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Original Article

Expression, purification, and cytotoxic evaluation of IL24-BR2 fusion protein

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Abstract

Interleukin (IL) 24 is a pro-inflammatory and tumor suppressor cytokine capable of inducing selective apoptosis in various cancer cells. BR2, on the other hand, is an anti-microbial peptide with selective penetrability to the cancer cells. In this study, we aimed to produce and purify a fusion protein containing IL24 as the toxic moiety fused to BR2, as targeting moiety, and then to evaluate its cytotoxic activities. For this purpose, the coding sequence of IL24-BR2 fusion protein and IL24 were cloned into the pET28a vector and used to transform *E. coli* BL21 (DE3) cells. Following induction of expression, protein purification performed using Ni-NTA chromatography. SDS-PAGE and western blotting were performed to confirm the expression and purification. Finally, cytotoxic effects of the purified proteins were evaluated on MCF-7 and HUVEC cell lines. Analysis of crude lysate of induced recombinant *E. coli* BL21 (DE3) bacteria and also purified proteins showed a band of approximately 22 and 18 KDa on SDS-PAGE and western blotting for IL24-BR2 and IL24, respectively. Finally, statistical analysis showed significant cytotoxic effects of IL24-BR2 on MCF-7 cells at 10, 20, and 40 µg/mL concentrations compared to IL24 alone, which showed no significant cytotoxic effects on cancer cells except in the highest concentration. In conclusion, production and purification of IL24-BR2 fusion protein with potential specific toxicity toward cancer cells was successfully achieved. However, further investigation of the cytotoxic effects of this fusion protein on other cell lines and *in vivo* cancer models must be performed.

Keywords: BR2; Cytotoxicity; Fusion protein; IL24; IL24-BR2.

INTRODUCTION

One of the most effective techniques to reduce side effects of cancer therapeutics is their targeted delivery to cancerous cells and tissues (1). In this regard, peptides with ability to bind to cancer cells selectively, but not normal cells, are under intensive investigation as targeting moieties for targeted therapy. Several promising types of such peptides have shown applicability for targeted treatment of cancer (2), such as cell penetrating peptides (CPPs), a group of cationic peptides with trans membrane transition capability (3). BR2, a CPP derived from a synthetic antimicrobial peptide, buforin IIb with 21 residues, has shown highly selective cytotoxicity on cancer cells without any hemolytic effects or non-specific toxicity on normal cells. Therefore, this 17 residues peptide can be used as a targeting moiety for targeted eradication of cancer cells without any cytotoxic effects on normal cells (4).

Interleukin (IL) 24, initially known as melanoma differentiation-associated gene-7 (MDA-7) protein, is a novel member of the IL10 family of cytokines (5). At low concentrations, IL24 functions as a cytokine and plays a role in autoimmune and infectious diseases and wound healing (6). At higher concentrations, on the other hand, it acts as an anticancer protein (7). Studies have shown that the concentration of this protein decreases during cancer progression (8), and it is almost impossible to be detected in metastatic stages (9).

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IL24 induces apoptosis, anti-angiogenesis, and inhibition of invasion and metastasis (6) in a wide variety of solid tumors without affecting normal cells (10). This selectivity and safety led to the successful entry of this protein into the phase I/II clinical trial as a gene therapy agent, (Ad.mda; adenovirus mediated expression of IL24), with a trade name of INGN 241 by introgen corporation (10,11). In addition, IL24 has been used successfully to enhance radiosensitivity of cancer cells such as lung and glioma cells (12), or used in conjunction with other conventional therapies (6). The limitation of Ad.mda-7 usage is that some cancer cells do not express coxsackie virus associated or adenovirus receptors; therefore, Ad.mda-7 is unable to infect them (13). Hence, one strategy to solve this problem is to target IL24 protein into the cancer cells via production of a cancer cell targeted fusion protein (14).

In the present study, we applied BR2, a new CPP, as a cell targeting moiety. CPPs are short peptides having a net positive charge due to being rich in lysine or arginine. These peptides, due to their positive charge, can attach to the cell membrane that has a negative charge and penetrate into the cell (3). The cancer cell membrane has more negative charge than normal cell because of different membrane composition. This property causes the selective penetration of these peptides into cancer cells (4,15).

Therefore, the aim of the present study was to produce a novel cancer targeting fusion protein containing IL24 sequence fused to BR2, a cancer specific cell penetrating peptide, in order to direct the pro-apoptotic and cytotoxic effects of IL24 toward cancer cells.

MATERIALS AND METHODS

Plasmids, bacterial strains, and reagents

Top 10 and BL21 (DE3) *Escherichia coli* (*E. coli)* strains, pET28a expression plasmid, MCF-7 (human breast carcinoma cell line), and HUVEC (human umbilical vein endothelial cell) from Pasteur Institute of Iran (Tehran, I.R Iran); Kanamycin and isopropyl β-D-1-thiogalactopyranoside from Sigma (San Diego, California, USA). Fast Digest™ restriction endonucleases, GenJET plasmid preparation kit, and GenJET gel extraction kit from Thermoscientific (Fermentas; Vilnius, Lithuania). Other materials in the molecular biology grade were in commercial form. All tests performed according to the Sambrook and Russell protocols unless otherwise mentioned (16).

Fusion protein design and synthesis

The IL24 sequence was obtained from NCBI (gene bank accession number: BC009681.1), and fused to BR2 sequence (RAGLQFPVGRLLRRLLR) via an eleven-residue rigid linker (P(AP)5). Since C-terminal end of BR2 is responsible for its cell penetrating capability, a 6X-His-tag coding sequence was placed at the N-terminal end of the whole construct. The three-dimensional model of this fusion protein was predicted using Modeller 9.14 program in order to make sure the freedom of action of each domain. The predicted models were evaluated in terms of energy distributionand finally visualized in PyMol software. VMD software used to determine the interaction between two moieties of fusion protein via salt bridge formation analysis. The nucleotide sequences of IL24-BR2 synthesized using GENEray (Hong Kong) and obtained in pGH-IL24-BR2 plasmid.

Cloning of the IL24-BR2 coding sequence

Both pGH-IL24-BR2 vector and pET28a plasmid were digested with *Nco*I and *Xho*I restriction endonucleases (45 min at 37°C), followed by gel extraction of the desired DNA fragments using GenJET gel extraction kit. Then, the IL24-BR2 coding fragment was ligated to the pET28a plasmid using T4 DNA ligase (Fermentase, USA) as instructed by the manufacturer. The fidelity of cloning was verified by restriction digestion of the obtained plasmid with the mentioned enzymes, and finally by DNA sequencing.

IL24 synthesis and cloning

IL24 coding sequence was amplified by suitable primers (Table 1) using pET28-IL24-BR2 plasmid as a template and then was gel purified and cloned in to the *Nco*I and *Xho*I restriction sites of the pET28a plasmid. Finally, the recombinant plasmid was authenticated by DNA sequencing and designated as pET28-IL24 plasmid.

Expression of IL24-BR2 and IL24

Competent *E. coli* BL21 (DE3) cells transformed with pET28a-IL24-BR2 or pET28a-IL24 plasmids used to induce the expression of IL24-BR2 or IL24 protein, respectively. In this regard, overnight cultures of the bacteria inoculated into 200 mL of fresh culture (15 µg/mL kanamycin to retain the recombinant bacteria) at 37 °C with shaking until OD 600 of 0.4 to 0.6 was reached. Then, the protein expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside at 37 °C for 4 h. Then, the cells were harvested by centrifuging at 7000 *g* for 10 min at 4°C. Finally, expression of the recombinant proteins was evaluated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western blotting

For further confirmation of bands observed on SDS-PAGE, western blotting was performed. Briefly, the protein bands separated on 15% SDS-PAGE gels were electro-transferred onto activated polyvinylidene difluoride membrane (Sigma, Germany). Then, the membrane was blocked in 3% skim milk in tris buffered saline (TBS buffer, 125 mM NaCl, 25 mM tris, pH 8) overnight at 4 °C. Afterward, the membrane was washed with 1% (v/v) Tween® 20 in TBS buffer (TTBS) for three periods of 10 min and then, the membrane was incubated at room temperature with anti-His tag antibody (Invitrogen, USA) (1:1000 in TB-Tween® buffer 0.1%) for 1.5 h, followed by three times of 10 min washing with the wash buffer mentioned above. Finally, H_2O_2 30% and diaminobenzidine (Sigma, Germany) was used to visualize protein bands.

Purification of the recombinant proteins

Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Invitrogen, California, USA) used to purify the recombinant proteins. Following induction of protein expression, bacterial cells pelleted and suspended in guanidine buffer (6 M guanidium HCl, 20 mM sodium phosphate pH 7.8, 500 mM NaCl) and sonicated on ice using three 30-second bursts and three 15-second at average intensity with a 2-min cooling period between each burst. Then, the samples centrifuged at at 3000 *g* for 15 min at 4°C. The column preloaded with Ni-NTA agarose and equilibrated with denaturing binding buffer (8 M urea, 20 mM sodium phosphate pH 7.8, 500 mM NaCl,). Next, the supernatant applied to the column and incubated for 30 min at room temperature while shaking. After two washes with 4 mL of the denaturing binding buffer, washing 3 times with denaturing wash buffer (8 M urea, 20 mM sodium phosphate pH 6, 500 mM NaCl and imidazole 20 mM) followed by 5 times of washing with denaturing wash buffer (same as before except the pH 5.3) was performed. Proteins were then eluted with denaturing elution buffer (8 M urea, 20 mM NaH_2Po_4 pH 4, 500 mM NaCl) and subjected to dialysis against phosphate-buffered saline (PBS) pH 7.4 for 24 h at 4 °C. Bradford assay with bovine serum albumin (Sigma, Germany) as a standard protein was used for measuring the protein concentration (17). The control protein, IL24, was expressed and purified in the same way.

Table 1. Primer sequences used for cloning of interleukin 24.

Primer	Sequence 5′-3′	Restriction enzyme	Restriction site
Forward	CATGCCATGGCCCATCATCATC	*Nco*I	CCATGG
Reverse	CCGCTCGAGCTATTACAGCTTATAAAATTTCTG	*Xho*I	CTCGAG

Cytotoxicity assay

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the cytotoxic effects of IL24-BR2 and IL24 proteins on cancer and normal cells. MCF-7 (as cancer originated cells), and HUVEC (as normal cells) cultured in RPMI-1640 or dulbecco's modified eagle medium (DMEM), respectively, containing 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) at 37ºC in a 5% CO_2 incubator. At the day of experiment, 180 µL of the medium containing 5×10^4 cells/mL of each cells were seeded in each wells of 96 microplates and incubated for 24 h at 37ºC in 5% CO_2 incubator. Afterwards, 20 µL of the purified proteins resulting in final concentrations of 0.001, 0.01, 0.1, 1, 10, 20, and 40 µg/mL or 20 µL PBS (as negative control) were added to each wells and incubated for 48 h at 37 °C in 5% CO2. After 48 h, 20 µL of MTT (5 mg/mL) was added to each well, and 3 h post incubation, well contents were replaced with 150 µL dimethyl sulfoxide (DMSO) to dissolve formazan crystals and finally the plates were subjected to absorbance read at 570 nm by a microplate reader (Bio-Rad, USA).

Statistical analysis

To ensure the accuracy and reproducibility of data, the MTT test was performed in three independent experiments of 4 replicate wells for each concentration. Cell culture media used as blank and PBS treated cells considered as negative control. SPSS 22 software was used for statistical analysis. Analysis of variance (ANOVA) followed by Tukey's post hoc test used to determine the differences between groups. $P < 0.05$ was considered significant. The IC_{50} of IL24-BR2 was determined by drawing the graph of cell survival percent against concentration using GraphPad Prism 5.0 software.

RESULTS

Fusion protein design and synthesis

Three-dimensional structure of IL24-BR2 fusion protein was designed using crystal structure of human interleukin 10, with a resolution of 1.6 Å (PDB id: 2ILK) for IL24, the solution structure of the second N terminal of FK BP38 (PDB id: 2MF9) for linker, and crystal structure of a histone octamer with a resolution of 1.9 Å (PDB id: 1TZY) for BR2. Between 100 predicted structures for fusion protein, the best structure based on the lowest discrete optimized protein energy (DOPE) score of -14249.82422, molpdf of 1877.5569 and GA341 (0.9997) selected. The structure of BR2 in the fusion protein showed a structure similar to the model prepared for buforin IIb. This part of the fusion protein consists of alpha helical structure in the BR2 C-terminal, which is needed for membrane penetration. Furthermore, three-dimensional structure of IL24 was predicted with 8 helices and without any beta sheets. Finally, the results of salt bridge formation between two segments of the fusion protein via VMD software showed that there is no interaction between two parts and this confirmed the independent activity of two domains (Fig. 1).

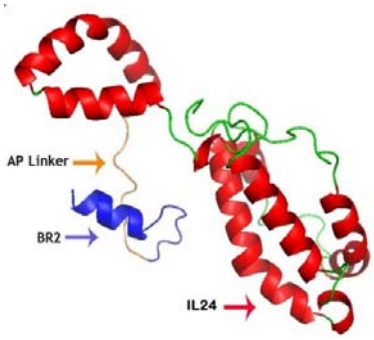

Fig. 1. Ribbon structure of IL24-BR2 fusion protein visualized by PyMol software. The presence of rigid linker between two segments of the fusion protein resulted in no interaction between two parts confirming the independent activity of two domains. IL, Interleukin; AP, Alanine-proline.

Cloning and expression of IL24-BR2 and IL24

The fidelity of cloning of IL24-BR2 or IL24 gene into pET28a plasmid was confirmed by restriction endonuclease digestion and DNA sequencing (data not shown). Expression of the corresponding proteins on SDS-PAGE showed bands of approximately 22 or 18 kDa for IL24-BR2 or IL24, respectively. Western blotting also authenticated the observed bands by revealing similar bands on the polyvinylidene difluoride membrane (Figs. 2 and 3).

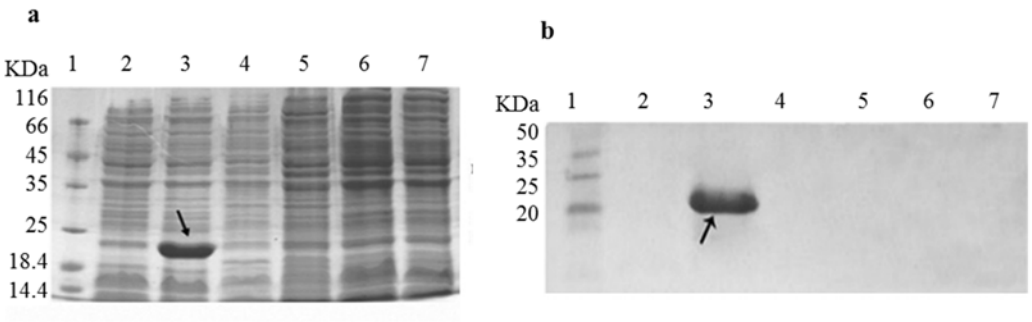

Fig. 2. (a) 15% SDS-PAGE analysis of IL24-BR2 expressed in *E. coli*, (b) western blot analysis of IL24-BR2 expressed in *E. coli*. Lane 1, protein marker; lane 2, un-induced *E. coli* BL21 (DE3) containing pET28a-IL24-BR2; lane 3, induced *E. coli* BL21 (DE3) containing pET28a-IL24-BR2; lane 4, un-induced pET28a containing non recombinant pET28a; lane 5, induced ET28a containing non recombinant pET28a; lane 6, un-induced *E. coli* BL21 (DE3) without pET28a; and lane 7, induced *E. coli* BL21 (DE3) without pET28a. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IL, interleukin; *E. coli, Escherichia coli*.

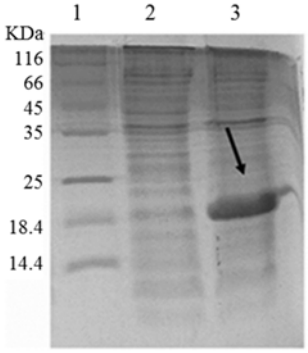

Fig. 3. 15% SDS-PAGE analysis of IL24 expressed in *E. coli*. Lane 1, protein marker; lane 2, un-induced *E. coli* BL21 (DE3) containing pET28a-IL24; and lane 3, induced *E. coli* BL21 (DE3) containing pET28a-IL24. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IL, interleukin; *E. coli*, *Escherichia coli*.

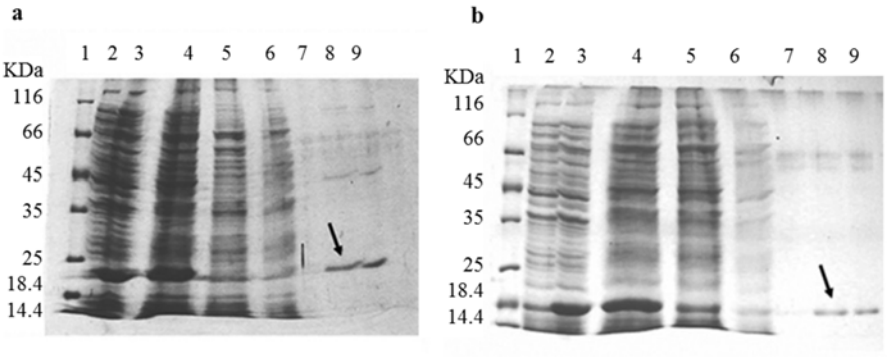

Fig. 4. (a) 15% SDS-PAGE analysis of the purification process IL24-BR2 fusion protein, (b). 15% SDS-PAGE analysis of the purification process IL24 protein. Lane 1, protein marker; lane 2, un-induced *E. coli* BL21 (DE3) containing recombinant pET28a; lane 3, induced *E. coli* BL21 (DE3) containing recombinant pET28a; lane 4, sample after sonication and centrifuging; lane 5, sample after loading protein to column; lane 6 sample after binding buffer; lane 7, sample after washing buffer; lane 8, sample after elution buffer, and lane 9, sample after dialyze. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IL, interleukin; *E. coli*, *Escherichia coli*.

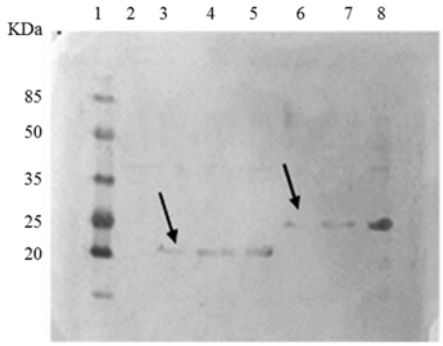

Fig. 5. Western blot analysis of purified IL24-BR2 and IL24. Lane 1, pre-stained protein marker; lane 2, un-induced *E. coli* BL21 (DE3) without pET28a; and lane 3, purify IL24 protein after dialyze; lane 4, purify IL24 protein before dialyze; lane 5, induced *E. coli* BL21 (DE3) containing pET28a-IL24; lane 6, purify IL24-BR2 fusion protein after dialyze, lane 7, purify IL24-BR2 fusion protein before dialyze, and lane 8, induced *E. coli* BL21 (DE3) containing pET28a-IL24-BR2. IL, Interleukin; *E. coli*, *Escherichia coli*.

Purification of IL24-BR2 and IL24

IL24-BR2 and IL24 proteins purified as mentioned in the materials and methods section and the purified bands were observed on SDS-PAGE (Fig. 4). The efficiency of purification procedure was about 48% of total expressed proteins. Western blotting confirmed the identity of the purified bands by showing the corresponding 22 kDa or 18 kDa on the polyvinylidene difluoride membrane following analysis of the purified proteins (Fig. 5).

MTT assay

The effect of various concentrations of IL24 and IL24-BR2 on MCF-7 and HUVEC cell lines was evaluated by MTT assay. As shown in Fig. 6, there was significant reduction in survival of MCF-7 cells treated with IL24-BR2 at 10, 20, and 40 µg/mL in comparison with the HUVEC cells ($P = 0.021$). Furthermore, the cytotoxic effects of IL24-BR2 was significantly higher than IL24 alone for these cancer cells in concentration above 10 µg/mL. For HUVECs, on the other hands, IL24-BR2, showed no significant differences in cell survival except in the final concentration when compared to the negative control (PBS treated cells). Furthermore, there were no significant differences in cell survival between IL24 and IL24-BR2 against HUVEC cells ($P > 0.05$).

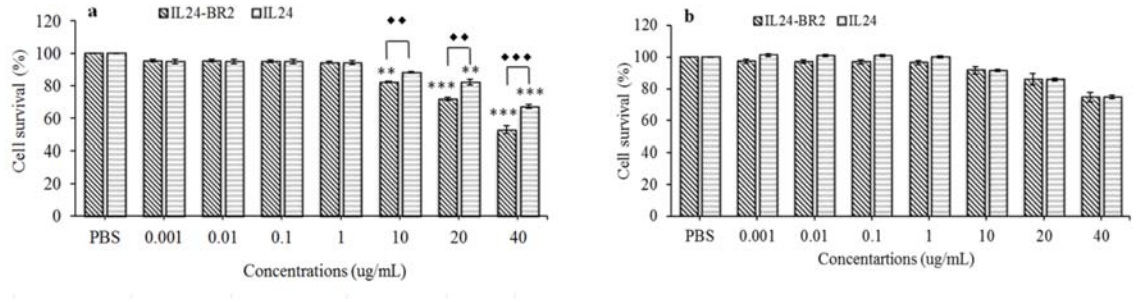

Fig. 6. Evaluation of cytotoxic effects of IL24-BR2 and IL24 against (a) MCF-7 and (b) HUVEC cells. Significant toxicity was observed when MCF-7 cells treated with IL24 at 20 and 40 µg/mL and IL24-BR2 at 10, 20, and 40 µg/mL. HUVEC cells did not show significant toxicity to IL24-BR2 and IL24, except at 40 µg/mL for both proteins. Data represent the mean + SEM of three independent experiments of triplicates. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ Indicate significant differences compared with negative control group (PBS). $\blacklozenge\blacklozenge P < 0.01$, and $\blacklozenge\blacklozenge\blacklozenge P < 0.001$ Indicate significant differences between the IL24-BR2 and IL24 alone. IL, Interleukin

DISCUSSION

IL24 showed inhibitory effects on replication of a many cancer cells *in vitro*, such as skin, prostate, breast, central nervous system, cervical, sarcoma, colorectal, and lung, without affecting normal cells (14). The precise mechanism of IL24 and its selective action is still not well defined. The mechanism by which IL24 inhibits the growth of cancer cells depends on the type of cancer; Different observations showed that in most cases, IL24 functions independent of JAK/STAT signal transduction pathways, which is the classical pathway for many cytokines (18,19).

Hitherto, several fusion proteins have been made using IL24 as a toxic moiety. For example, in one study, glutathione S-transferase (GST)-MDA-7 fusion protein expressed by *E. coli* and used for treatment of a renal cell carcinoma. Although, the cell line had shown resistance to Ad.mda-7, revealed dose-dependent cell killing activity when treated with the GST-MDA-7 (14). This fusion protein inhibited proliferation of the cancer cells at concentrations ranging from 0.25 to 2.0 nmol/L, but showed cell killing effects at 20-fold greater concentrations (9). In another study, anti-cancer effects of RGD-IL24 (Arg-Gly-Asp-IL24) fusion protein produced in *E. coli* were evaluated. In the mentioned study, MTT assay was performed following treatment of MCF-7, and normal human lung fibroblast cell line, NHLF, with various concentration of the RGD-IL24. Their results showed that RGD-IL24 and IL24 can suppress the growth of cancer cells without affecting normal cells proliferation. The concentration of purified protein of RGD-IL24 used in this assay was 6 µg/mL (1).

In the case of BR2, on the other hand, its ability as a drug delivery vehicle to cancer cells was investigated by its fusion to antiRAS-scFv. The mentioned fusion protein showed apoptotic effects at 2 µM concentration (20). Furthermore, the cellular uptake of BR2 was investigated and showed good distribution to HeLa and HCT116 without any hemolytic activity (20). DT386-BR2 is another fusion protein in which BR2 has been used as the targeting moiety and the first 386 amino acid residues of the diphtheria toxin as a toxic moiety. Whereas DT386-BR2 protein showed cytotoxic effects on HeLa and MCF-7 cancer cell lines with IC_{50}s of 0.55 and 2.08 µg/mL, respectively, it did not have any toxic effects on HUVEC and HEK 293 normal cell lines. The evaluation of cytotoxic effects of BR2 on cancer and normal cells showed no inhibitory effects on both cancer and normal cells (21).

In our study the IC_{50} of IL24-BR2 was calculated about 2 µM against MCF-7 cancer cells, which was higher than the obtained amount in previous studies for IL24. These differences can be due to the probable conformational changes in IL24 during the production and purification process.

BR2, which used for the enhancement of protein penetration, showed no cytotoxic effects against even cancer cells. So, the observed cytotoxic effects of IL24-BR2 could be attributed to the IL24. On the other hand, the cytotoxic effects of IL24-BR2 were significantly more than IL24 against MCF-7 cell line at least at higher concentrations and this is due to the more penetration ability of the fusion protein to cancerous cells.

IL24 undergoes post-translational modification such as protein phosphorylation and ubiquitination during expression (19). In one study, the effect of glycosylation on IL24 activity was investigated. After expression of IL24 in *E. coli* and its purification with affinity chromatography, the anticancer effect of non-fusion rIL24 protein without glycosylation was studied on the lung cancer cells (A549) and the normal human lung fibroblast (NHLF); the results of this study showed that non-glycosylated protein induced apoptosis in cancer cell without affecting normal cell. The IC_{50} of rIL24 was 0.8 µg/mL (11). According to this study and others studies, such as GST-MDA-7 (14) RGD-IL24 (1), and DT386-BR2 (21) that express IL24 or BR2 in *E. coli* successfully, we used *E. coli* BL21(DE3) as the host for the expression of IL24-BR2. However, one of the problems that have always been posed with *E. coli* expression system is the production of insoluble protein, which is mis-folding aggregates called inclusion bodies (22). In this study, IL24-BR2 was also expressed as inclusion bodies, which reduced protein activity. Therefore, to make IL24-BR2 fusion protein a soluble protein and enhance the expression level, the medium was supplemented with various chemicals including glucose (0.5% w/v), sodium chloride (0.5 M), sucrose (0.5 M), glycerol (10% v/v), ethanol (1% v/v), PBS (instead of using water), or $MgCl_2$ (1 mM). Moreover, in order to give enough time for protein refolding, we used stepwise dialysis by decreasing urea concentration in dialysis buffer performing dialysis experiment in an order of 6, 4, and 2 M urea for 2 h and then 300 mL PBS pH 7.4 overnight. However, these various chemicals showed no increase in solubility of this fusion protein. Otherwise, the addition of NaCl could increase expression of insoluble protein. Therefore, further studies are needed to produce soluble IL24-BR2 in order to increase the effectiveness of the protein.

CONCLUSION

In this study, the recombinant IL24-BR2 protein produced and purified as a novel fusion protein in order to enhance the cancer specific activity of IL24 by addition of a cancer specific cell penetrating peptide (BR2) with ability to penetrate to cancer cells without penetration to normal cells. However, efforts for production of soluble protein and investigation of its cytotoxic effects against other cell lines must be performed.

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