

## RESEARCH ARTICLE

# Anti-ROR1 scFv-EndoG as a Novel Anti-Cancer Therapeutic Drug

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### Abstract

**Aim:** Immunotoxins are proteins that consist of an antibody fragment linked to a toxin, used as agents for targeted therapy of cancers. Although the most potent immunotoxins are made from bacterial and plant toxins, obstacles which contribute to poor responses are immunogenicity in patients and rapid development of neutralizing antibodies. In the present study we proposed a new therapeutic immunotoxin for targeted cancer therapy of ROR1 expressing cancers: an anti ROR1 single chain fragment variable antibody (scFv)-endonuclease G (anti ROR1 scFv-EndoG). **Methods:** The three-dimensional structure of anti ROR1 scFv-EndoG protein was modeled and structure validation tools were employed to confirm the accuracy and reliability of the developed model. In addition, stability and integrity of the model were assessed by molecular dynamic (MD) simulation. **Results:** All results suggested the protein model to be acceptable and of good quality. **Conclusions:** Anti-ROR1 scFv-EndoG would be expected to bind to the ROR1 extracellular domain by its scFv portion and selectively deliver non-immunogenic human endonuclease G enzyme as an end-stage apoptosis molecule into ROR1-expressing cancer cells and lead rapidly to apoptosis. We believe that anti ROR1 and other anti-tumor antigen scFv-EndoG forms may be helpful for cancer therapy.

**Keywords:** Cancer therapy- ROR1- immunoconjugate- scFv- EndoG- apoptosis

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### Introduction

Cancer is a leading cause of death worldwide and imposes significant psychological and economic impact in the world (Dolatkhah et al., 2015). Today, several methods are used for cancer therapy, including chemotherapy and radiation therapy; however, these methods are associated with side effects as they not only affect cancer cells but also normal dividing cells (Gerber, 2008). Targeted therapy is a new generation of cancer treatment drugs designed to cope with a specific target protein that is believed to have a critical role in tumor growth or progression (Wu et al., 2006). The definition of cell surface antigens that are expressed by human cancers has revealed a broad array of target antigens that are overexpressed, mutated or selectively expressed in comparison with normal tissues (Loo and Mather, 2008; Scott et al., 2012). One of these antigens, which has recently attracted the attention of many scientists, is ROR1 (Receptor tyrosine kinase-like orphan receptor 1). ROR1 belong to the receptor tyrosine kinase (RTK) family (Borcherding et al., 2014; Rebagay et al., 2012) which are known to be key regulators of normal cellular processes such as proliferation, survival, differentiation and migration (Baskar et al., 2012). This protein is expressed on many cancers including B-cell chronic lymphocytic leukemia (B-CLL), mantle cell

lymphoma (MCL), acute lymphoblastic leukemia (ALL) (Baskar et al., 2008; Dave et al., 2012) also lung, colon, pancreas, renal, bladder, prostate, breast and ovarian cancers; while its expression was not detectable on normal tissue counterparts (Zhang et al., 2014; Zhang et al., 2012a; Zhang et al., 2012b). Thus, its unique expression profiles making it as an ideal therapeutic target for targeted based therapy.

Over the past decade, the efficacy of antibodies as targeted therapy tools in treating patients with cancer has been increasingly recognized (Weiner et al., 2010) and this strategy is now one of the most successful strategies for treating patients with hematological malignancies and solid tumors. Single-chain variable fragment (scFv) antibodies are one of the most popular recombinant antibody (rAb) formats (Weisser and Hall, 2009). It consists of variable regions of heavy (VH) and light (VL) chains which are joined together by a flexible peptide linker. Lacking the Fc region and Fc glycosylation, lead to low immunogenicity and these two properties prevent immune-mediated neutralization of scFv antibodies and therefore by improving their half-life making them better therapeutic agents compared to the full-length mAbs (Ahmad et al., 2012; Monnier et al., 2013). Furthermore, antibody fragments can be fused to a range of toxins such as cytotoxic proteins, radionuclides, or drugs. Once

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fused, these immunotoxins could specifically deliver their agents towards antigen-expressing cancer cells (Ahmad et al., 2012).

Among the different proteins that participate in the various stages of apoptosis processes, EndoG (Endonuclease G) is released from the mitochondria in a pro-apoptotic Bcl-2 family-dependent and caspase-independent manner after which is translocated to the nucleus where it cleaves DNA into large fragments, likely due to cooperation with DNase I (Li et al., 2001; van Loo et al., 2001; Widlak et al., 2001). In addition, several studies have shown the role of EndoG in tumor growth inhibition (Hamada et al., 2014; Winnard et al., 2008; Yoshida et al., 2006).

In this work, we have built a recombinant immunoconjugate construct consist of anti-ROR1 scFv and EndoG enzyme which are joined by furin sequence as a linker. Indeed, after binding and ROR1-mediated endocytosis of our immunoconjugate, it can be processed by furin enzyme, and EndoG escapes from endosome into cytosol. Furin is a ubiquitous, Ca<sup>2+</sup>-dependent, transmembrane serine endoprotease (Thomas, 2002) that plays an active role in the maturation of many cellular proteins, and its prevalence is frequently exploited by bacterial toxins and viruses during intoxication and infection (48). Here, we used the *Pseudomonas* Exotoxin A (PEA) furin cleavage sequence (RHRQPRGWEQL), to after immunoconjugate internalization, furin cleaving enzyme separate the two immunoconjugate domains, and EndoG domain escapes into cytosol of tumor cell and lead to cancer cell apoptosis. In the present study anti-ROR1 scFV-EndoG immunoconjugate was constructed and evaluated by in silico approaches.

## Materials and Methods

### *Amino acids sequences retrieval of anti ROR1 scFv and EndoG*

The amino acid sequences of patent anti ROR1 scFV antibody retrieved from United States Patent Application Publication (US 2013/0101607 A1) and the amino acid sequences of human EndoG with accession number Q14249 was retrieved from Uniprot (<http://www.Uniprot.org>) Database.

### *Homology modeling*

The process of homology modeling of protein structure usually needs first existing defined templates structure. To build three dimensional structures of the anti-ROR1 scFv-EndoG, the structure of anti ROR1 scFv and mature form of EndoG separately were predicted using Modeller 9.11. The PDB files of selected templates were retrieved from the RCSB Protein Databank at <https://www.rcsb.org/pdb> (Berman et al., 2000). In order to align each target sequence against template sequences Clustal Omega program at <http://www.ebi.ac.uk/Tools/msa/clustalo> was used (Sievers et al., 2011).

### *Domains assembling and building final construct; Anti-ROR1 scFv-EndoG*

Inorder to assemble domains to build the final construct

including Anti ROR1 scFv, linker (furin sequence) and EndoG, AIDA (ab initio domain assembly server) at <http://ffas.sanfordburnham.org/AIDA/> was used. As the anti-ROR1 scFV-EndoG contains two different functionally and structurally independent units (antibody and enzyme) which are linked to each other's by furin sequence as linker, and the arrangement and orientation of these two domains against each other is important for proper functionality; we applied AIDA. AIDA combines steps of identifying individual domains, predicting (separately) their structures and assembling them into multiple domain complexes using an ab initio folding potential to predict domain– domain interaction and relative domain positions and orientations (Heo et al., 2013; Xu et al., 2015).

### *Model refinement*

For refinement of final built model, the Galaxy web server at <http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE> was used. GalaxyRefine performs repeated structure perturbation and subsequent overall structural relaxation by molecular dynamics simulation (Heo et al., 2013).

### *Tertiary structure validation*

To evaluate the quality of predicted 3D models, ERRAT server at <http://services.mbi.ucla.edu/ERRAT> (Colovos and Yeates, 1993) and PROCHECK web server at <http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html> were used (Laskowski, 2001).

### *Molecular dynamic simulation*

For MD simulation, modeled PDB structure of anti-ROR1 scFv-EndoG protein was embedded in a box with proper dimensions equal to 1 nm from the edges of the fusion protein. Afterwards, the system was solvated with spc216 water model and then, structure was relaxed through the energy minimization (EM) process. Finally, the main MD run was performed after removing all restrains from the system. GROMOS force field implemented in Gromacs 4.5.3. Calculation of RMSD plot was applied using the commands implemented in Gromacs program.

## Results

### *Tertiary structure prediction*

In the present study, we used homology modeling methods to predict 3D structure of anti ROR1 scFv-EndoG protein. The 3D structure of anti ROR1 scFv and Endo G were predicted separately by modeller. For anti ROR1 scFv five templates were selected (which were including 2KH2, 3AUV, 1X9Q, 1QLE, 1D5I) and they all are antibody (Harrenga and Michel, 1999; Midelfort et al., 2004; Mundorff et al., 2000; Wilkinson et al., 2009; Yu et al., 2012), and for EndoG six templates were selected (which were including 3ism, 3s5b, 4qn0, 4a1n, 2O3B, 1zm8) (Ghosh et al., 2005; Ghosh et al., 2007; Hanczyc et al., 2013; Lin et al., 2012; Lin et al., 2016; Loll et al., 2009). For domains assembling and final construct building of anti ROR1 scFV-linker (furin sequence) –EndoG, AIDA was used. As anti ROR1

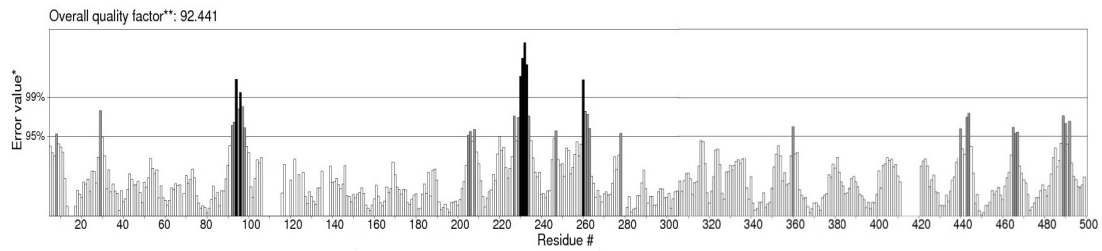


Figure 1. ERRAT for Validating the 3D Model of Anti ROR1-EndoG. Error values are plotted as a function of the position of a sliding 9-residue window. In the ERRAT plot, the overall quality factor is 92.44%.

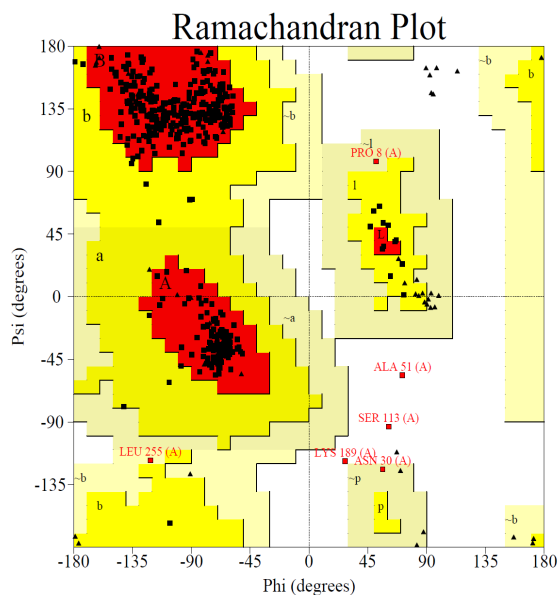


Figure 2. PROCHECK Result for Validating 3D Model of Anti ROR1-EndoG. Red, yellow, light yellow and white regions are most favored regions (94.1 %), additionally allowed regions (4.8%), generously allowed regions (0.5%) and disallowed regions (0.7%) respectively.

scFv-EndoG is a two domains protein, in order to predict the best orientation and arrangement of these two domains against each other, AIDA web server was applied.

#### Tertiary structure validation

To evaluate the quality of predicted protein structure, ERAAT and PROCHECK web servers were used. ERRAT is a program for verifying protein structures determined by crystallography. Error values are plotted as a function of the position of a sliding 9-residue window. The error function is based on the statistics of non-bonded atom-atom interactions in the reported structure (compared to a database of reliable high-resolution structures) (Colovos and Yeates, 1993). The ERRAT result for initial model indicated the overall quality factor of model was 61.12. Good high resolution structures generally produce values around 95% or higher. For lower resolutions (2.5 to 3 Å) the average overall quality factor is around 91%. ERRAT demonstrated that the initial 3D model requires refinement processes. The best preliminary assembled model built by AIDA was used for refinement at Galaxy refine. After structure refinement and energy minimization, high-quality improved 3D model was achieved. After all refinement steps, ERRAT factor of

the best-quality refined model improved from 61.12 to 92.44 (Figure 1).

The stereochemical quality of the predicted model and accurateness of the protein model was evaluated with Ramachandran's map calculations using PROCHECK. In the present study, the stereochemical evaluation of backbone psi and Phi dihedral angles of the Anti ROR1 scFv-EndoG revealed that 94.1 %, 4.8%, 0.5% and 0.7% of residues were located in the most favored regions, additionally allowed regions, generously allowed regions and disallowed regions respectively (Figure 2). Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions. Therefore, The Ramachandran plot of PROCHECK analysis results suggest that the predicted model was of good quality.

Therefore all protein structure validation programs show that the predicted Anti ROR1-EndoG protein model is having acceptable structure. The 3D structure of final model of anti ROR1 scFv-EndoG is shown in Figure 3.

#### Molecular dynamic simulation

Root mean square deviation (RMSD) was calculated for finding the deviations and changes of the anti-ROR1

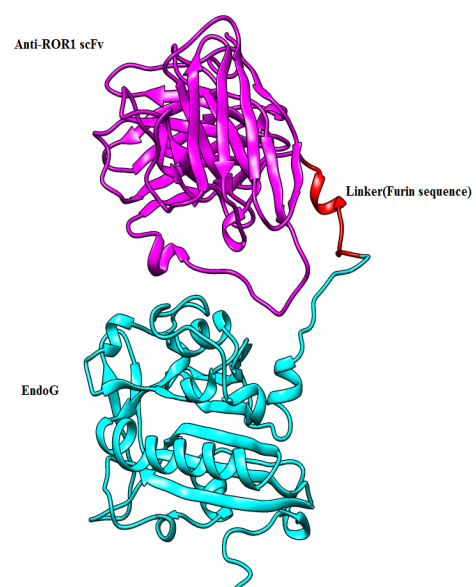


Figure 3. 3D Representations of Anti-ROR1 scFv-EndoG Construct. Anti ROR1 scFv domain, linker and EndoG domain are shown in magenta, red and cyan, respectively.

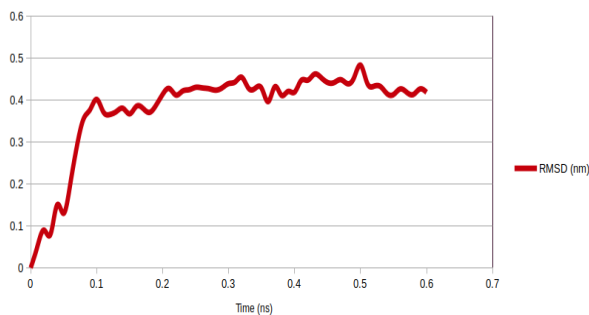


Figure 4. MD Simulation Result. RMSD of simulated anti ROR1 scFv-EndoG structure versus time shows low deviation values and the stability of protein

scFv-EndoG protein structure over the MD simulation period. As observed in Figure 4, there was a minimum deviation (RMSD<1Å) from the 0.1th nanosecond until end of simulation course. These deviation values indicated that our protein has had a suitable behavior through the simulation and it has a stable conformation.

## Discussion

Chemotherapy and radiation therapy have been developed for therapy of cancer. However, these methods often cause undesirable side effects as they have very little or no specificity. Currently, antibody-based targeted therapy against tumor antigens is widely used for treating patients with cancer. scFvs are format of antibodies which have several advantages compared to the full-length mAbs. They show improved pharmacokinetic properties, such as better tissue penetration as a result of smaller size in comparison to the whole antibodies; can be produced easily, fast and inexpensively in *E. coli* (Ozaki et al., 2015). mAb also in both formats (scFvs and full length Abs) are used as immunoconjugate to deliver toxin, enzyme, siRNA and other anti-tumor agents into cancer cells; and many preclinical and clinical studies in this regards have shown success (Becker and Benhar, 2012; Oberoi et al., 2013b). One of the most important obstacles to using the immunoconjugates in vivo is the using foreign immunogenic toxins such as bacterial or plant toxins, which is associated with decrease in its half-life and need to frequent dosing schedules. Baskar et al., (2012) have shown that employing ROR1-immunotoxins such as Bt-1 could serve as targeted therapeutic agents for ROR1-expressing cell malignancies. But, one factor which contributing to the poor responses is the rapid development of neutralizing antibodies. Because the immune system is intact in these patients and most of the immunoconjugates consist of bacterial or plant toxins and toxin domain is immunogenic (Alewine et al., 2015; Onda et al., 2011). Thus it is usually necessary to give repeated and continuous doses of a drug to obtain an effective response in cancers (Onda et al., 2011). In order to resolve this problem, we have proposed endonuclease G (EndoG), an enzyme which naturally is presented in the human cells and plays a critical role at the end stage of apoptosis processes.

In the present study we proposed and built an

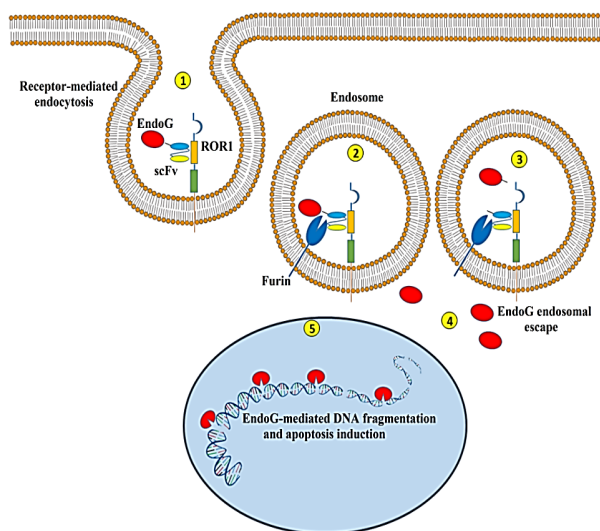


Figure 5. Schematic Presentation of Anti-ROR1 scFv-EndoG in Cancer Therapy. A: 1) scFV-EndoG structure. scFV-EndoG structure consist of a scFv binding domain against ROR1 antigen, furin sequence and EndoG (Endonuclease G). Anti-ROR1 scFV-EndoG binds to the ROR1 receptor via the scFv binding moiety and is internalized via receptor-mediated endocytosis 2) and trapped in the endosomal vesicle. 3) Furin enzyme digests furin sequence and EndoG is released in endosomal vesicle. 4) The EndoG is then released from the vesicles into the cytosol 5) and translocates into the cell nuclei and eventually leads to cancer cell apoptosis.

immunoconjugate construct, anti-ROR1 scFv-EndoG, by using insilico approaches. This fusion protein can be a safer and more effective delivery agent in comparison with conventional immunotoxins and has a potential use in cancer therapy.

EndoG is one of the most active cell death endonucleases (DNase/RNase) residing in the intermembrane space of mitochondria. This mitochondrial endonuclease has a unique site selectivity, initially attacking poly(dG). poly(dC) sequences in double-stranded DNA (Cote et al., 1989; Cote and Ruiz-Carrillo, 1993; Ohsato et al., 2002; Ruiz-Carrillo and Renaud, 1987)

It is synthesized as an inactive 32 kDa propeptide and after cleavage of signal peptide, mature active 27 kDa EndoG can be released from mitochondria during apoptosis, moves to the nucleus and cleave nuclear DNA (Li et al., 2001). Several studies have been proved the cytotoxicity of EndoG and its role in the apoptosis induction (Apostolov et al., 2007). Overexpression of extramitochondrially active EndoG in CV1 and HeLa cell lines have shown to induced cell death, while the expression of an inactive mutant form of EndoG did not induce cell death (Ghosh et al., 2005). In consistent with these data the loss of EndoG activity in *C.elegans* resulted in increased cell survival (Hengartner, 2001). The expression of EndoG in poorly differentiated invasive cancer cells in comparison with well-differentiated non-invasive cancer cell lines was extremely lower. In consistent human invasive carcinoma tissues have shown a decreased expression of EndoG and decreased endonuclease-mediated apoptosis (Basnakian et al., 2006).

Thus, in the present study we proposed and built an immunoconjugate construct, Anti-ROR1 scFv-human EndoG 3D structure by using bioinformatics tools as a novel drug for cancer therapy. In this regards, we predicted and evaluated the 3D structure of anti-ROR1 scFv-EndoG protein. Stability of the construct also evaluated by Gromacs program. The results showed good quality of model and the MD simulation showed good stability of our protein. In this immunoconjugate construct we used a human end stage apoptosis inducing protein, EndoG, to circumvent the foreignness and immunogenicity problem related to toxin moiety of conventional immunotoxins. The structure of anti ROR1 scFv-EndoG and its mechanisms in tumor cell killing is shown in Figure 5.

Granzyme B also has been used as apoptosis inducing protein in fusion format with different anti-cancer antibodies for targeted killing of cancer cells (Dälken et al., 2006; Kurschus et al., 2004; Oberoi et al., 2013a). This cytotoxic protein induces apoptotic cell death by inducing different signaling pathways in a both caspase-dependent and caspase-independent manner. Granzyme B induces early event of apoptosis process by interaction with different initial signaling proteins (Boivin et al., 2009); In contrast, EndoG induces late phase of apoptosis that occurs after that the cell has committed to die and it doesn't need to activate any early-phase proteins of apoptosis. EndoG is released from mitochondria and directly enters into the nuclei and induces cell death (Li et al., 2001). Thus, antibody based targeted delivery and accumulation of EndoG in the cytoplasm of cancer cell could lead to direct and fast apoptosis induction. Although GrB is one of the most promising candidates and this enzyme has already revealed its potential for targeted cancer therapy, however, the clinical application of GrB may be limited because it is inactivated by the overexpression in tumors of its specific inhibitor serine protease inhibitor PI-9/SPI-6 (Hehmann-Titt et al., 2013; Medema et al., 2001) which is important mechanisms of escape by tumors. Another problem with GrB in comparison with EndoG is its very high isoelectric point which resulting in a positive surface charge contributing to nonspecific binding to normal healthy cells and off-target effect (Hehmann-Titt et al., 2013).

The large molecular size of most mAb-drug immunoconjugates often results in poor penetration into solid tumors. Using scFv instead of whole antibody in immunotoxin conjugate take advantage of smaller size of immunotoxin also better tumor penetration. Today many scFv antibodies against different tumor antigens on the surface of cancer cells have been produced and are evaluating in different phase of clinical trials (Allahyari et al., 2017; Becker and Benhar, 2012). As the structure of scFv antibodies are conserved, the scFv domain in this immunoconjugate construct can be replaced by any other approved scFv against cancer antigens, without any changes in the anti-cancer antibody-EndoG structure and function.

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