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Original Article

The effect of mutation on neurotoxicity reduction of new chimeric reteplase, a computational study

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Abstract

Background and purpose: Excitotoxicity in nerve cells is a type of neurotoxicity in which excessive stimulation of receptors (such as N-methyl-d-aspartate glutamate receptors (NMDAR)) leads to the influx of high-level calcium ions into cells and finally cell damage or death. This complication can occur after taking some of the plasminogen activators like tissue plasminogen activator and reteplase. The interaction of the kringle2 domain in such plasminogen activator with the amino-terminal domain (ATD) of the NR1 subunit of NMDAR finally leads to excitotoxicity. In this study, we assessed the interaction of two new chimeric reteplase, mutated in the kringle2 domain, with ATD and compared the interaction of wild-type reteplase with ATD, computationally.

Experimental approach: Homology modeling, protein docking, molecular dynamic simulation, and molecular dynamics trajectory analysis were used for the assessment of this interaction.

Findings/Results: The results of the free energy analysis between reteplase and ATD (wild reteplase: -2127.516 ± 0.0, M1-chr: -1761.510 ± 0.0, M2-chr: -521.908 ± 0.0) showed lower interaction of this chimeric reteplase with ATD compared to the wild type.

Conclusion and implications: The decreased interaction between two chimeric reteplase and ATD of NR1 subunit in NMDAR which leads to lower neurotoxicity related to these drugs, can be the start of a way to conduct more tests and if the results confirm this feature, they can be considered potential drugs in acute ischemic stroke treatment.

Keywords: Chimeric reteplase; Docking; Molecular dynamic simulation; Excitotoxicity; Neurotoxicity.

INTRODUCTION

Plasminogen activators (PAs) are a group of serine protease enzymes that can fibrin clot lyses by converting plasminogen to plasmin. Following the formation of vascular thrombi, the epithelial cells secret tissue plasminogen activators (tPA) that bind to fibrin and plasminogen as a tertiary complex. Then, tPA cleaves Arg/Val bond in the plasminogen and converts it to plasmin. Finally, with the fibrinolytic action of plasmin, the fibrin clot is degraded. PAs are utilized in several disorders such as pulmonary embolism, myocardial infarction, and acute ischemic stroke that are linked to clot formation (1). So far, three generations of PAs have been developed. The first generation (urokinase and streptokinase) lacks fibrin specificity (2), a weakness that increases the risk of hemorrhage and plasminemia (3).

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Alteplase is a recombinant form of tPA that belongs to the second generation of PAs, a fibrin-specific medication. For the treatment of patients with pulmonary embolism, acute myocardial infarction, and acute ischemic stroke, the Food and Drug Administration (FDA) has approved alteplase (2). The finger domain, the epidermal growth factor, the kringle1 (K1) and kringle2 (K2) subunits and the protease domain are the five domains that constitute the alteplase. It has also three glycosylation sites. The finger domain and K2 play roles in fibrin binding (4,5). However, alteplase demonstrates fibrin specificity as a favorite feature compared to the first generation of PAs, it has certain disadvantages, including a short half-life, the possibility of hemorrhage, also neurotoxicity such as excitotoxicity (6-8). Alteplase and every plasminogen activator with a K2 domain can cause excitotoxicity as a side effect (8,9). Alteplase binds to NR1 subunit from N-methyl-d-aspartate glutamate receptors (NMDAR) in brain parenchyma *via* the lysine binding site that includes aspartate 148 and tryptophan 253. Then, by cleaving the amino-terminal domain (ATD) of this subunit, calcium influx to the neural cells increases which ultimately results in excitotoxic necrosis (Fig. 1). Despite this side effect, alteplase is still the only FDA-approved medication for the treatment of ischemic stroke (10). However, research continues to find more effective drugs. Reteplase, tenecteplase, and desmoteplase are examples of the third generation of PAs that were developed to improve favorable features, such as enhancing fibrin specificity and expanding half-life. Among these drugs, reteplase has no essential glycosylation site, so it can be produced in *Escherichia coli* as a cost-effective production approach (11). Reteplase is a truncated form of alteplase that only has K2 and serine protease domains, compared to alteplase, so it has a longer half-life (11). Reteplase has been approved for acute myocardial infarction but not for acute ischaemic stroke (11). Since the greater fibrin specificity, is one of the main goals for designing novel drugs for acute ischemic stroke treatment (12), the lower fibrin specificity of reteplase compared to alteplase is a disadvantage that can hinder its success as a candidate in treating this disorder. Also, it can exhibit an excitotoxic effect in the presence of its K2 domain (1). Nevertheless, due to its desirable properties, including a prolonged half-life and cost-effective production procedure, reteplase can be improved with the aid of protein engineering to increase fibrin selectivity and reduce excitotoxicity. Previously, we designed new chimeric reteplases (M1-chr and M2-chr) with some desirable features such as enhanced fibrin affinity (13). These two chimeric reteplases are consisting of mutations in the lysine binding site of the K2 domain to decrease its interaction with the NR1 subunit. These mutations (D102N, D104N, and W119N for M1-chr and D102A, D104N, and W119N for M2-chr) decrease the drug's neurotoxicity without disrupting its fibrinolytic activity (14). Therefore, they can be potential candidate for acute ischemic stroke treatment. In this study, the interaction between these chimeric reteplases and the ATD of NR1 was assessed using a computational technique. Molecular dynamics (MD) simulation is a relevant computational tool in the biomolecules study because of its predictive potency and trustworthy analysis of the structure, functions, and dynamics of proteins (15,16). Furthermore, MD simulation is an accurate method in conception of the correlation between the proteins *via* the simulating of protein-protein interactions in the presence of water and analysis of the features in this interaction (17-19).

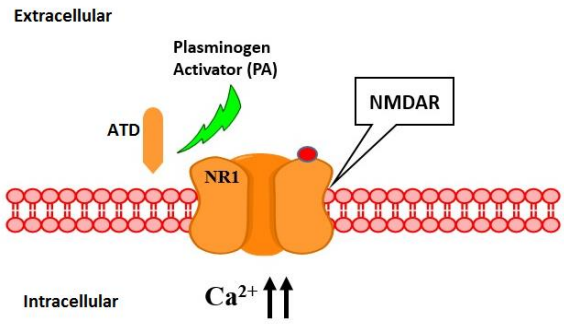

Fig. 1. The interaction between the kringle 2 domain of plasminogen activators such as reteplase with ATD of NR1 subunit in NMDAR leads to cleavage and increasing influx of Ca^{2+} and high levels of this ion in neurons. ATD, Amino-terminal domain; NMDAR, N-methyl-d-aspartate glutamate receptors.

MATERIALS AND METHODS

Homology modeling

In order to obtain an exact assessment of the interaction between chimeric reteplase and wild reteplase with the ATD of NR1, the three-dimensional structure of this domain in the site of interaction (20) was constructed by Modeller 9.17 software. PDB ID 6MM9 was used as a template and among 1000 constructed PDB models using Modeller software, the model with the lowest discrete optimized protein energy (DOPE) value was considered the best model. This 3D structure was improved by MD modeling and prepared for protein docking.

Protein docking

Interaction between the ATD subunit with M1-chr, M2-chr, and wild reteplase was assessed by protein docking *via* the HADDOCK web server (21). The docking process in HADDOCK web tools is directed by ambiguous interaction restraints, which are obtained from the available experimental data on the residues involved in the intermolecular interaction (22). Given the site of interaction (20), for ATD residues 230-255, wild-reteplase residues 60-96, and chimeric reteplase residues 98-134 were considered as active radiuses, whereas residues neighboring were used as passive residues in the docking procedure.

MD simulation

The structure of wild and chimeric reteplase was simulated in the previous study (13). In this study, the GROMACS (version 5.5.1) software package was used to simulate the 3D structure of ATD and its protein complex with reteplase. This simulation used the GROMOS96 43a1 force field and a basic point charge model of water as the solvent using 3D periodic boundary conditions. A 10 Å distance between the box's borders and the protein's surface was taken into account in the triclinic solvation box. Depending on the overall charge of each protein, Cl ions are added to the system to neutralize it as much as necessary. The initial phase was utilizing the steepest descent algorithm to minimize energy use. The system was calibrated in the second phase using a 500 ps MD simulation in the canonical (NVT) ensemble at 100 Kelvin (K) and a 2000 ps MD simulation in the isothermal-isobaric (NPT) ensemble at the same temperature. To have a better structure, NPT was achieved with a temperature coupling constant and pressure coupling constant of 0.1 and 1 ps, respectively. In this phase, the pressure was set using the Berendsen technique and the linear constraint solver algorithm was utilized to manage the bond lengths in interactions with the H atoms. In the production phase, 20 ns for ATD and 50 ns for protein complexes, simulation was performed with a duration time of 0.001 ps in 300 K temperature systems. The temperature was set at 300 using the Nose-Hoover thermostat and the pressure was maintained at 1 bar using the Parrinello-Rahman pressure coupling method. The cutoff for electrostatic and van der Waals interactions was specified as 12 Å.

MD trajectory analysis

To analyze docking complexes, the interaction between ATD and each reteplase was analyzed after simulation. These analyses are including root mean squared fluctuation (RMSF), root means squared deviation (RMSD), average solvent accessible surface area (SASA), the radius of gyration (Rg), the minimal distance between two proteins in each docking complex, number of contacts between residues of two proteins in each docking complex, the total number of intermolecular hydrogen bonds formed between two proteins (23-25). Also, The molecular mechanics Poisson-Boltzmann surface area (MMPBSA) method was used which has been broadly applied as an effective and trustworthy free energy simulation method for the assessment of molecular interactions like protein-ligand binding or protein-protein interactions (26).

RESULTS

Homology modeling

Using the homology modeling, several models were presented by the Modeller software for ATD of the NR1 subunit, which was selected based on the lowest DOPE value, and this model was then optimized through MD simulation (Fig. 2).

Before the MD simulation of ATD structure, the assessment of this structure with the PDBsum web server (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html), showed that 86.4% of residues are located in the most favored regions of Ramachandran plot which indicated a usual structure (Fig. 3)

Molecular docking

Three structures of reteplase (M1-chr, M2-chr, and wild type) were docked with ATD by the HADDOCK web server. The docking procedure was performed in the site of the K2 domain of reteplase and around arginine 260 of ATD (20). Docking results showed that the M1-chr interacted better than the M2-chr and even the wild type, with ATD. But the interaction of M2-chr with ATD was weaker than the others (Table 1).

Figure 4 illustrates the schematic of docked complexes and the amino acids involved in the interaction. In particular, the three amino acids asparagine 102, 104, and 119 of chimeric reteplase are found in the interaction between them and the ATD of the NR1 subunit.

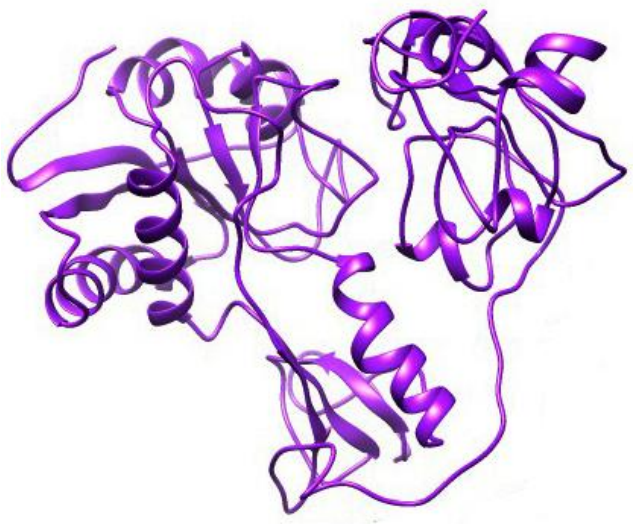

Fig. 2. The best model for the *a*mino-terminal domain from the NR1 subunit was obtained through modeller and optimized by molecular dynamics simulation.

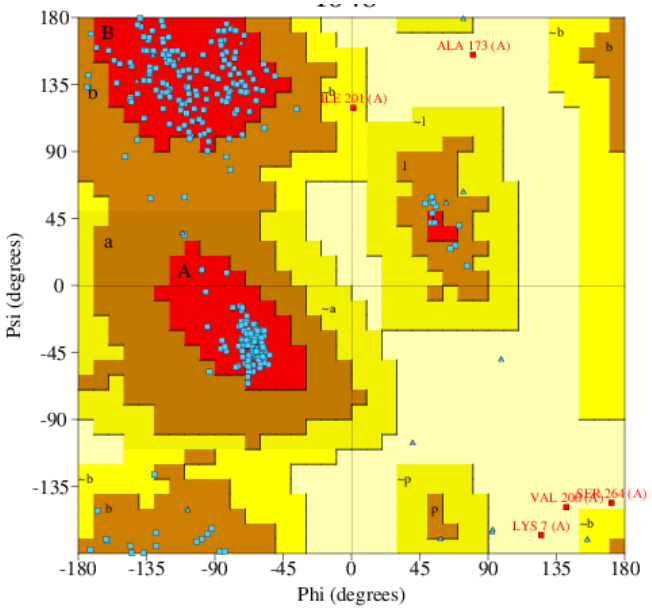

Fig. 3. Ramachandran plot of amino-terminal domain structure.

Table 1. The molecular docking results between ATD of the NR1 subunit of the NMDA receptor and reteplase (wild and mutants).

	Wild reteplase-ATD	M2-chr_ATD	M1-chr_ATD
HADDOCK score	-61.6 ± 3.3	-36.0 ± 5.3	-78.8 ± 2.1
Cluster size	268	113	47
RMSD from the overall lowest-energy	0.6 ± 0.4	0.5 ± 0.3	0.7 ± 0.4
Van der Waals energy	-95.3 ± 8.0	-97.7 ± 2.7	-101.4 ± 3.2
Electrostatic energy	-371.4 ± 60.6	-339.8 ± 34.5	-392.8 ± 44.7
Desolvation energy	11.1 ± 9.0	33.8 ± 6.1	41.5 ± 11.7
Restraints violation energy	969.0 ± 77.77	958.6 ± 23.30	595.8 ± 34.52
Buried Surface Area	3423.6 ± 168.1	2691.8 ± 88.2	2855.5 ± 94.0
Z-Score	-1.7	-1.3	-1.7

ATD, Amino-terminal domain; NMDAR, N-methyl-d-aspartate glutamate receptors; RMSD, root means squared deviation; M-chr, chimeric reteplases.

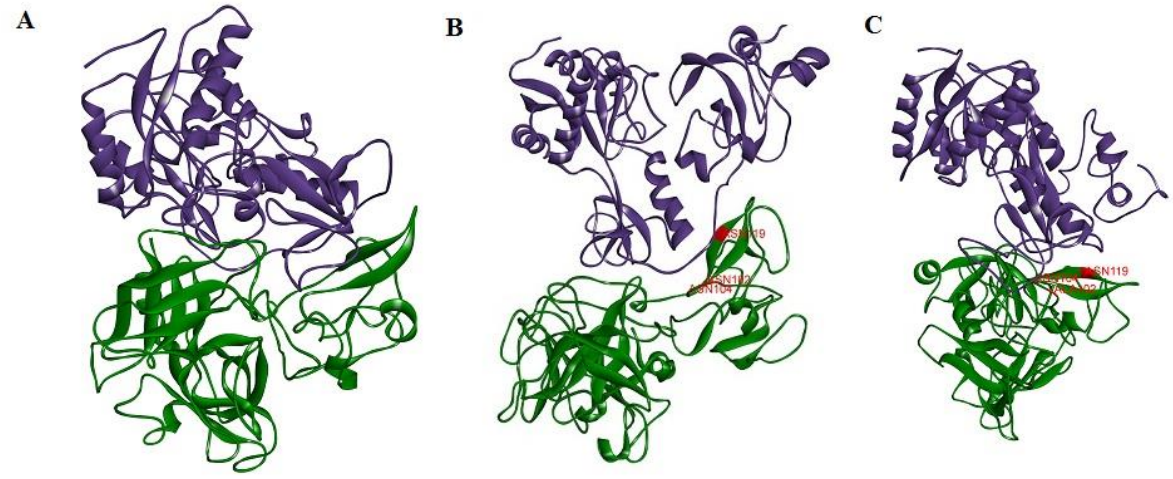

Fig. 4. Schematic representation of docked complexes including (A) wild reteplase complex; (B) M1-chr; and (C) M2-chr. The green structure represents reteplase and the purple structure represents the amino-terminal domain of the NR1 subunit of the N-methyl-d-aspartate glutamate receptors receptor, M-chr, chimeric reteplases.

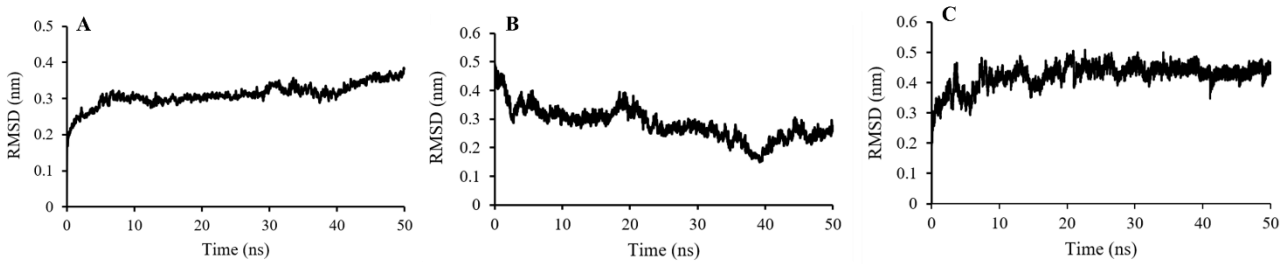

Fig. 5. RMSD plot analysis of wild-type and mutants of reteplase. (A) Wild-type form; (B) M1-chr; and (C) M2-chr. RMSD, root means squared deviation; M-chr, chimeric reteplases.

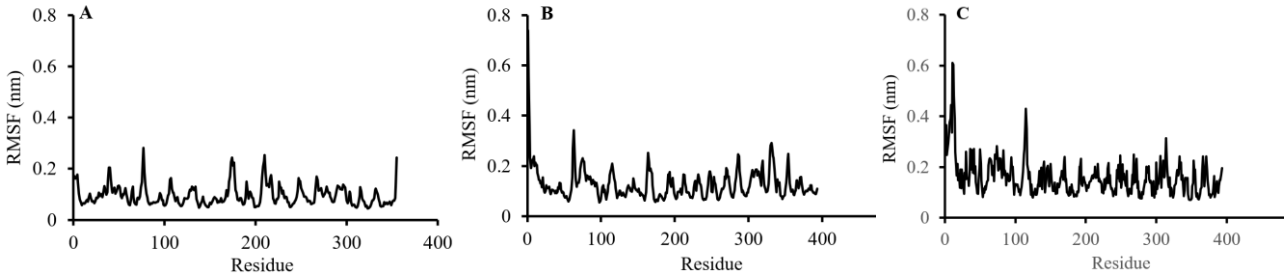

Fig. 6. Backbone RMSF of wild-type reteplase and mutants. (A) wild-type form; (B) M1-chr; and (C) M2-chr. The score of RMSF related to critical residue in the interaction is marked on each graph. RMSF, Root mean squared fluctuation; M-chr, chimeric reteplases.

MD simulation

MD simulation was performed for the accurate assessment of protein-protein interaction. All three complexes were examined for 50 ns. According to RMSD analysis, the complexes reached a stable status after 10 ns (Fig. 5).

The mean values of RMSD for 20% of the final nanoseconds were 0.34 ± 0.01, 0.24 ± 0.02, and 0.43 ± 0.01 for wild-type, M1-chr, and M2-chr, respectively.

When a simulation is equilibrated, the protein structure fluctuates around a stable average conformation. The computation of the fluctuations relative to the reference structure and the number of atoms showed residue flexibility as RMSF. Figure 6 showed the RMSF graph of reteplase for each of the complexes. The score of RMSF related to critical residue in the interaction is mentioned on each graph. The value of two residues in chimeric reteplase was higher compared to the

wild-type corresponding residues. Also, the Rg of all three does not show many changes over time, which indicates the compactness and relative stability of the proteins (Fig. 7). These parameters and other parameters for all three complexes are summarized in Table 2.

MMPBSA analysis

The interaction-free energies between two moieties of the complexes were evaluated by MMPBSA as an efficient method to determine structural stability and predict binding affinities. Table 3 shows different energies between wild type and both chimeric reteplases and ATD of NR1 subunit also their total binding energy. The result indicates that the total binding energy of wild reteplase and ATD (-2127.516 ± 0.0 kJ/mol) was stronger than the binding of two chimeric reteplases with this domain. However, the M2-chr_ATD complex showed lower binding energy than the M1-chr_ATD complex, indicating that M2-chr establishes weaker interaction with the NR1 subunit and can be a better choice with lower neurotoxicity.

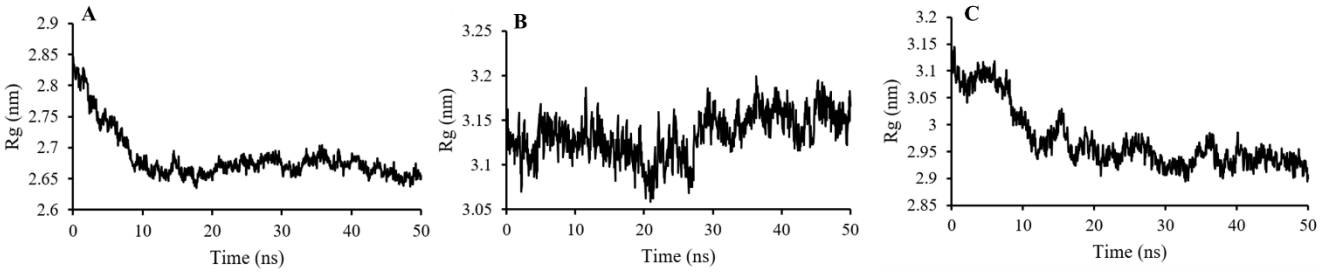

Fig. 7. The Rg of (A) M1-chr_ATD complex; (B) M2-chr_ATD complex; and (C) wild reteplase_ATD complex. Rg, radius of gyration; M-chr, chimeric reteplases; ATD, Amino-terminal domain.

Table 2. The average parameters of molecular dynamics analysis for wild-type, M1-chr_ATD complex and M2-chr_ATD complexes.

Analysis	Wild reteplase-ATD	M1-chr_ATD	M2-chr_ATD
RMSD reteplase (nm)	0.34 ± 0.01	0.24 ± 0.02	0.43 ± 0.01
RMSD NR1 subunit (nm)			
RMSF reteplase (nm)			
RMSF NR1 subunit (nm)			
The sum of electrostatic and van der Waals energy between reteplase and NR1 (KJ/mol)	-1784.68	-1513.25	-771.83
The minimum distance between reteplase and NR1	0.16 ± 0.0	0.16 ± 0.0	0.14 ± 0.0
Number of contacts between reteplase and NR1	568 ± 32	446 ± 15	462 ± 15
Number of hydrogen bonds between reteplase and	26 ± 3	18 ± 3	19 ± 3
hydrogen bonds between complex protein and solvent	1195 ± 23	1309 ± 23	1244 ± 23

RMSD, root means squared deviation; M-chr, chimeric reteplases; ATD, Amino-terminal domain.

Table 3. The calculated free energies of binding (kJ/mol) of the reteplase and NR1 subunit of the N-methyl-d-aspartate glutamate receptor using molecular mechanics Poisson-Boltzmann surface area.

Analysis	Wild reteplase-ATD	M1-chr_ATD	M2-chr_ATD
Van der Waal energy (kJ/mol)	-1084.37	-776.40	-546.90
Electrostatic energy (kJ/mol)	-2867.25	-2607.08	-626.83
Polar solvation energy (kJ/mol)	1930.16	1693.30	704.83
Solvent accessible surface area energy	-106.05	-71.33	-52.99
Binding energy	-2127.51	-1761.51	-521.90

M-chr, Chimeric reteplases

DISCUSSION

Despite the fibrinolytic effect, tPA displays life-threatening activity in the brain, such as excitotoxicity, that can diminish the overall advantage from thrombolysis during the stroke. Regarding the complications related to tPA, the next generations of this drug were designed to improve as much as possible. One of them is that reteplase has a prolonged half-life than tPA. Because of the K2 domain involved in the interaction of these proteins with NMDAR and the indication of an excitotoxic effect (27), reteplase can have such an effect on the brain. Previously we designed two new chimeric reteplase (M1-chr and M2-chr) with enhanced fibrin affinity which can be potential drugs for ischemic stroke with some other desirable properties in particularly decreased neurotoxicity. These chimeric reteplase consist of three mutations in the K2 domain which disrupts their interaction with the NR1 subunit. In this study, we examined the interaction of these two chimeric reteplase with ATD of NR1 subunit *in silico*. The 3D structure of ATD was constructed at the site of interaction according to the sequence reported by Fernández-Monreal and co-workers (20). The interaction was assessed with the HADDOCK web server. This docking result showed weaker interaction (greater HADDOCK score) between M2-chr and ATD compared to M1-chr and wild type (-36 ± 5.3 compared to -78.8 ± 2.1 and -61 ± 3.3, respectively), but the wild type score was greater than M1-Chr. For a more accurate assessment of these interactions, an MD simulation analysis was performed. According to the various analysis with MD, both mutants showed weaker interaction with ATD compared to wild type. However, M2-Chr showed the weakest interaction with ATDPreviously, some methods were conducted to reduce the neurotoxicity caused by the use of tPA (as an ischemic stroke drug). Macrez and coworkers used polyclonal immunoglobulins against the aminoterminal domain of the NMDAR NR1 subunit (termed αATD-NR1) in mice. They demonstrate that this immunotherapy leads to decreasing excitotoxic damage, BBB leakage, and related brain injury and neurological debits (28). Parcq *et al.*, designed some mutated tPA in the kringle2 domain to interfere in tPA interaction with the NR1 subunit they reported these mutants to show fibrinolytic activities similar to that of wild-type tPA without promoting NMDAR-mediated neurotoxicity (14). A non-neurotoxic tPA variant was also designed by Goulay and his coworker. This mutant was named OptPA and contained two single-point mutations, a substitution R275S to prevent the processing of tPA by plasmin (sc-tPA to tc-tPA), and a second substitution (W253R) in the K2 domain to prevent its interaction with NMDA receptor. Their results showed that this mutant prevent excitotoxicity and also improved neurobehavioral effects in intracerebral hemorrhage pig models (14). In another study, it was reported that applying an NMDAR antagonist which is much more selective to the NR2D unit, can rectify tPA-enhanced excitotoxicity in a thrombotic stroke model in mice, effectively. Administration of UBP145 prevented the deleterious effect of late thrombolysis by tPA (29). Our result also showed weaker interaction between chimeric reteplase and ATD of NR1 subunit compared to wild reteplase. RMSF value which is indicating residue fluctuation can show variability in the residues involved to the interaction. High value in RMSF indicates that such amino acids do not create strong interactions with the receptor (30). In our study we performed mutation in residues in lysine binding site of K2 domain which are involved to the interaction with ATD (D102N, D104N, W119N for M1-chr and D102A, D104N, W119N for M2-chr). The results of RMSF showed an increase in value of two residues in chimeric reteplase compared to wild type corresponding residues (Fig. 2). Since the mutations are designed to disrupt the interaction between the chimeric ratplases and the receptor, this can be an expected result. Decreasing in the number of hydrogen bonds between reteplase and ATD in the case of two chimeric reteplase compared to wild type (18 ± 3 in M1-chr, 19 ± 3 in M2-chr and 26 ± 3 in wild type reteplase) and the number of contacts (446 ± 15 in M1-chr, 462 ± 15 in M2-chr and 568 ± 34 in wild type reteplase) can also indicate less interaction between two chimeric reteplase and ATD of NR1 subunit. Finally, more positive

binding energy for chimeric reteplase and ATD calculated in MMPBSA analysis confirmed weaker interaction between them compared to wild type reteplase (-1761.510 ± 0.0 for M1-chr, -521.908 ± 0.0 for M2-chr and -2127.516 ± 0.0 for wild type reteplase).

In a previous study, we showed the enhanced fibrin affinity of these mutants compared to wild reteplase (13). According to the advantage of reteplase compared to tPA for example prolonged half-life and the possibility of affordable production in bacteria, these mutants are designed to be a potentiate drug for ischemic stroke. The present study showed these mutants can be considered safe fibrinolytic drugs in terms of neurotoxicity and considered potential alternatives to tPA in ischemic stroke treatment. However, an experimental study is suggested to prove this claim.

CONCLUSION

In this study, we assessed the interaction of two new chimeric reteplase with NMDA receptors. These mutants have been designed to have lower interaction with the receptor leading to lower excitotoxicity. Results showed such decreased interaction between mutated reteplase and NMDA receptor compared to wild type. The experimental assessment also proposed verification of this feature and considering these chimeric reteplases a potential cost-effective drug in the treatment of ischemic stroke.

Acknowledgments

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

P. Mohammadipour wrote the article; K. Mahnam conducted scientific guidance and revised the manuscript; M. Taherzadeh contributed to article writing and editing; Sh. Ahangarzadeh contributed to article writing and editing; A. Alibakshi contributed to MD simulation analysis and visualization of results; E. Mohammadi conceptualized the project and performed MD simulation and analysis. The finalized article was approved by all authors.

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