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Use of Molecular Dynamic Tools in Engineering of Onconase Enzyme to Increase Cellular Uptake and Evade RI

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Abstract
Onconase is a distinctive RNase due to a prominent feature that enables this protein to exhibit selective toxicity against cancer cells. Therefore, in this study, we aimed to modify Onconase and then assess engineered Onconase characteristics such as cellular uptake, cytotoxicity, the ability to evade RNase inhibitor, and protein functionality. For this purpose, six models of Onconase enzyme were designed based on protein docking and Molecular Dynamic simulation results. For this purpose, five mutations (K31A, K55A, S72C, R73A and K76A) were selected, because MD results showed that this combination was effective for the goal of this study. Then, the engineering Onconase was synthesized and cloned into the pET22b (+). Recombinant protein was produced in *E. coli* BL21 (DE3) and after protein purification, enzyme properties and cytotoxicity were investigated. Modeling results revealed that replacement of lysine with alanine could lead to an increase in surface charge and cellular uptake. Furthermore, these changes had no influence on the structural stability of Onconase and the enzyme could still evade RI. Experimental results revealed that the activity of the recombinant Onconase produced in this study was 213 ± 19 U/mol, which is 3.5 times lower than RNase A (746 ± 13 U/nmol); however, this enzyme is resistant to RI. Also, our recombinant Onconase was able to induce apoptosis in HeLa cancer cells at 1.99 µg/uL Onconase, whereas RNase A had no cytotoxic effects on these cells under similar conditions. Our findings suggested that an in silico study could be highly useful for enzyme engineering and drug discovery.

Keywords Onconase · Recombinant protein · Molecular dynamics · Protein engineering

Introduction

The Onconase enzyme (Onc) has been widely studied because it is presently in phase III of clinical trials to test its potential as an antitumor drug (Fagagnini et al. 2017). It has been previously revealed that Onc has an uncommonly high denaturation temperature, thus making Onc one of the most thermostable mesophilic proteins (Leland et al. 1998). However, this thermostability of Onc has also led to its low adaptability to the substrate and the transition state (Notomista et al. 2006). Therefore, to eliminate the defects of the natural enzyme, researchers engineered amino acids of Onc (Sundlass and Raines 2011; Turcotte et al. 2009). The cytotoxicity of ribonucleases is highly correlated withthe net positive charge and the distribution of that charge in ribonucleases (Futami and Yamada 2008; Johnson et al. 2007). Therefore, increasing the positive charge by mutagenesis (Ilinskaya et al. 2002; Notomista et al. 2006), making chemical alterations, and adding protein transduction domains to toxic or nontoxic ribonucleases can increase their cytotoxicity (Turcotte et al. 2009). Previous studies have shown that using arginine residues in bullfrog sialic acid-binding lectin and bovine pancreatic ribonuclease could increase the internalization in these homologues of RNase (Ogawa et al. 2002; Fuchs et al. 2007). However, another study investigated

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the effect of lysine and arginine residues on the cellular internalization and functioning of Onc and replaced ten non–active-site lysine residues with arginine (Sundlass and Raines 2011). Among 104 residues of Onc, three are arginine and twelve are lysine. They reported that an increase in arginine content greatly affects cellular uptake. It has also been previously shown that this protein is highly cationic (leading to $Z = +5$ and $pI > 9$) (Ardelt et al. 1991). Based on an electrostatic potential map, it could beseen that, other than the active site, three other regions with high positive charge are present in Onc at neutral pH. In another report, Turcotte et al. (2009) changed the wild-type Onc by the use of systematic alteration of twelve lysine and three arginine residues to alanine.

Today, protein molecular dynamics and computer modeling have advanced and protein modeling in dynamic situations has been made possible. Therefore, we evaluated previous research on Onc amino acid engineering to assess characteristics such as cellular uptake, cytotoxicity, the ability to evade RI and protein functionality. Also, we modeled six structures of Onc with the aim of investigating the molecular dynamics of the protein for the first time. Here, we present a new way in silico study that could be so useful for enzyme engineering and drug discovery.

Materials and Methods

Selection of Amino Acid for In Silico Analysis

According to Turcotte et al. (2009) and Sundlass and Raines (2011), six models of amino acid replacement were designed (Table 1). All the structures needed for this study were extracted from Protein Data Bank (PDB) website. The Pdb file for (http://www.rcsb.org) Onc enzyme was chosen using two access points. One of the files is related to the natural Onc and the other is related to the mutational Onc (M23L). The Pdb file related to the ribonucleic controller is available with the access point https://doi.org/10.2210/pdb2bnh/pdb, the file related to the ribonucleic controller attached to RNase enzyme is https://doi.org/10.2210/pdb1dfj/pdb, and the RNase enzyme file is available with the access point https://doi.org/10.2210/pdb2k11/pdb.

Protein Docking

For docking of Onc proteins to RI, we used Pymol and the online Boston University website called ClusPro (Yueh et al. 2017). In Pmyol software, Onc enzyme was manually substituted with RNase type A enzyme with its related Pdb file (https://doi.org/10.2210/pdb2bnh/pdb). Also, RI was attached to the RNase type A enzyme. Moreover, Onc enzyme and RI were introduced to ClusPro as ligand and receptor. Then, protein docking was done. Finally, the results of Pymol and Cluspro docking were compared and saved to be utilized in MD research.

Prediction of Protein Structure of Models

Presently, several computational biology tools are commonly used to determine protein structure. In this study, I-TASSER online software was used for protein modeling (Yang et al. 2015). First, amino acid replacement in Onc gene sequences were done and a I-TASSER analysis was performed to obtain the Pdb file. The Pdb files were used to assess the impact of amino acid variants and mutations on Onc structure using structural and/or sequence information. These techniques were also adopted by numerous recent studies using default parameters.

MD Analysis

All MD simulation levels occurred in Linux 17/2 using Gromacs software version 5 (Merlino et al. 2005; Van Der Spoel et al. 2005). Briefly, protein structures in this study were in cube of water with more than 6700 water molecules. Ionization status was assessed to reach natural pH of the environment. Extra system charge was optimized by adding a proper number of ions in a 7 angstroms surface. System energy minimization occurred for a 20 picosecond period and under 300 Kelvins temperature. Lengths of all bonds were limited by *Lincs*. Newton's equations of motion were synchronized with $2f_s$ rate and atomic information for analysis was saved for each 0/5 ps. Dielectric 1 stability and simulation temperature of 300 kelvins were considered. 40 ns

Table 1 Residue alterations in models

Candidate residue	K31	K45	K49	K55	K76	R73	S72
Model 1	–	A	A	A	A	–	–
Model 2	–	R	R	R	R	–	–
Model 3	–	R	R	R	–	A	–
Model 4		–	–	–	A	A	C
Model 5	–	A	A	A	–	–	C
Model 6	A			A	A	A	C

simulation were used to check the dynamics of Onc condition and ribonucleic inhibitor (Jurrus et al. 2018).

Analysis of MD Results

Stability of structural simulation done in this study was assessed with many geometrical factors in unit of time. Average of root mean square deviations (RMSD), radius of gyration (Rg) and root mean square fluctuation (RMSF) were extracted (Liu et al. 2018).

Gene Synthesis and Production of Recombinant Protein

The nucleotide sequence of mutant Onc was optimized for *E.coli* host by GeneRay codon optimization and sent to GeneRay Company (Shanghai, China) for synthesis. The Onc gene was subcloned in the pGH vector and then cloned into the pET22b (+) vector using specific primers of 5′ CCATGG GACAGGACTGGCTGACATTCCAG 3′ and 5′ CTCGAG TTAGCAAGAGCCCACGCCCAC 3′ between *Nco*I and *Xho*I sites. Cultivation of *E. coli* cells containing recombinant pET22b (+) was done in LB broth overnight at 37 °C followed by shaking at 180 rpm. Subsequently, 200 mL of overnight culture was inoculated into 2 L of LB broth medium containing 100 µg/mL ampicillin and grown at 37 °C until an optical density (OD) at 600 nm was attained. To induce expression, isopropyl β-D-thiogalactoside (IPTG) was added to the culture broth medium to a final Onc of 1.5 mM and the culture was incubated at 37 °C for 5 h. To collect the induced cells, centrifugation at 12,000×*g* and 4 °C was used for 20 min, then the supernatant was discarded. The cells were initially assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Protein Extraction and Sepharose Chromatography

The *pelB* signal peptide secreted recombinant protein into the periplasmic environment; therefore, osmotic shock methods were used for extraction of protein. Briefly, 25 mL of hypertonic buffer (Tris–Hcl 30 mM, EDTA 1 mM and Saccharose %20, pH 8) was added to the induced cells. The solution was incubated for 30 min on ice and centrifuged for 20 min in 8000×*g*. Then, the supernatant was collected, after which the pellet was suspended in 25 mL of hypotonic solution containing 5 mM MgSo4. The supernatants from the hypertonic and hypotonic solution were combined and dialyzed in lysis solution (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH: 8.0) for 16 h. To condense the proteins, the solution was filtered by Amicon Ultra-0.5 mL (Merck, USA). Extraction of the target protein was done by Q-Sepharose chromatography. In short, the Q-Sepharose column was washed by lysis solution three times and 5 mL of the supernatant containing soluble recombinant protein was filtrated three times in 4 °C. Then, the lysis solution was washed with six dosages of NaCl Onc (0.1, 0.2, 0.3, 1, 2, 3 Molar) in the Q-Sepharose column and was then collected in tubes for SDS-PAGE analysis. The best Onc of NaCl for extraction of the target protein was used for the whole amount of the soluble recombinant proteins. The protein Onc was estimated by Bradford analysis.

Enzyme Activity

The ribonucleolytic activity of the recombinant Onc was compared with the native RNase A according to the method used by Tripathy et al. (2013). Briefly, the yeast RNA and EtBr solutions were initially combined and the mixture was incubated for 30 min. Finally, 200 µL of 2 µM ribonuclease was appended to the solution and any changes in its fluorescence intensity were examined for 300 s with an emission and excitation wavelengths of 600 and 510 nm, respectively, and a slit width of 5/5 nm. To assess the effect of RI on Onc, the enzymatic activity test was carried out with five concentrations of RI (Thermo, USA).

MTT Assay

To examine the antitumor activity of recombinant proteins, HeLa cells were prepared for seeding in a 96-well flask and incubated in 37 °C and 5% CO_2 for 72 h. Then, five concentrations of mutant Onc (100 ng, 500 ng, 1000 ng, 1500 ng, 2000 ng, 2500 ng and 3000 ng) were assessed in three replications. To assess cell viability, 20 mL of a 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) diluted in PBS was added. Cell viability was defined as the percentage of viable cells treated with protein, while IC50 was expressed as the concentration at which 50% cell viability reduction occurs.

Results and Discussion

RMSD Analysis

RMSD values of the native and recombinant proteins were evaluated to determine the possible impact of amino acid replacements on the structure of the proteins. RMSD values for all protein backbones were examined during the MD simulation and were compared with the initial structure of the native protein. Figure 1 demonstrates that RMSD values of the recombinant proteins (Sample 1–Sample 5) are rather unstable in comparison to the native protein. Nevertheless, the native protein was stabilized at around 3 ÅRMSD value. The majority of RMSD values of the recombinant proteins were higher compared to the native protein, particularly

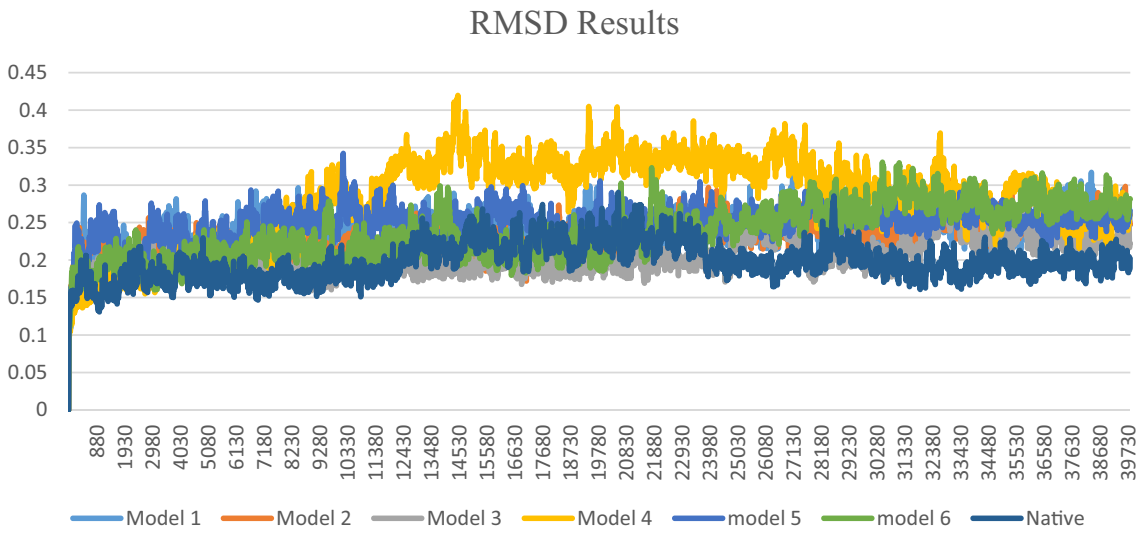

Fig. 1 RMSD analysis of six models designed for engineering of Onc enzyme. Model 6 had a similar pattern with the native model

Model 4 which showed the highest value. Also, Fig. 1 indicates that the amino acid replacements in Model 6 had no significant destabilizing effects on the protein structure.

RMSF Analysis

Furthermore, we examined RMSF variations to estimate the effect ofamino acid replacementon the dynamic behavior of each residue. As shown in Fig. 2, it could be seen that fluctuations of Model 6 coincided with that of the native enzyme. Other samples were high up to4 Å for residues between 10 and 37 positions, whereas the second highest peak occurred at 73 position. Increase in RMSF slope is an indication of the flexibility of amino acids, but this parameter could also lead to protein destruction. Therefore, it seems that the best amino acid replacement occurred successfully when RMSF slopes of native and recombinant proteins were similar.

Based on our results, the highest level of was observed for S72C mutant protein. Analysis of energy parameters of the MD simulation for both recombinant and native proteins

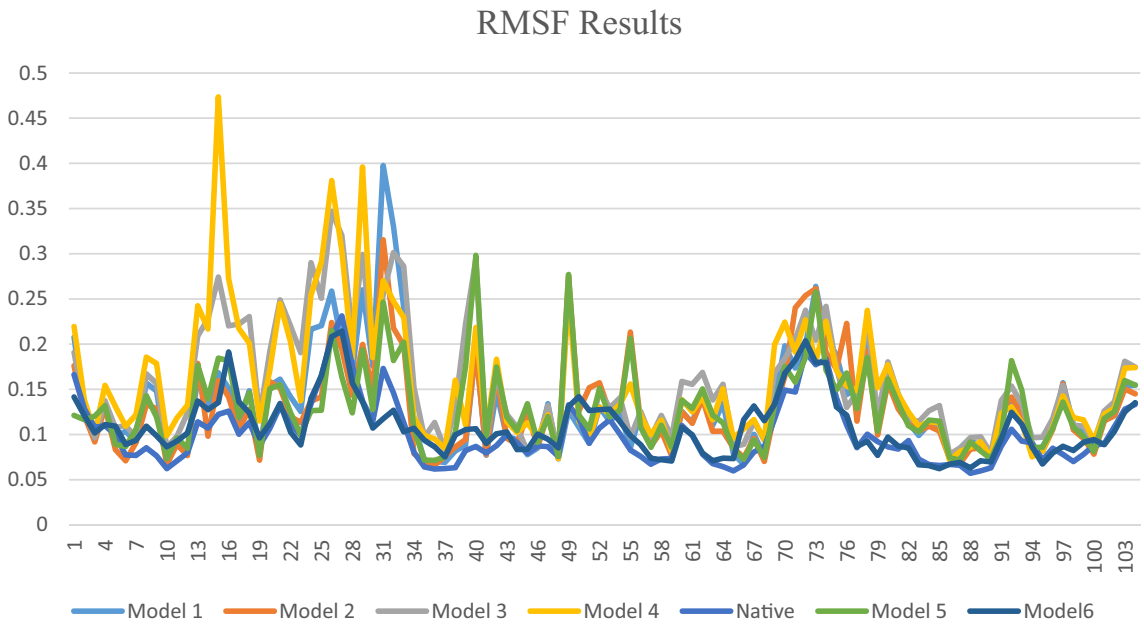

Fig. 2 RMSF analysis of six models designed for engineering of Onc enzyme

showed that the total energy remained between − 2434 and − 2122 kcal/mol, while this number was − 2326 kcal/mol for the native protein. Moreover, it is evident that increasing the position of the mutant residue also increases the total energy. In other words, mutations that occur in the center of the protein structure could reduce the total energy of the system, in contrast to mutations that occur close to the 3′ end of the protein.

Radius of Gyration

Finally, we analyzed the Rg for the native protein as well as the mutations that are involved in compactness of the protein. As a result, S72C showed the lowest compactness of the protein structure (10.5 Å), and K76A had the highest (11.3 Å). Also, the native protein showed quite high compactness (11.1 Å). Our results suggest that all mutant proteins contributed to destabilizing the structure of the protein, which eventually limits protein compactness.

Protein Analysis and Docking

When the 3D structure of Onc was analyzed to assess protein stability, it was shown that three models (Model 1, Model 2 and Model 4) of protein were destroyed among MD simulations (Fig. 3). The MD results revealed that mutations that occurred in closely located residues could disrupt protein functionality and structure. Therefore, Model 6 was designed with mutation K31 for this purpose. This test generated positive results and model 6 was found to be a stable protein that matches with the native protein. MD simulation of Model 6 with RI is illustrated in Fig. 4. The Onc with five amino acid alterations (K31A, K55A, R73A, K76A and S72C) can completely evade RI. Futami and Yamada (2008) reported

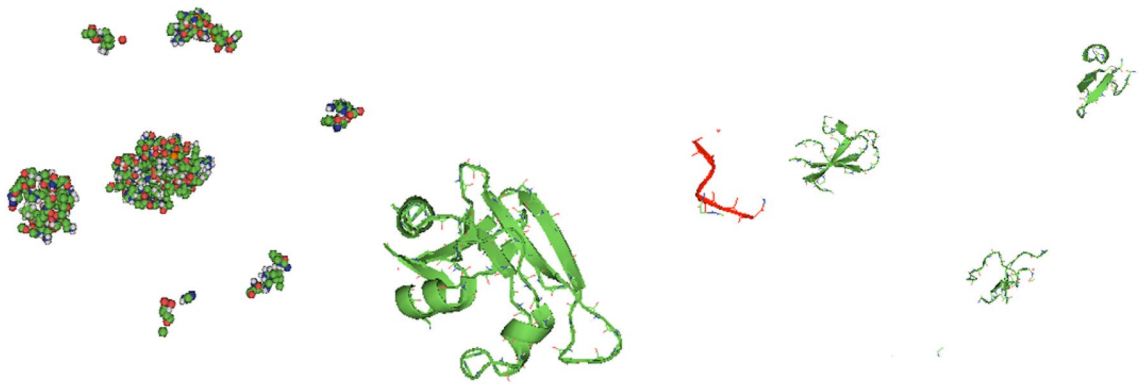

Fig. 3 3D structure of Onc in models (Model 1, Model 2 and Model 4). The PDB file in 40 ns showed destruction of proteins

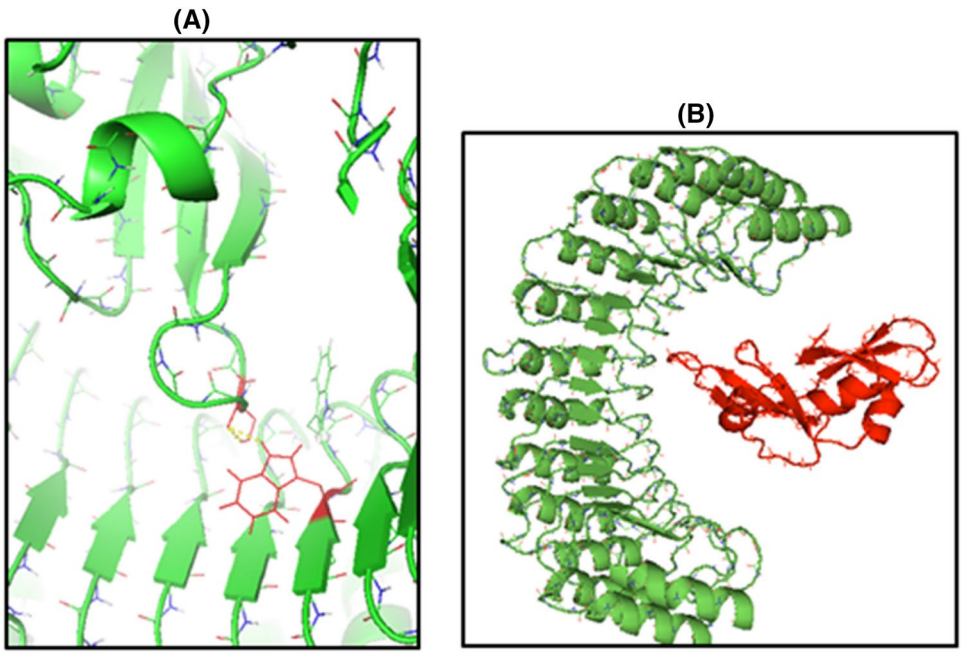

Fig. 4 Protein docking and MD simulation of native and recombinant Onc with RI. **a** Protein docking and MD simulation of native Onc with RI, **b** Protein docking and MD simulation of recombinant Onc with RI

that distribution of net charge is of high importance. Results in this study showed that imbalance in charge distribution could destabilize Onc structure. Although Sundlass and Raines (2011) considered the proper distribution of surface charge in their study (10 of 12 lysine residues were altered to arginine), but they lost protein stability because the recombinant protein was more susceptible in presence of protease. Goel and Chauhan (1997) reported that tumor cells could overexpress proteases which are capable of degrading susceptible proteins. Therefore, the recombinant Onc produced in Sundlass and Raines (2011) study is not qualified for cancer therapy.

In most studies on Onc production, RI activity was not considered. MD analysis of Onc in this study showed that RI activity could be more effective on cytotoxicity of the enzyme. Therefore, we optimized protein sequences of Onc that could evade RI which are very important.

Production of Onc protein

The production of mutant Onc protein was confirmed by the use of SDS PAGE and protein purification was successfully done by osmotic shock in the Q-Sepharose column (Fig. 5). Though our results proved protein expression, no proteins were found in the LB medium. In other words, nearly all the proteins were expressed either inside the bacterial cell or secreted into the periplasmic area. The Onc of the recombinant proteins extracted was 1.5 mg/mL by Bradford analysis.

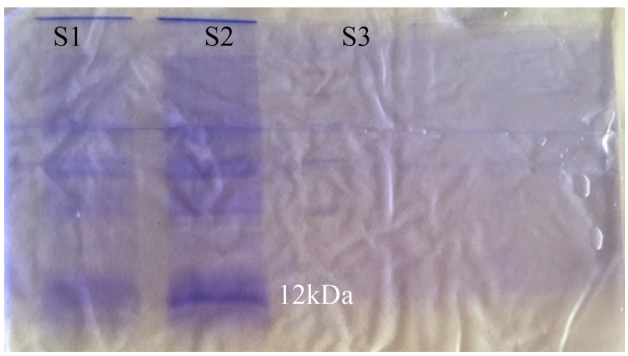

Fig. 5 SDS-PAGE of Onc enzyme. *S1* pET 22b vector, *S2* pET22b + ONC and *S3* Protein marker

Enzymatic Features

The cytotoxicity of a ribonuclease is highly associated with its enzymatic activity (Rutkoski and Raines 2008). It was shown in our results that the ribonucleolytic activity of Onc was 213 ± 19 U/mol, which was lower compared to RNase A (676 ± 23 U/nmol). In our study, the commercial native RNase A was used as a control and the value obtained was similar to a previously reported value (Lee and Raines 2008). Also, the mutant Onc was incubated with pepsin in order to determine its susceptibility to protease degradation. It was revealed that Onc showed resistance to pepsin degradation throughout the entire incubation process (22 h), during which 90% of Onc remained undamaged. The results also demonstrated that the Onc activity did not differ significantly in presence of RI, while RNase A hardly showed any activity at Onc of 50 ng/µL.

The Cytotoxic Effect of the Mutant Onc on HeLa Cell Line

To examine the cytotoxic effect of mutant Onc on the HeLa cell line, MTT assay was done following incubation with recombinant Onc. As shown in Fig. 6, the Onc concentration required for 50% cell survival (the surviving fraction is 50%, SF0.5) was estimated. The commercial RNase A was used as a control. Following co-incubation with the HeLa cell line for 36 h, the Onc concentration for SF0.5 was ~ 1990 ng; however, RNase A had no cytotoxic effect on HeLa cells.

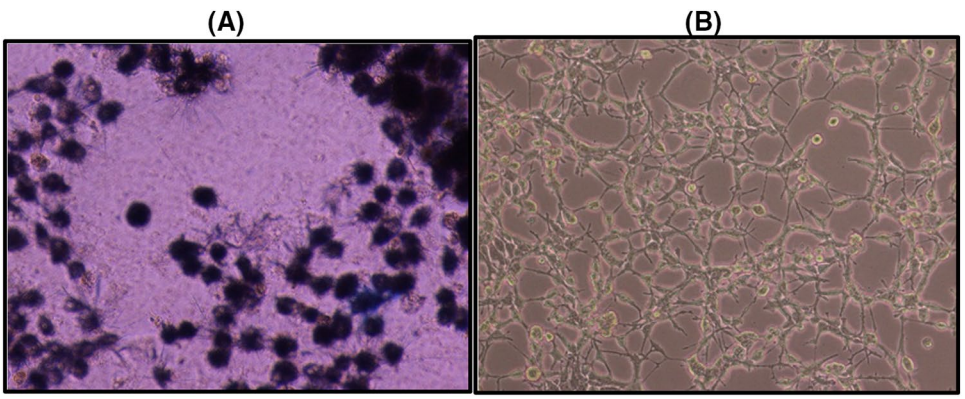

Fig. 6 Effect of recombinant Onc on HeLa cell in MTT assay. **a** Normal HeLa cell, **b** HeLa cell with treatment of Onc

Conclusion

In this study, Onc engineering was done using MD simulation analysis of protein and unique features such as increasing the cellular uptake and evading RI were introduced to Onc. Furthermore, the suitable activity and thermostability of the recombinant enzyme produced in the present study revealed that Onc could be an alternative candidate for immunotoxin design and bonding to scFv antibody.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

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