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Apoptotic Effects of a Recombinant Rovalpituzumab-Granzyme B Immunotoxin in A549 Lung Cancer Cells

Iman Shakarami[1], Hamideh Mahmoodzadeh Hosseini[2], Jafar Amani[2], Zohreh Jangravi[3], Seyed Ali Mirhosseini[2]*

[1]Student Research Committee, Baqiyatallah University of Medical Sciences, Tehran, Iran; [2]Applied Microbiology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran; [3]Department of Biochemistry, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background: Although Delta-like ligand 3 (DLL3)-targeted therapies have shown promise for lung cancer, limited information exists on recombinant immunotoxins using Granzyme B as the cytotoxic moiety.

Objectives: This study evaluated the effects of the recombinant immunotoxin named Rovaliptuzumab-Granzyme B on apoptosis in the lung cancer cell line A549.

Methods: The A549 lung cancer cell line was treated with three different concentrations (150, 350, and 700 nanomolar (nM)) of Rovaliptuzumab-Granzyme B. A lactate dehydrogenase (LDH) activity assay was performed to measure cell membrane integrity and cytotoxicity. In addition, caspase-3/7 activation was studied as a marker of apoptosis. Quantitative real-time PCR was used to quantify *BAX* and *BCL-2* gene expression levels.

Results: Our data show that Rovaliptuzumab-Granzyme B, at all studied concentrations (150, 350, and 700 nM), significantly causes cytotoxicity in the A549 cells, as characterized by increased LDH release (48, 120, 206.8%, respectively), increased caspase activity (0.77, 1.33 and 2.34 nmol/min/mL, respectively), and enhances in the *BAX/BCL-2* gene expression ratio compared with the negative control (1.97, 2.68 and 4.03, respectively) (p<0.05). In addition, the *BCL-2* gene expression showed a 0.74-, 0.67-, and 0.58-fold decrease at concentrations of 150, 350, and 700 nM, respectively, while a 1.48-, 1.8-, and 2.34-fold increase in *BAX* gene expression was seen at concentrations of 150, 350, and 700 nM, respectively, compared with the negative control (p<0.05).

Conclusion: Overall, these findings suggest that the recombinant Rovalpituzumab/Granzyme B immunotoxin exerts pro-apoptotic activity in the A549 lung cancer cells.

Keywords: Lung cancer, Immunotoxin, Granzyme B, Apoptosis

*Corresponding author:
Seyed Ali Mirhosseini, Applied Microbiology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences, Vanak Sq. Molasadra St., Tehran, Iran
Email: ali.mirh@gmail.com

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INTRODUCTION

Lung cancer is one of the leading causes of cancer-related deaths worldwide and has posed numerous challenges for physicians and researchers due to its complex characteristics and resistance to common chemotherapy methods (1, 2). Accordingly, the need for new and effective strategies for treating patients with lung cancer has become a necessity. One promising approach is targeted therapy, such as immunotoxins, which specifically target cancer cells (3, 4).

Immunotoxins combine an antibody and a cytotoxic agent, designed to target and kill cancer cells (5). These compounds bind specifically to proteins on the surface of cancer cells and then deliver the toxin into the cell, inducing cell death (6). Immunotoxins offer high precision in targeting cancer cells and cause less damage to healthy cells (7). Because of their targeted nature, immunotoxins have fewer side effects than conventional treatments such as chemotherapy and radiation. Immunotoxins can be used to treat a variety of cancers, but their immune response can reduce their effectiveness, in addition to being expensive to use (5). Immunotoxins are being researched and developed as a new approach to cancer treatment, and they hold great promise for improving treatment outcomes in cancer patients (8).

Although considerable progress has been made in developing immunotoxins, several challenges continue to limit their broader clinical application in lung cancer. Many immunotoxins are based on bacterial or plant toxins such as Pseudomonas exotoxin A, Diphtheria toxin, ricin, and gelonin. Although they have high cytotoxic potency, their clinical application faces significant problems due to high immunogenicity, the development of neutralizing antibodies, off-target cytotoxicity, and limitations in repeated treatment (9). For this reason, human cytotoxic proteins such as Granzyme B have emerged as promising alternatives to conventional bacterial and plant toxins. Granzyme B is usually secreted by cytotoxic T cells and plays a central role in cell death by activating caspases and the mitochondrial apoptotic pathway. This mechanism may allow for a more controlled, apoptosis-specific mode of cell death rather than the necrotic or non-specific cytotoxicity associated with some first- and second-generation immunotoxins (10). Compared with bacterial or plant-derived toxins, Granzyme B offers several advantages for designing recombinant immunotoxins. First, Granzyme B is an endogenous human serine protease and intrinsically less immunogenic, reducing the risk of neutralizing antibody formation that limits repeated dosing of bacterial- or plant-toxin-based immunotoxins. Second, unlike bacterial toxins, which irreversibly inhibit protein synthesis and cause off-target cytotoxicity and vascular leak syndrome, it is less likely to cause these effects. However, its endogenous inhibitor, serpin B9/PI-9, poses a recognized challenge in some tumor tissues (11). *BAX* and *BCL-2* genes are members of a family of proteins involved in apoptosis, and can induce and inhibit apoptosis, respectively (12). Delta-like ligand 3 (DLL3) is a Notch-inhibitory ligand, expressed in 70–95% of tumor specimens collected from small cell lung cancer (SCLC) cells, but its expression in normal tissue and in non-SCLC subtypes (NSCLC) remains low (13). This restricted distribution makes DLL3 such an interesting cell-surface target for antibody-based and immunotherapies in SCLC and related high-grade neuroendocrine carcinomas. This rationale led to the development of the Rovalpituzumab antibody, engineered to bind DLL3 with high specificity (14). Most previous studies have focused on evaluating clinical trials of Rovalpituzumab Tesirine or investigating its cytotoxicity, and little information exists on its use in designing the immunotoxin (15). However, limited information is available regarding the use of Granzyme B as the toxic moiety of a

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Rovalpituzumab-based immunotoxin or the molecular mechanisms underlying its apoptotic activity.

In our previous study, we designed and produced a recombinant immunotoxin consisting of the targeted antibody Rovalpituzumab and the human cytotoxic molecule Granzyme B, and we evaluated its preliminary anticancer activity *in vitro* (16, 17). This study was developed to provide additional molecular evidence of apoptosis-associated cellular responses following treatment with the recombinant immunotoxin Rovalpituzumab-Granzyme B. For this purpose, LDH enzyme activity as an indicator of cell damage, caspase-3/7 activity as an indicator of apoptosis pathway activation, and *BAX* and *BCL-2* gene expression were evaluated.

MATERIAL AND METHODS

Cell culture

In this study, we used the A549 cells, a human NSCLC cell line with previously reported detectable DLL3 expression. Previous studies have demonstrated DLL3 expression in the A549 cells by quantitative RT-PCR, and our previous work further demonstrated specific binding of the recombinant Rovalpituzumab-based immunotoxin to the A549 cells using a Cell-ELISA assay (17, 18). The cell line was obtained from the Genetic Resources Center Bank, Pasteur, Iran, and is a human lung carcinoma cell line. The necessary measures for the growth of this cell line include cell growth in RPMI-1640 (Hyclone) culture medium with necessary supplements such as 10% fetal bovine serum (FBS) (Hyclone) and 1% antibiotic (penicillin-streptomycin) (Gibco Life Technologies, Thermo Fisher Scientific), maintained in T25 flasks at 37°C and 5% CO_2. Every 2-3 days, the culture medium was changed to remove waste materials and metabolites, and when the cells covered 70-80% of the well surface, they were subcultured.

Expression, purification and behavior of immunotoxin components

Briefly, in our previous study, we designed Rovaliptuzumab-Granzyme B by fusing two parts: the single-chain variable fragment (scFv) of the Rovalpituzumab antibody to human Granzyme B via a flexible $(G_4S)_3$ linker, and validated it by bioinformatic analysis. To express recombinant Rovaliptuzumab-granzyme B, we cloned the synthetic gene construct into the expression vector pET28a and transformed it into *Escherichia coli* BL21(DE3). After induction with 1 mM IPTG, we purified the Rovaliptuzumab-Granzyme B protein from bacterial lysates by nickel-affinity chromatography. Expression and purification (a product of approximately 72 kDa) were assessed and confirmed by SDS-PAGE and Western blotting using an anti-His-tag antibody. The secondary structure of the purified protein was verified by circular dichroism (CD) spectroscopy. The recombinant Rovaliptuzumab-Granzyme B demonstrated specific, concentration-dependent binding to the DLL3-positive A549 lung cancer cells by Cell-ELISA, whereas no binding was observed on DLL3-negative HUVEC cells, confirming its target specificity. In addition, Cytotoxicity assessment using the MTT test revealed an IC50 of 338 nanomolar (nM) in the A549 cells at 24 h. Although the MTT assay was performed at 24, 48, and 72 h, there were no significant differences in cytotoxic concentrations across the three time points. The immunotoxin showed no cytotoxic effect on HUVEC cells at low concentrations, but non-specific cytotoxicity was observed at 700 nM and higher. Furthermore, flow cytometric Annexin V/PI staining showed that Rovaliptuzumab-Granzyme B induced apoptosis in 31.8% of the A549 cells at its IC50 concentration (16, 17). Since the Cytotoxic effects of Rovaliptuzumab-Granzyme B on the A549 cells at 48 and 72 h were not significantly different from those observed at 24 h, the 24 h time point was selected for assays.

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Evaluation of the cytotoxic effects of immunotoxin by the LDH method

Lactate dehydrogenase (LDH) enzyme is present in the cytoplasm of cells in a soluble form. During cell damage, this enzyme is released and passes through the cell membrane, indicating cytotoxicity. In this method, a kit from Kia Zist Company, Iran, was used to monitor this enzyme's activity with high precision and sensitivity using calorimetry (570nm) and fluorometry (Em/Ex = 540/592nm). In this experiment, we used recombinant immunotoxin at concentrations of 150, 350, and 700 nM (from our previous study) for 24 h to assess LDH activity (40.000 per well). All steps were performed according to the kit instructions. To further ensure data accuracy, this test was repeated three times, and PBS was used as a negative control. At the end of this section, cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity\%} = \frac{(Absorption\ of\ test\ substance) - (Absorption\ spontaneous\ release)}{(Absorption\ Maximum\ Release) - (Absorption\ Spontaneous\ Release)}$$

Evaluation of the apoptotic effects of immunotoxin by measuring caspase 3/7 activity

To assess caspase 3/7 activity, the A549 cells (500.000 per well) were treated with recombinant immunotoxin Rovaliptuzumab-Granzyme B for 24 h at different doses (150, 350, and 700 nM). After treatment, the cells were collected using trypsinization and placed in microtubes. We followed the manufacturer's instructions (Invitrogen) to investigate caspase 3/7 activity, which generally included separating the cells with PBS, lysing them with lysis buffer, and adding caspase substrates (DEVD-pNA for caspase 3 and IETD-pNA for caspase 7). Next, the samples were incubated for 2 h at 37°C. Finally, absorbance was read at 405 nm. For data analysis, absorbance values were recorded, and caspase 3/7 activity levels were calculated using the positive and negative controls. Caspase activity was calculated according to the following formula:

$$\text{Caspase Activity} = \frac{M \times 105.5}{T \times 50} \times Dilution\ coefficient\ of\ the\ sample$$

M: µmol from standard curve plot
T: Incubated time for each sample in minutes

Quantitative Real-time PCR

To examine the expression of *BAX* and *BCL-2* genes in the A549 cell line (500.000 per well) treated with recombinant immunotoxin Rovaliptuzumab-Granzyme B, the following protocol was implemented: The A549 cell line was maintained under standard culture conditions, then cells were treated with immunotoxin Rovaliptuzumab-Granzyme B at different concentrations (150, 350, and 700 nM). After 24 h, cells were collected, and total RNA was extracted using an RNA extraction kit (Kimia Andisheh Teb Company, Iran). The quantity and quality of extracted RNA were examined using a NanoDrop (Microplate spectrophotometer, INNO-M, South Korea) and agarose gel electrophoresis, respectively. Extracted RNAs were converted to cDNA using the Pars Tous Company kit Iran). To assess gene expression, a Rotorgene device (Qiagen, Germany) was used. Reaction amounts and protocols were determined according to the instructions in the Cybergreen Master Mix brochure (Pars Tous Company, Iran). The results were analyzed using the ΔΔCT method, and the expression levels of *BAX* and *BCL-2* genes were calculated relative to the control gene *GAPDH*, as a reference gene. Allele ID version 6.01 software and the NCBI BLAST database were used, respectively, to design and check primer specificity (Table 1).

Statistical analysis

Data analysis was performed using SPSS version 11, one-way ANOVA, and the Tukey test. A *p*-value of less than 0.05 was considered significant.

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Table 1. Sequences of the primers used in this study

Gene	Primer sequence (5'-3')	Product length	Accession	Ta (℃)
GAPDH	F: AAGGCTGTGGGCAAGGTCATC R: GCGTCAAAGGTGGAGGAGTGG	248	NM_001289746.2	58
BAX	F: TCAGGATGCGTCCACCAAGAAG R: TGTGTCCACGGCGGCAATCATC	103	NM_004324.4	56
BCL-2	F: ATCGCCCTGTGGATGACTGAGT R: GCCAGGAGAAATCAAACAGAGGC	127	NM_000633.3	54

RESULTS

The effect of different concentrations of Rovaliptuzumab-Granzyme B on LDH enzyme activity

The LDH test is an important biochemical assay, and its activity level can indicate cellular status and tissue damage. In this study, the activity of the LDH enzyme was tested under different concentrations of recombinant immunotoxin (Fig. 1). At all studied concentrations, 150, 350, and 700 nM, the activity of the LDH enzyme significantly increased as a dose-dependent manner (48, 120, and 206.8%, respectively) compared with the baseline (12% in the group without treating with immunotoxin) (p<0.01, 0.001, and 0.001, respectively). This indicates that the recombinant immunotoxin affects enzyme activity and can have a significant effect.

Recombinant Rovaliptuzumab-Granzyme B plays a role in apoptosis induction by stimulating caspase 3/7 activity

In this study, the effect of recombinant immunotoxin on caspase 3/7 activity in the A549 cells was investigated. The A549 cells were exposed to recombinant immunotoxin at different concentrations (150, 350, and 700 nM), and caspase 3/7 activity was measured (Fig. 2). The results were analyzed to compare changes in enzyme activity. At 150, 350, and 700 nM, the activity of caspase 3/7 was approximately 0.77, 1.33, and 2.34 nmol/min/mL, respectively, compared with the negative control (0.43 nmol/min/mL), and the increase in all groups was significant (p<0.05, 0.01, and 0.01, respectively).

Recombinant immunotoxin modulates the expression of genes involved in apoptosis

As shown in Figure 3(a), our results

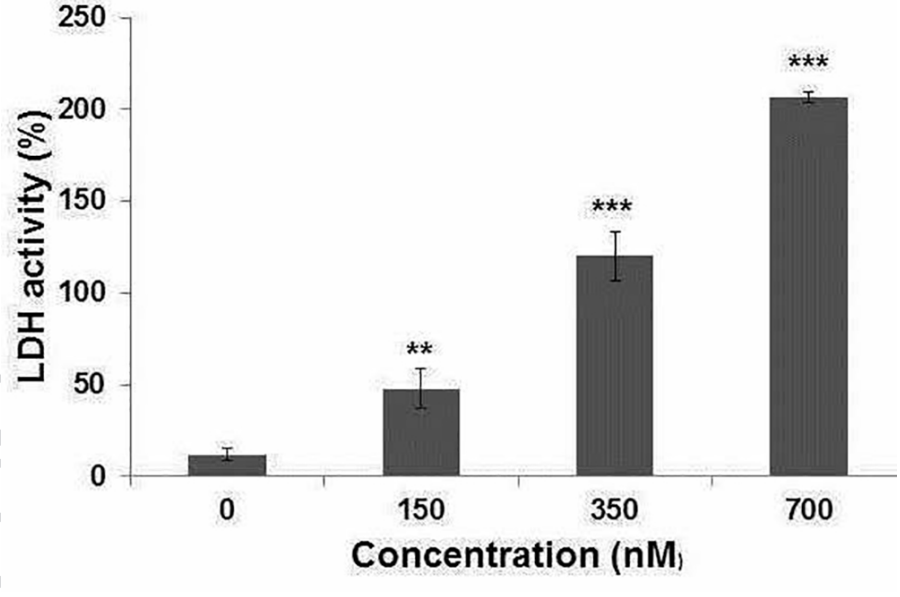


Fig. 1. Effects of Different Concentrations of Rovaliptuzumab-granzyme B on LDH activity. For LDH, we found that 150, 350, and 700 nM Rovaliptuzumab-granzyme B significantly increased LDH activity compared with the baseline (12% in the group without immunotoxin treatment) (**p < 0.01, *** p < 0.001).

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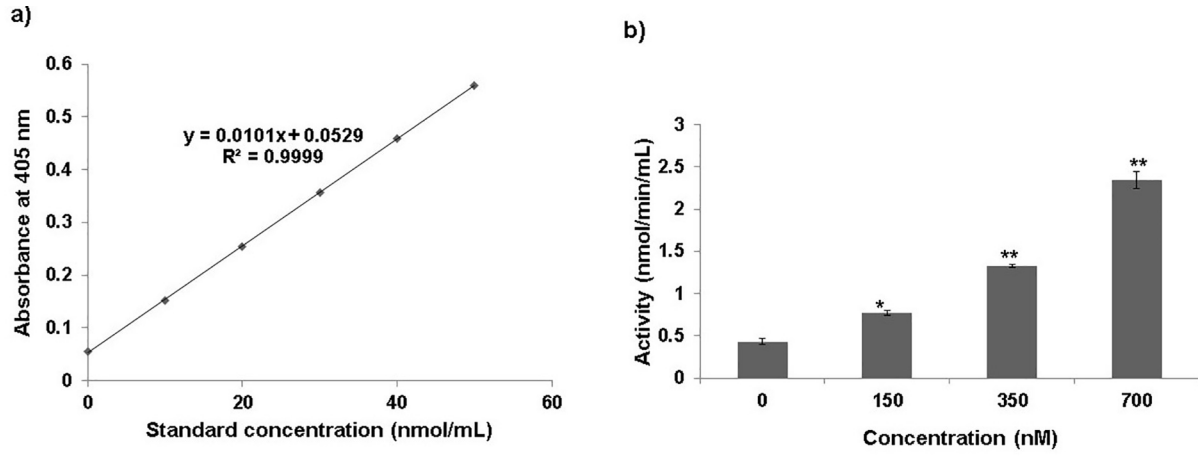


Fig. 2. The level of caspase 3/7 activity after treatment with 150, 350, and 700 nM Rovaliptuzumab-granzyme B for 24 h in the A549 cell line. **a)** Standard curve **b)** Caspase 3/7 activity rates (*p < 0.05, ** p < 0.01)

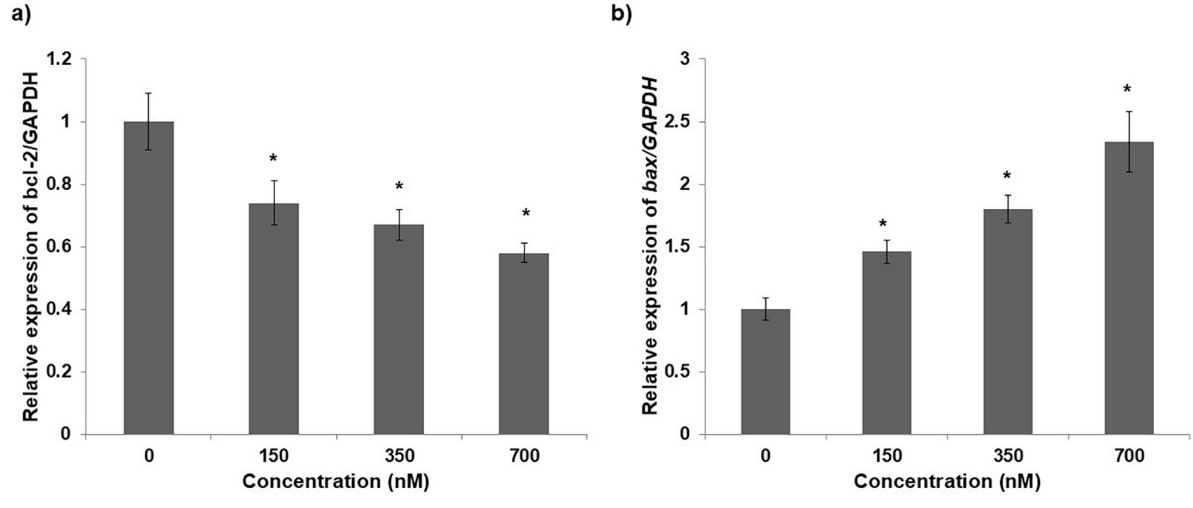


Fig. 3. Relative mRNA expression of a) *BCL-2* and b) *BAX* at different concentrations of recombinant Rovaliptuzumab-granzyme B on A549 cells**.** (* p < 0.05)

showed a 0.74-, 0.67-, and 0.58-fold reduction in *BCL-2* mRNA expression after exposure to 150, 350, and 700 nM, respectively (p<0.05). In addition, *BAX* mRNA expression (Figure 3(b)) at concentrations of 150, 350, and 700 nM of recombinant immunotoxin showed 1.48-, 1.8-, and 2.34-fold increases, respectively, compared with the negative control (p<0.05). Furthermore, the *BAX/BCL-2* expression ratio at concentrations of 150, 350, and 700 nM increased by approximately 1.97, 2.68, and 4.03-fold, respectively, compared with the negative control. This indicates a positive effect of immunotoxin on the activation of genes related to apoptosis induction.

DISCUSSION

Immunotoxins as a targeted strategy could be applicable for the treatment of cancers, especially those resistant to conventional therapies. Lung cancer is one of the types of cancer that does not respond well to conventional treatments. The topic of designing immunotoxins for this type of cancer dates back almost 40 years, with Whatman's research group presenting it in 1987. Because specific cancer biomarkers play an important role in cancer cell categorization, progression, and properties, selecting and applying these biomarkers could benefit

the design and development of targeted compounds (19). DLL-3 overexpression on lung cancer cells and its rare expression on normal cells make this protein a desirable candidate for immunotoxin design (20). In our previous study, we designed a new immunotoxin composed of an ScFv from Rovaliptuzumab as an anti-DLL-3 antibody and Granzyme B as a toxic moiety, and demonstrated specific cell binding and dose-dependent cytotoxic and apoptotic effects on the A549 cells. In the current study, some of the cell death pathways are examined in more detail (17). Our findings demonstrated that treatment with this immunotoxin increased LDH release and caspase-3/7 activity, while simultaneously upregulating *BAX* and downregulating *BCL-2* gene expression in a dose-dependent manner.

Serum levels of LDH are useful in the prognosis of many types of cancer, which can be interpreted in different ways. Oncologists consider LDH a useful biomarker for poor prognosis, while biologists recognize it as a factor influencing signaling pathways in carcinogenesis and the immune response (21, 22). Studies have shown that recombinant immunotoxins exert their lethal effects by binding to a surface antigen on cancer cells, delivering the toxin into the cytosol, and triggering programmed cell death (23).

One of the main findings of this study was the dose-dependent increase in LDH release following treatment with the recombinant immunotoxin. LDH is widely used as an indicator of membrane integrity, and its release into the extracellular environment reflects the loss of plasma membrane integrity during cell death. Similar increases in LDH activity have been reported in studies evaluating the cytotoxic effects of anticancer agents, supporting LDH as a reliable marker of treatment-induced cytotoxicity (24). The elevated LDH levels observed in the present study indicate that the recombinant immunotoxin effectively induces cellular damage in the A549 cells.

The increased LDH release observed following treatment with the recombinant Rovalpituzumab-Granzyme B immunotoxin suggests that this construct induces substantial cellular damage in the A549 cells. LDH is a well-established marker of plasma membrane disruption, widely used to evaluate treatment-induced cytotoxicity (24). The dose-dependent increase in LDH activity observed in the present study is consistent with the reduction in cell viability we previously reported for this immunotoxin, indicating that higher concentrations of the construct result in greater cytotoxic effects. Similar findings have been reported in NSCLC models, where increased LDH release was associated with enhanced cytotoxicity and apoptosis following treatment with targeted anticancer agents (25, 26). Although LDH release alone cannot distinguish apoptosis from other forms of cell death, its concurrent increase with apoptotic markers observed in the present study suggests that membrane damage results from ongoing cell death rather than nonspecific toxicity.

Immunotoxins can induce apoptosis through various intrinsic and extrinsic pathways in cancer cells (27, 28). These compounds can lead to programmed cell death by targeting apoptotic signaling pathways. In our previous study, annexin/PI staining showed that recombinant Rovalpituzumab-Granzyme B induces and increases apoptosis. In addition, the results from the present study showed increased caspase-3/7 activity following treatment with the recombinant Rovalpituzumab-Granzyme B immunotoxin. Caspase-3 and caspase-7 are the main caspases of the apoptotic pathway, and their activation causes irreversible progression of apoptosis in the cells. Previous studies have shown that Granzyme B induces apoptosis by directly activating caspases and the mitochondrial apoptotic pathway, leading to target cell death (29, 30). Therefore, the increased caspase-3/7 activity observed in the present study suggests that the recombinant immunotoxin activates apoptotic signaling in the A549 cells. Furthermore, the gene expression analysis

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supported the pro-apoptotic activity of the recombinant immunotoxin. Treatment of the A549 cells resulted in a dose-dependent increase in *BAX* expression accompanied by a reduction in *BCL-2* expression, leading to a marked increase in the *BAX/BCL-2* ratio. The balance between these two members of the Bcl-2 family is a main factor in mitochondrial outer membrane permeabilization and the subsequent activation of the intrinsic apoptotic pathway. An increased *BAX/BCL-2* ratio promotes cytochrome c release from mitochondria, resulting in caspase activation and apoptotic cell death (31). Therefore, the observed changes in gene expression agree with the increased caspase-3/7 activity detected in the present study and suggest that apoptosis induced by the recombinant immunotoxin may be associated with activation of the mitochondrial pathway. Similar changes in *BAX* and *BCL-2* expression have been reported after treatment with targeted anticancer agents and recombinant immunotoxins, where apoptosis induction was accompanied by upregulation of pro-apoptotic genes and suppression of anti-apoptotic genes (32, 33). Although only mRNA expression was evaluated in the present study, these findings provide additional molecular evidence supporting the pro-apoptotic effect of the recombinant Rovalpituzumab-Granzyme B immunotoxin in the A549 cells. However, the present study did not evaluate upstream apoptotic mediators such as caspase-8, caspase-9, or cytochrome c release; the concurrent increase in caspase-3/7 activity, together with changes in apoptosis-related gene expression, could support involvement of the intrinsic apoptotic pathway.

A limitation of the present study is that LDH release, caspase-3/7 activity, and *BAX/BCL-2* expression were assessed only at a single 24 h time point. However, our previous work showed no significant difference in overall cytotoxicity between 24, 48, and 72 h (16, 17). Future studies at additional time points (e.g., 6, 12, and 48 h), assessment of upstream apoptotic mediators such as caspase-8 and caspase-9, evaluation of mitochondrial involvement and cytochrome c release, and confirmation of apoptosis-associated changes at the protein level are needed to better understand the pattern of these molecular events. Furthermore, it is suggested that researchers investigate the precise molecular mechanisms as well as the combined effects of immunotoxins with other treatments. These studies can help clarify how recombinant Rovalpituzumab-Granzyme B immunotoxins work. Despite the relatively favorable apoptotic effects of the recombinant immunotoxin in this study, further studies are needed to evaluate its effects on other molecular mechanisms in different high-DLL3-expressing SCLC cell lines, DLL3-negative cell lines, and animal models.

CONCLUSION

In summary, the results of this study show that recombinant immunotoxin can increase LDH activity, caspase 3/7 activity, and *BAX* gene expression, and reduce *BCL-2* gene expression in the A549 cell line, indicating the potential of immunotoxins to induce apoptosis and cell death. These findings suggest that the recombinant Rovalpituzumab-Granzyme B immunotoxin merits further evaluation as a potential targeted therapeutic candidate for lung cancer.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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