Original Article

Evaluation of Cytotoxic and Apoptotic Effects of DT386–BR2: A Promising Anticancer Fusion Protein

Fatemeh Shafiee, Mohammad Rabbani, Ali Jahanian-Najafabadi

Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Purpose: In the previous studies, we designed an anticancer immunotoxin containing the catalytic and translocation domains of diphtheria toxin fused to BR2, a buforin II-derived antimicrobial peptide as a cancer-specific cell penetrating peptide, in order to target various cancer cells. The aim of this study was to evaluate the *in vitro* cytotoxicity of DT386–BR2 against K-562 cells as the most famous cell line for leukemia. **Materials and Methods:** MTT and flow-cytometry assays were used for determining the cytotoxic effects and cell death mechanism of DT386–BR2, respectively, against K-562 cell line. The recombinant DT386 and synthetic BR2 were used as the negative control in cytotoxicity assay. **Results:** The results of this study showed a significant reduction in survival of K-562 cells caused by DT386–BR2 as compared with BR2 and DT386 fragments. On the contrary, the flow-cytometry results showed apoptosis induction by DT386–BR2 after 12 h in a dose- and time-dependent manner. **Conclusion:** DT386–BR2 fusion protein can be used for further preclinical studies for determining its pharmacokinetic/pharmacodynamic profiles and evaluating its anticancer efficacy in suitable animal models.

Keywords: *Apoptosis, cytotoxicity, DT386–BR2, flow cytometry, K-562*

Introduction

Immunotoxins are recombinant proteins comprising a toxic moiety conjugated to a targeting moiety in order to target a very potent cytotoxic agent to cancer cells.[1] Buforin is an antimicrobial peptide (AMP) that forms transient pores in cancer cell membrane and subsequently enters the cells, without any toxic effects on normal cells, which makes it suitable for designing of new immunotoxins.[2-4] On the contrary, diphtheria toxin (DT) is the most frequently used bacterial toxin for constructing of fusion proteins via replacement of its receptor-binding domain with different targeting moieties.[5] Hence, in a previous study, we constructed a new fusion protein comprising BR2 (a short 17 residues peptide derived from buforin II, which targets cancer cells more specifically)[6] as a targeting moiety, and the catalytic and translocation domains of the DT (DT386) as the cytotoxic moiety.[7] Preliminary studies on specific cytotoxicity of the DT386–BR2 showed reasonable specific cytotoxic activities on MCF-7 and HeLa tumor cell lines as compared with the normal cells, including HUVEC and HEK293 cell lines. It has been shown that buforin IIb has selective cytotoxic effects against 62 various cancer cells regardless the type of cancer including K-562 cells.[4] In this study, we evaluated the cytotoxic effects of the recombinant DT386–BR2, as well as DT386 alone and BR2 on K-562 (human myelogenous leukemia-derived) cells by MTT assay in order to determine the probable anticancer effects of DT386–BR2 against K-562 cells and subsequently the mechanism of the induced cell death was determined by flow-cytometry using annexin V/PI staining method.

Materials and Methods

Materials

DT386–BR2 and DT386 proteins, and BR2 peptide were produced and purified according to our previous studies.[7-9] K-562 cell line was purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Annexin-V-FLUOS staining kit was obtained from Roche, Germany. All other chemicals were obtained from known commercial sources and were of molecular biology grade.

Production and purification of recombinant proteins

As shown in previous studies,[7-9] for production of DT386, BR2, and DT386–BR2 recombinant

Received: 04 Feb 2019
Accepted: 01 Oct 2019
Published: 26 Jun 2020

Address for correspondence:
Dr. Ali Jahanian-Najafabadi, Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Hezar Jarib Ave., Isfahan, Iran.
E-mail: jahanian@pharm.mui.ac.ir

Access this article online

Website: www.jrpsjournal.com

DOI:10.4103/jrptps.JRPTPS_15_19

Quick Response Code:

How to cite this article: Shafiee F, Rabbani M, Jahanian-Najafabadi A. Evaluation of cytotoxic and apoptotic effects of DT386–BR2: A promising anticancer fusion protein. J Rep Pharm Sci 2020;9:68-72.

proteins, *Escherichia coli* BL21 (DE3) was used. In this regard, the bacteria were transformed with the recombinant pET28a–DT386–BR2, pET28a–DT386, or pTXB1–BR2 plasmids in order to express DT386–BR2, DT386, or BR2, respectively. Induction of expression was performed using 1 mM isopropyl-β-D-thiogalactoside and finally, purification of DT386 and DT386–BR2 or BR2 was performed by nickel affinity chromatography, or IMPACT system, respectively. In each stage, confirmation of the expression and purification was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Sample preparation for *in vitro* assays

Endotoxin removal of the purified proteins was performed using Triton X-114 method.[10] The endotoxin free protein solution was dialyzed against phosphate-buffered saline (PBS; pH 7.4) for *in vitro* assays for each protein or peptide. Protein concentration was determined using Bradford's[11] method toward various concentrations of bovine serum albumin (BSA).

Cytotoxicity assay

To determine the cytotoxic effects of DT386–BR2, DT386, and BR2, MTT assay was used. Cell suspension with 5×10^4 cells/mL concentration of K-562 cells in RPMI 1640 in the final volume of 180 µL was seeded in each well of a 96-well plate and incubated at 37 ºC in a CO_2 incubator. In the next day, 20 µL of various concentrations of the fusion protein, DT386 or BR2 (final concentrations: 0.1, 0.5, 0.75, 1, 5, and 10 µg/mL), were added to each well. After 48 h of incubation, 20 µL of MTT solution (5 mg/mL) was added to each well, and the plate was further incubated for 3 h. Finally, the formazan crystals were dissolved in 150 µL dimethyl sulfoxide, and the plate was subjected to absorbance read at 570 nm using a microplate reader.

Flow-cytometry analysis

About 5×10^5 cells/well of K-562 cells was cultured in a 6-well microplate. After 12 h, the cells were incubated with the IC50 concentration of DT386–BR2 for 12 or 24 h and subsequently subjected to flow-cytometry analysis. Briefly, all cells were collected, washed with PBS, and then incubated with annexin-V-FITC and propidium iodide according to the manufacturer's instructions (Roche, Germany) for 15–20 min. Finally, the cells were centrifuged at 300× *g* and washed using PBS, resuspended in 500 µL PBS, and analyzed by flow cytometry on a BD FACSCalibur, BD Company (San Jose, California, US).

Statistical analyses

Cytotoxicity assay was performed in three independent experiments and four replicate wells for each concentration of every recombinant peptide and proteins. PBS-treated cells were considered as negative control and results were expressed as cell viability % ± SD. The Statistical Package for the Social Sciences software version 22.0, SPSS Inc (South Wacker Drive, Chicago, US) was used for statistical analysis. Analysis of variance (ANOVA) followed by *post hoc* test was used to distinguish the differences between groups. The significance was assumed as $P < 0.05$. Finally, the IC50 of each protein was determined by drawing the graph of cell survival percent against concentration using GraphPad Prism 6.0 software, GraphPad Software (San Jose, California, US).

Results

DT386–BR2 showing cytotoxic effects on K-562 cell line

Cytotoxic effects of DT386, BR2, and DT386–BR2 proteins on K-562 cells were measured *in vitro* by MTT assay.

The analyzed data showed that the cytotoxic effects of DT386 and DT386–BR2 against K-562 cells were concentration-dependent; that is, increasing concentration of the recombinant proteins leads to increased cytotoxicity on the cells.

The analyzed data showed that DT386-BR2 has significant cytotoxic effects in concentration above 0.75 µg/mL in comparison to the PBS-treated cells as the negative control [Figure 1]. On the contrary, the differences between the cytotoxic effects of DT386 and DT386–BR2 in all concentrations were significant ($P < 0.05$). Furthermore, significant differences were observed between the cytotoxic effects of DT386–BR2 and BR2 in the same concentrations ($P < 0.05$). The final conclusion of MTT test is the fact that BR2 showed no significant cytotoxic effects against the mentioned cell line as compared with the negative control ($P > 0.05$).

Finally, based on the graph of concentration/percent of cell survival drown by GraphPad Prism 6.0, the IC50 of DT386–BR2 fusion protein and DT386 was determined to be as 0.8 and 2.05 µg/mL, respectively. The IC50 of BR2 was not calculable in the concentrations which were used.

DT386–BR2 fusion protein showing proapoptotic effects on K-562 cell line

The flow-cytometry analysis of the K-562 cells treated with 0.75 and 1 µg/mL of DT386–BR2 fusion protein for 12 and 24 h, and subsequently stained with the annexin-V-FITC and propidium iodide, showed that the percent of apoptotic cells increased with time and elevated concentrations of DT386–BR2 fusion protein. In fact, the untreated cells showed 74.63% of live cells, 25.17% apoptotic cells, and approximately 0% necrotic cells. In the case of the cells treated with 0.75 µg/mL of the fusion protein for 12 h, results showed 31.8% apoptotic cells and 0% necrotic cells. Furthermore, the apoptotic and necrotic percent of the cells treated with 1 µg/mL of the fusion protein after 12 h of incubation was showed to be 59.24% and 0.02%, respectively. The amount of apoptotic (early and late) cells increased by increasing the incubation time to 24 h. The results showed 62.8% of apoptotic cells in the case of 24 h of treatment with 0.75 µg/mL of DT386–BR2 fusion protein. In addition, the percent of necrotic cells was determined to be 0.094%. These results for cells treated with 1 µg/mL of the fusion protein were about 78.97% and 0.04% for apoptotic and necrotic cells, respectively.

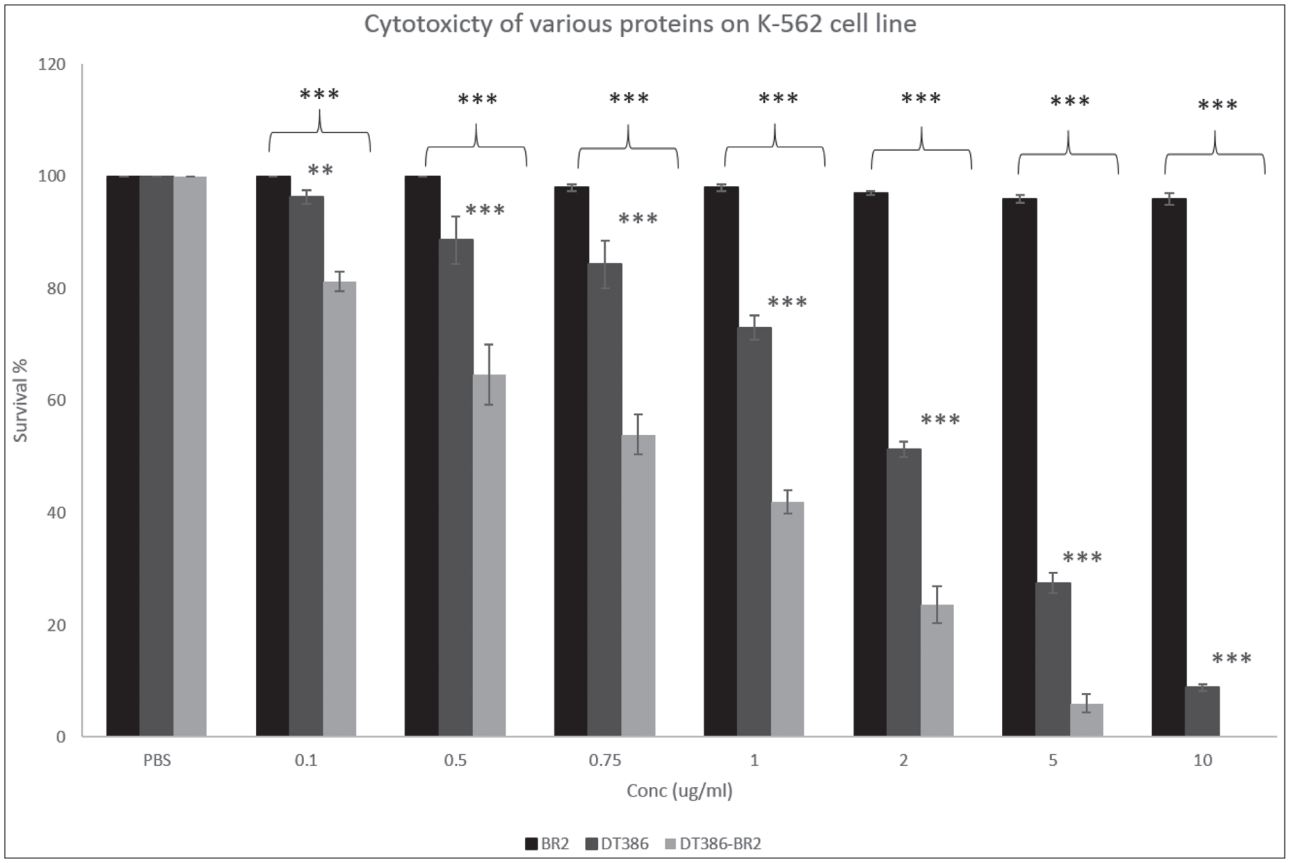

Figure 1: Evaluation of the cytotoxic effects of BR2, DT386, and DT386–BR2 against K-562 cells. Treatment of K-562 cells that are permeable to BR2 and DT386–BR2 but not to DT386 recombinant protein showed significant toxicity at concentrations of 1 µg/mL and higher for DT386–BR2, and 2 µg/mL and higher for DT386. However, no significant reduction was found in viability of the cells exposed to BR2. Data represent the mean percent of three independent experiments of triplicates. Error bars represent ± SD. *n* = 3, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 for comparison of the same concentrations of DT386 and DT386–BR2 or DT386-BR2 and BR2.

Taken together, the results of flow-cytometry assay confirmed that DT386–BR2 fusion protein induces cell death via apoptosis [Figures 2 and 3].

Discussion

The aim of this study was to evaluate the cytotoxic effects of DT386–BR2 fusion protein as a targeted chemotherapeutic agent against K-562 cell line and also to determine the mechanism of induced cell death.

Results showed that DT386–BR2 fusion protein was cytotoxic against K-562 cell line in a concentration-dependent manner. We previously showed similar effects of this fusion protein on two other cancer including MCF-7 and HeLa cell line, without any statistically significant effects on normal HUVEC and HEK 293 cells.[7] These findings established that this fusion protein might be a targeted chemotherapeutic agent for different cancer cells.

The observed cytotoxicity of DT386–BR2 fusion protein was statistically higher than DT386 alone. It must be noted that DT386 lacks receptor-binding domain so it cannot enter the cells via receptor-mediated endocytosis. Zhang *et al.*[12] evaluated the cytotoxicity of recombinant DT385 protein on 35 cancer and normal cell lines. Their results showed that this protein is cytotoxic for six cancer cell lines because of nonspecific endocytosis. Therefore, this nonspecific endocytosis of DT386 could be the reason for the observed cytotoxicity in our study. Furthermore, we observed that the apoptotic effects of the DT386–BR2 fusion protein started less than 12 h post-treatment according to the results of flow-cytometry assay, while the killing effects of DT386 was shown 48 h after treatment of the K-562 cells. In consistent with our results, Zhang *et al.*[12] also observed that maximum apoptosis and loss of membrane integrity occurred 72 h after treatment with the DT385. In addition, Lim *et al.*[7] showed that BR2 can enter cancer cells (HeLa, HCT116, and B16/F10) within 30 min after treatment and distribute throughout the cytoplasm and nucleus of cancer cells with very poorly internalization into normal cells (HaCat, BJ, and NIH 3T3) under the same experimental conditions. Considering these results, the reason for much more potent and faster cytotoxicity of DT386–BR2 against K-562 cell line as compared with DT386 is attributed to the ability of BR2 for its specific permeation to cancer cells.

The usage of buforin as a delivery vector was shown as follows: in one study, buforin covalently attached to the green

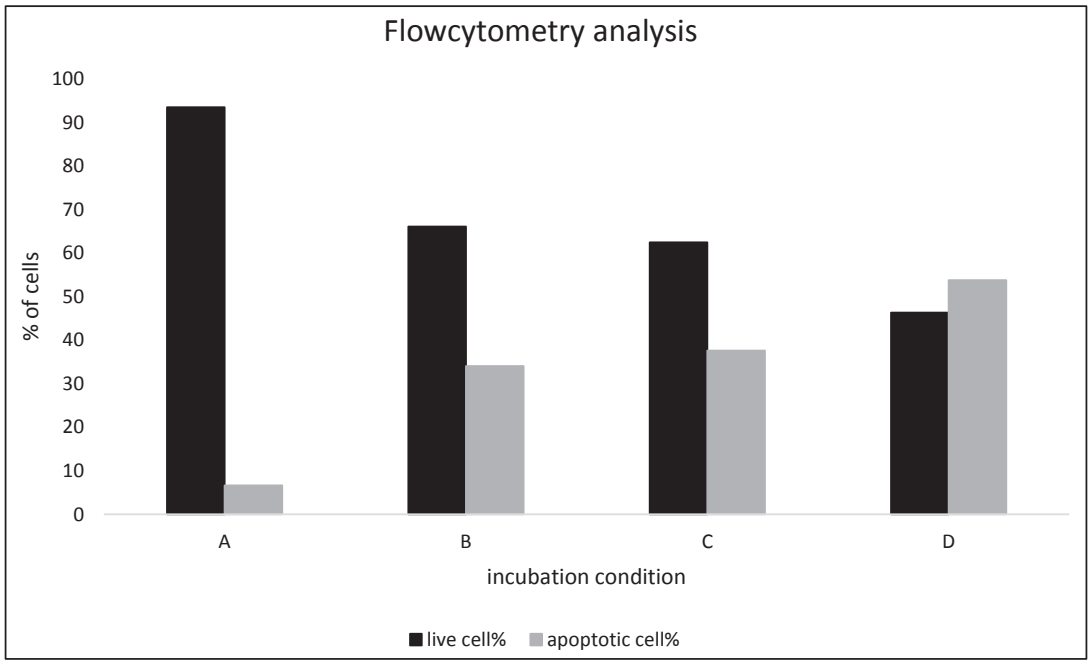

Figure 2: Assessment of the DT386–BR2 cytotoxicity on K-562 cells for different time lengths and different concentrations by flow cytometry. (A) Cells treated with 0.75 μg/mL of DT386–BR2 for 12 h. (B) Cells treated with 1 μg/mL of DT386–BR2 for 12 h. (C) Cells treated with 0.75 μg/mL of DT386–BR2 for 24 h. (D) Cells treated with 1 μg/mL of DT386–BR2 for 24 h.

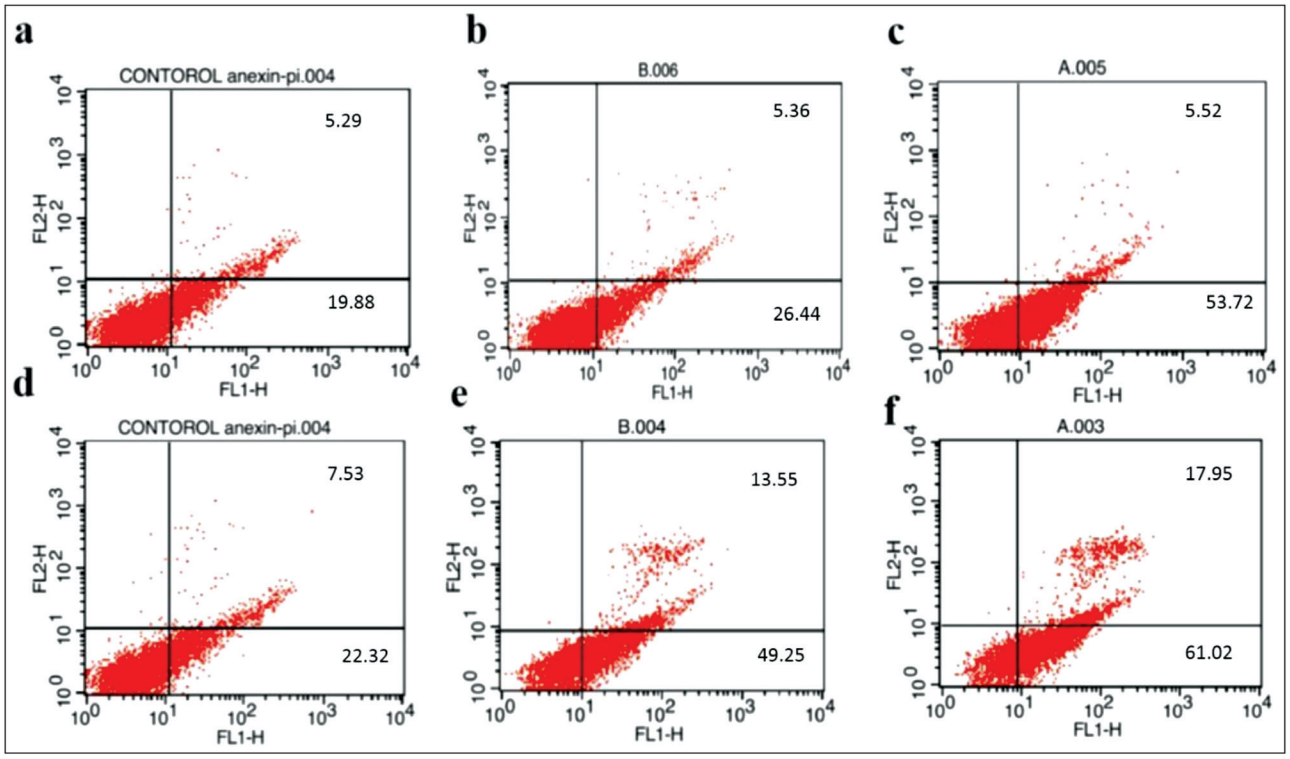

Figure 3: Determination of cell death mechanism by flow cytometry. (a) Untreated control cells after 12 h of incubation. (B) Cells treated with 0.75 μg/mL of DT386–BR2 for 12 h. (C) Cells treated with 1 μg/mL of DT386–BR2 for 12 h. (D) Untreated control cells after 24-h incubation time. (E) Cells treated with 0.75 μg/mL of DT386–BR2 for 24 h. (F) Cells treated with 1 μg/mL of DT386–BR2 for 24 h. Lower left chamber: live cells (annexin V^-/ PI^-). Lower right chamber: early apoptotic cells (annexin V^+/ PI^-). Upper left chamber: dead cells (annexin V^-/ PI^+). Upper right chamber: late apoptotic cells (annexin V^+/ PI^+).

fluorescent protein (GFP: 28 kDa), could enter to HeLa cells, and suggested the potency of this peptide as a vector for macromolecular delivery into cells.[13]

The capability of BR2 as a specific drug carrier was shown by fusion to a single-chain variable fragment against K-ras, which induced apoptosis in K-ras-mutated HCT116 cells with IC50

about 2 μM.[7] These results suggest that BR2 has great potential as a useful drug delivery carrier with cancer cell specificity.

On the contrary, in terms of cytolethal activity of BR2 our results showed no significant reduction in the K-562 survival rate when treated with the concentrations of 0.1, 0.5, 0.75, 1, 2, 5, and 10 μg/mL. Similar results were obtained in our previous study, in which increasing concentrations of BR2 (1, 10, and 100 μg/mL) were used for the treatment of HeLa, MCF-7, HUVEC, and HEK 293 cells, which showed no significant reduction in survival of neither cancer nor normal cells.[7] Furthermore, Lim *et al.*[6] used BR2 at final concentrations of 1, 2, and 5 μM (2, 4, and 10 μg/mL) for treatment of HCT116—human colon cancer cell line. Similar to our results, it was shown that BR2 has no significant cytotoxicity against HCT116 cell line as compared with PBS.[6] So, the observed cytotoxic effects of DT386-BR2 in our study attributed to the presence of DT386 which enters the cells trough BR2 as the cell penetrating peptide.

Our results showed that DT386–BR2-induced apoptosis in K-562 cell line in a time- and concentration-dependent manner. Although it has been shown that buforin IIb can induce cell death via mitochondrial-dependent apoptosis,[4] by considering non-cytolethal effect of BR2 (which is a buforin IIb derivative) on cancer cell lines after even 48 h of incubation, the observed apoptotic effect of DT386–BR2 must be attributed to its toxic moiety. The apoptotic effects of DT without the receptor-binding domain were also determined in the case of DT385, which showed 85% of apoptosis after 72 h of incubation. These finding showed that DT386 can enter cells via nonspecific endocytosis but has the potential to kill cells because of apoptosis. However, DT386–BR2 with the ability to penetrate to cancer cells showed these effects in less time and only after 12 h. The concentration-dependent apoptosis was also observed in the case of ONTAK™, Seragen, Inc (Boston, Massachusetts, US) (an Food and Drug Administration [FDA]-approved immunotoxin which is DT389-IL2) against dendritic cells.[14] In addition, in the case of DT388-GMCSF, this apoptosis-inducing effect on leukemia cell line was also observed.[14] In the mentioned study, HL-60/VCR, a multidrug-resistant human myeloid leukemia cell line and wild-type HL-60 cells were used to evaluate the apoptotic effects of DT388-GMCSF. The results of this study showed that after 48 h of treatment with DT388-GMCSF (10 nM), ceramide levels (an apoptosis modulator) in both cell lines rose six-fold and cell survival decreased to 10%, whereas treatment with GMCSF alone showed no effect. Furthermore, ceramide levels increased approximately about 80% and activation of caspase-9 and chromatin fragmentation was occurred after 6 h of DT388-GMCSF addition.[15]

Conclusion

Taken together, DT386–BR2 fusion protein showed significant specific cytotoxic effects on K-562 cell line, which is mediated by apoptosis. Therefore, considering the promising anticancer effects of this fusion protein, it will be further evaluated in upcoming preclinical *in vitro* and *in vivo* studies regarding its detailed mechanism of action, and eradication of tumor cells in animal cancer models.

Ethical conduct of research

This paper does not contain any studies with human and animal participants.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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