset of cancer is associated with some mutations in certain genes in normal cells; therefore, cancer is a genetic disease. The developed tumor cell mass is apparently and functionally different from healthy cells, being benign (with progressive, exponential, and non-aggressive growth to other tissues) or malignant (rapid, metastatic growth) eventually resulting in patient's death.[1]

The conventional procedures for treating cancer include surgery and removing the tumor mass, followed by radiation and/or chemotherapy to destroy the remaining cancer cells.[2] Surgical treatment is not considered in the late stages of cancers or highly metastatic cancers with poor prognosis, but rather chemical drugs are solely applied which requires high doses without enough specificity for the altered cancerous cells.[3]

Given the high expenditures of treating cancer patients which requires specialized units, medications, instruments, and various disciplines and facilities, it seems essential to pay attention to the prevention/prognosis of different cancers and focusing on novel and effective treatments.[4] Different studies have applied many viruses and their encoded proteins to discover some solutions to encounter the complexities and problems involved in cancer treatment.[5]

The virus-specific capabilities for attachment to cancer cells have become interesting to researchers as a new approach which may compete with other techniques such as radiation, chemotherapy, and surgery.[6] Multiple applications of these recombinant proteins have recently attracted huge attention in terms of understanding the specific tumor cell physiology. Therefore, novel therapeutic methods for cancer treatment will be developed with different mechanisms to influence cancer cells with our growing knowledge of these proteins in the future.

The apoptin protein has been extracted from chicken anemia virus (CAV) among other apoptotic proteins.[7] CAV is an important immunosuppressive virus found in newborn chickens and natural poultry hosts, consisting of

*Corresponding Author: Gholamreza Olad, PhD; Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran. Tel: +98-9128043492. Email: grolad@gmail.com

three protein sequences: VP1, VP2, and VP3.[8]

The 50 kDa viral protein VP1 is the only protein isolated from purified viral bodies. The non-structural 30 kDa protein VP2 probably functions as a scaffold protein when virions accumulate. The 16 kDa viral protein VP3 locates in the nucleus of infected cells, but it cannot be identified in the purified viral bodies,[9] The product of the VP3 gene has been retitled apoptin due to its death-related capabilities.[10] The mentioned protein is capable of inducing apoptosis without using the suppressor pathway of the p53 tumor, but it is dependent on Bcl-2 tumor suppressor. Apoptosis induced by apoptin involves caspase induction leading to mitochondrial alterations and cytochrome c release.[11]

The C-terminal portion of apoptin consists of a bipartite nuclear localization sequence (NLS). The NLS1 includes amino acids No. 88-82 and the NLS2 includes the remaining amino acids No. 111-121.[12] Moreover, apoptin includes an assumed export sequence putative nuclear sequence in the remaining amino acid No. 75-105. This identification sequence makes apoptin move inside/outside of the nucleus. The apoptin protein specifically induces apoptosis in the transfected cells with no effects on healthy cells. This specificity feature is mainly due to its site in the cell; in healthy cells, there is cytoplasmic apoptin, but it is localized to the nucleus of transformed cells. It has been widely reported that the regulation of apoptin nuclear localization needs phosphorylation.[13]

Nowadays, it has been indicated that the expression of apoptin induces apoptosis in a broad range of cancer cells, while having no effects on normal cells.[14] The exact mechanisms of the specific apoptosis caused by CAV-apoptin in cancerous cells are not yet clearly understood. However, based on the existing evidence, the property of apoptin's tumor-specific killing activity is the consequence of several molecular events.[15] Apoptin acts through the intrinsic mitochondrial pathway, in a caspase-3 and caspase-9 dependent and p53 independent manner.[16] The selective toxicity of apoptin might be attributed to its differential subcellular localization in tumor and normal cells, which is controlled by phosphorylation.[17]

The aim of this paper was to examine the impact of recombinant apoptin on proliferation of lung cancer and breast cancer cell lines in order to provide further evidence regarding the cytotoxicity of apoptin in cancerous and normal cell lines.

Material and Methods
Preparation of Protein-Encoded Gene
The gene synthesis was ordered in the PET-28a vectors using the sequences existing in the GenBank sequence database.

Bacterial Culture
Escherichia coli BL21 DE3 was cultured in LB Broth medium containing the kanamycin antibiotic incubated overnight with 180 rpm at 37°C.

Preparation of Competent Cells
The liquid culture of bacteria (200 µL) was left to grow overnight in a new flask (5 mL) and incubated under previous conditions until OD = 0.5–0.7 was reached. Then, it was centrifuged at 700 rpm for 8 minutes at 4°C. The supernatant was discarded, and 1 mL of cold $CaCl_2$ was added to the sediment and kept in the refrigerator for 20 minutes. Next, it was centrifuged at 600 rpm for 4 minutes at 5°C with the removal of supernatant and followed by added cold $CaCl_2$ (600 µL) to the remaining sediment. Then, it was put in the refrigerator for 20 minutes, centrifuged at 500 rpm for 5 minutes, followed by added cold $CaCl_2$ (300 µL) to the remaining sediment and placed again in the refrigerator for 20 minutes.[18]

Transformation of Competent Cells
Recombinant plasmid encoding the synthetic apoptin gene (1 µL) was added to 100 µL competent cells, maintained on ice for 40 minutes, transferred to a thermoblock for 2 minutes at 42°C, and finally stored at 4°C for 15 minutes. After adding 600 µL of antibiotic-free culture medium to the prepared sample, it was incubated on a shaker at 200 rpm at 37°C for 37 minutes. Finally, the obtained microbial suspension was cultured on LB agar plate supplemented with kanamycin.[19]

Protein Expression
After the cultured colonies were counted on LB agar plate, the single colony was grown on LB broth medium tube, supplemented with kanamycin and incubated overnight for screening purpose. Then, 100 µL of the bacterial suspension was added to 5 mL of kanamycin-containing medium (20 µg/mL) and incubated to reach OD = 0.5-0.7. Next, it was incubated with 0.01 mM IPTG on a shaker at 200 rpm for 6 hours at 37°C. Finally, the sediment was separated after supernatant removal using centrifugation at 700 rpm for 7 minutes at 4°C.[20]

Confirmation of Protein Expression with SDS-PAGE Assay
In each sample, the separated sediment was mixed with 20 µL of 8 M urea and 2 µL of sample buffer, treated for 10 minutes at 95°C and finally centrifuged for 15 minutes at 13 000 rpm. The supernatant (20 µL) in each sample was transferred on SDS-PAGE in 14% polyacrylamide gel wells, separately, and also, it was put into one well along with a molecular weight marker. Electrophoresis was run at a voltage of 200 V in a tank for 35 minutes. After this step, the electrophoresis gel was separated, followed by washing, Coomassie Brilliant Blue was used to stain for 35 minutes, and the stain was washed for 30 minutes.[21]

Protein Purification Using Affinity Chromatography Column

The obtained cell precipitate was dissolved in 30 mL of lysis buffer and then sonicated. Next, it was centrifuged at 13 000 rpm for 1 hour at 4°C. After centrifugation, the clear supernatant was collected and transferred into an NTA-Ni column to purify the protein. Then, the column was washed using a progressive imidazole gradient (20, 80, and 200 mM). The output solutions were collected separately for each column step. Qualitative analysis of purified protein was performed using the SDS-PAGE. All purified fractions containing the target protein were combined and dialyzed with PBS buffer at concentrations of 0, 2, 4, and 6 mM for 12 hours, separately.[22] The NanoDrop instrument was used for measuring protein concentrations.

Culture of Cell Lines

The mentioned cells were cultured in a T25 flask with RPMI medium supplemented with 10% bovine fetal serum (FBS) in an incubator at 37°C, 5% CO_2, and 80% moisture content. After the density reached 80%, they were put into a 96-well culture plate (as 106 cells per well) for treating A-549 (lung cancer) and MCF-7 (breast cancer) cell lines with apoptin concentrations at 0.05, 0.1, and 0.2 mg/mL and treating the Vero (primary) cell line with apoptin concentrations at 0.0325, 0.065, and 0.13 mg/mL.[23]

Cell Proliferation Measured by MTT Assay

RPMI-1640 medium (100 µL) was added to wells of a 96-well microplate with a flat bottom containing 10^6 K562 cells. Then, the cells were incubated for 48 hours at 37°C with 5% carbon dioxide and 80% moisture content. After the incubation was completed, 20 µL of 5 mg/mL MTT solution was added to wells. Subsequently, the cells were incubated for another 4 hours. Since formazan dye crystals are precipitated in the cell cytoplasm, 100 µL of DMSO was added to each well to dissolve them; then, the color intensity was measured using ELISA at 492 nm.[24]

Statistical Analysis

The present study is an experimental study. Data were expressed as means ± standard deviations (SD) and were analyzed using one-way ANOVA to make comparisons between the groups. Whenever the ANOVA analysis indicated significant differences, Tukey's multiple comparison post-hoc test was performed to compare the mean values between the different groups. Differences between the groups were considered significant if P values were < 0.05.

Results

Apoptin Protein Expression

Some samples were taken from the transgenic bacterial culture to evaluate the expression of this recombinant protein, before and after IPTG-induced expression, and assayed using the SDS-PAGE procedure. The results showed that after IPTG-induced expression, this recombinant protein was expressed as nearly 14 kDa (Figure 1).

Protein Purification

Based on the H-polyhistidine sequence presented in the related protein, affinity chromatography with a Ni-NTA resin column was applied for protein purification, and the obtained results verified protein purity (%) by SDS-PAGE analysis (Figure 2).

MTT Assay

The purified apoptin protein was added to three cell line including Vero, A-549, and MCF-7 with the following results: After the MTT assay was performed in the prepared solutions with three serial diluted concentrations (0.0325, 0.065, and 0.13 mg/mL) on the Vero cell lines, the obtained results showed the highest cytotoxic effect at 0.0325 mg/mL (Figure 3).

After the MTT assay was performed in the prepared solutions with three serial diluted concentrations (0.05, 0.1, and 0.2 µg/mL) on A-549 cell lines, the obtained
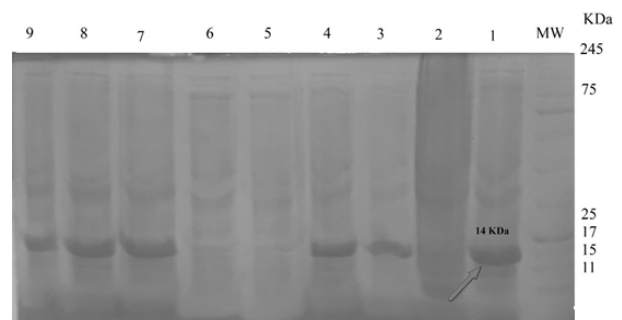

Figure 1. Gene Expression of Apoptin Recombinant Protein. Lane MW prestained protein ladder, Lane 1, 3, 4, 7, 8 and 9 test samples (positive clones), Lane 6 and 5 control samples.
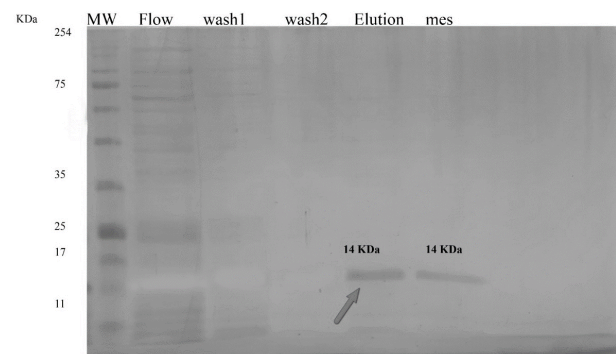

Figure 2. Purification of Apoptin Recombinant Protein. MW Lane prestained protein ladder, Flow lane, and wash1 and wash2 lanes with 20 and 80mM of imidazole, Elution lane containing 200mM imidazole, MES buffers lane.
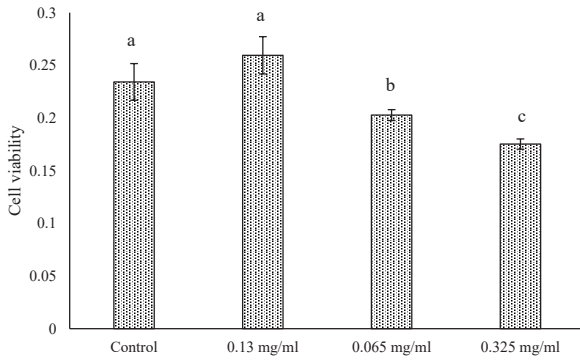
Figure 3. The Results of Purified Apoptin Protein on Vero Cells Proliferation. Different superscript letters show statistically significant differences between the groups (*P*<0.05).
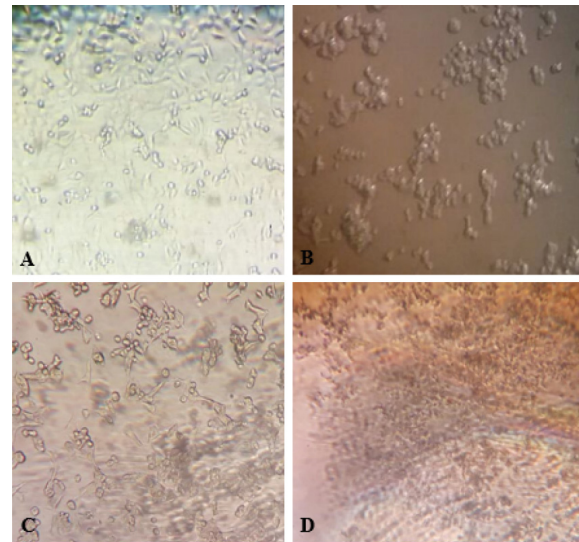

Figure 4. Images of A-549 Cells after Treatment with Different Concentrations of Apoptin Protein of CAV Virus Under Laboratory Conditions (Inverted microscope, 400x). A) No treatment with Apoptin. B) Treatment with 0.2 µg/mL concentration. C) Treatment with concentration of 0.05. D) Treatment with concentration of 0.1.

results showed the highest cytotoxic effect at 0.2 µg/mL (Figures 4 and 5).

After the protein assay was performed in the prepared solutions with three serial diluted concentrations (0.05, 0.1, and 0.2 mg/mL) on MCF-7 lines (Figures 6 and 7), the obtained results showed the highest cytotoxic effect at 0.2 mg/mL.

Discussion

Mainly due to the advent of widespread treatment techniques developed for cancers, most conventional therapies encounter certain troublesome constraints, necessitating innovations to enhance the capabilities of the therapeutic methods. Previous studies suggest that these limitations include severe side effects caused by their nonspecific cytotoxicity, development of drug resistance, and increased apoptosis resistance in tumor cells. Also, there are many side effects reported for the conventional agents applied in cancer therapy.[1]

Alkylating agents, antimetabolite drugs, and antibiotics are the most commonly used drugs in tumor therapies, and their proposed mechanisms include cell infiltration involved in the immune response against cancer through increased induction of apoptosis in cancer cells.[25]

The mutations in p53 gene are involved in the development of cancer. In most studies about resistant anticancer drugs, it has been proposed that the removal of the mentioned mutations can act as the best alternative pathway. Hence, in recent studies, most researchers have emphasized p53-independent treatments. The best features of an anti-oncogenic therapy that shows specific and selective cytotoxicity are as follows:

1) The proposed method is required to function with specificity and selectivity in cytotoxicity. In fact, the inhibited proliferation of abnormal cells (without normally regulated cell cycle) is intended, for instance, in checkpoint mutations; 2) The method is required to block the cell cycle for the induced cell death or apoptosis; 3) The method is required to prevent drug resistance to
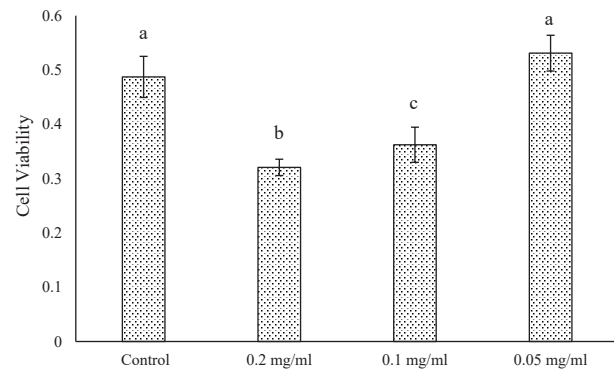

Figure 5. Toxicity effects of purified Apoptin recombinant protein on cell proliferation of A-549 cells. Different superscript letters show statistically significant differences between the groups (*P*<0.05).

the apoptosis process; 4) The process of cell cytotoxicity should be independent of common oncogenic mutations including the mutations in p53; 5) Finally, it should demonstrate high absorbability.[26]

Various studies show that apoptin can meet the above-mentioned conditions of inducing full apoptosis in cancer cells, while the surrounding highly proliferative cells do not show any decreased viability, including endothelial cells, hepatocytes and hematopoietic stem cells. Therefore, it seems that the cell sensitivity to apoptin and the consequent lethal results can be explained with some changes associated with the cell transformation process (into cancerous cell) and apoptin ability to induce apoptosis independently of p53. Apoptin is an important pharmacological agent and candidate for cancer therapy by induction of a pro-apoptotic message. Recent researches have demonstrated
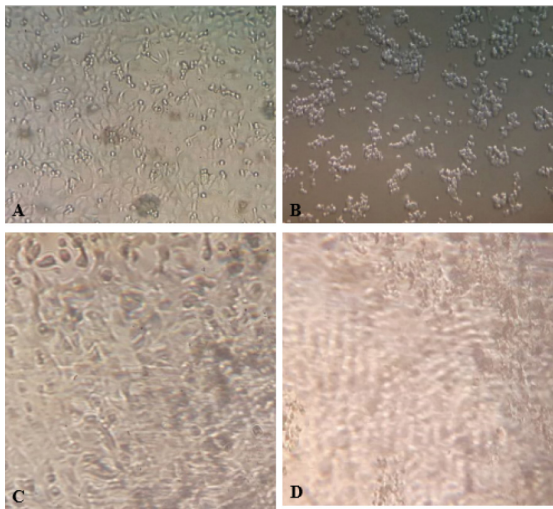
Figure 6. Images of MCF-7 Cells after Treatment with Different Concentrations of Apoptin Protein of CAV Virus Under Laboratory Conditions (Inverted microscope, 400x). A) Without treatment with Apoptin. B) Treatment with 0.2. C) Treatment with concentration of 0.05. D) Treatment with concentration of 0.1.
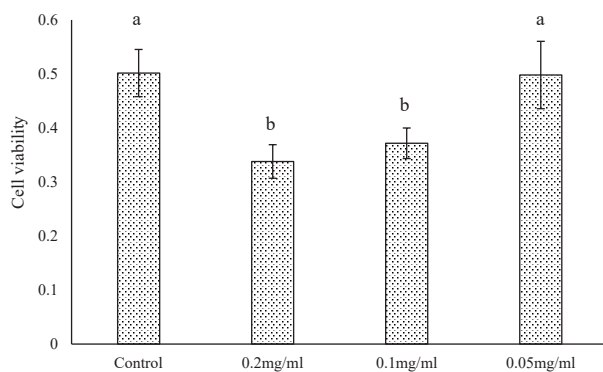

Figure 7. Toxicity Effects of Purified Apoptin Recombinant Protein on Cell Proliferation of MCF-7 Cells. Different superscript letters show statistically significant differences between the groups ($P<0.05$)..

that the negative feedback of Survivin was able to increase the apoptotic cells significantly when applied along with apoptin compared with treatment alone.[27] Another study reported that the integrated application of apoptin with interleukin-18 led to a severe Th1 immune response to Lewis lung carcinomas which resulted in significant growth inhibition of these tumor cells.[28]

The most critical challenge in studies of apoptin is related to the development of a technique for efficient apoptin delivery to the tumor lesion site. Olijslagers et al injected the adenoviral vectors to express and transmit the apoptin gene, suggesting that the injected adenoviruses with the apoptin gene resulted in a substantial regression of the tumors.[29]

Also, some studies applied other viruses to increase the induction and effectiveness of the apoptin protein. For example, a recent study indicated that the infection induced by avian smallpox virus (without the ability of

proliferation, functioning as a vector for the apoptin gene) resulted in significant apoptosis induction in hepatoma cells, suggesting the inhabitation of subcutaneously implanted tumor cells in mice.[30]

While the apoptin protein can function with specificity in the apoptosis induction of the transformed (cancerous) cells, the healthy cells are not affected by this process.[1] The specificity of apoptin depends on where it is found inside the cell; therefore, its specificity to the cancer cell is increased compared to other healthy cells, due to the transmission of this molecule from the outside of the cell to the nucleus during the process of attachment to the cancer cells. In cancer cells, this protein localizes inside the cell nucleus regulated through phosphorylation.

Apoptin specifically induces apoptosis in tumor cells through a pathway distinct from p53. Apoptosis is generally mediated by the intracellular contents of cysteine proteases (like caspases) functioning as both initiators and executors in the apoptosis process.

In the present study, after purification of apoptin recombinant protein, we first assessed the antiproliferative effects by MTT assay at three concentrations (0.05, 0.1, and 0.2 mg/mL) on both MCF-7 and A549 cancer cell lines. MTT test results indicated that apoptin recombinant protein decreased the proliferation of these cancer cells at the highest treated concentration (0.2 mg/mL) in a dose-dependent manner. The interesting finding was related to the highest and lowest effective concentrations, being similar in both MCF-7 and A549 cancer cells. The conventional chemotherapy drugs mostly do not have functional selectivity. In fact, they not only induce apoptosis in the cancer cells of the tumor tissue, but also impose some damages on surrounding healthy cells. Therefore, the apoptin recombinant protein was evaluated at three concentrations (0.032, 0.065, and 0.13 mg/mL) regarding the corresponding values of lethal selectivity in the 'Vero' healthy fibroblast lines (kidney epithelial cells extracted from an African green monkey) using MTT assay. The results indicated some reverse lethal effects on the Vero lines compared to the assayed cancer cells (A-549, MCF7) for apoptin recombinant protein. Given these findings, the assayed protein could not influence the proliferation of the Vero lines significantly at higher concentrations, but it functioned conversely at lower concentrations with a significant decrease observed in the Vero line's proliferation. As shown in Table 1, the maximum lethality in Vero cells was associated with the lowest concentration of apoptin, while inversely, the maximum lethality occurred at the highest relative concentration of apoptin in the cancer cells. These findings can prove the selective effects of apoptin recombinant protein on both healthy and cancerous cells.

In conclusion given the obtained results in the present work, it appears that the apoptin recombinant protein may efficiently kill various tumor cell types and thus

Table 1. Comparison of the Effects of the Optimum Apoptin Toxicity Concentrations in the Three Cell Lines

Cell lines Treated with Different Apoptin Concentration	Optimum Concentration of Toxicity (mg/mL)	Cell Viability (%)
Vero	0.032	0.17
A-549	0.200	0.32
MCF-7	0.200	0.33

serve as a potential candidate anti-tumor immunotoxin. However, it is necessary to design further molecular and immunological studies to evaluate the apoptotic-related mechanisms of this protein and find an appropriate and efficient vector for in vivo transmission. Therefore, it is possible to introduce the apoptin agent for combating cancers.

Authors' Contribution
All authors equally contribute to this study.

Conflict of Interest Disclosures
The authors report no conflicts of interest.

Ethical Statement
Ethical approval was granted by the Medical Ethics Committee of the Baqiyatallah University of Medical Sciences (IR.BMSU. REC.1396.558).

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