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Original Article

Trypanothione Reductase Gene Mutations in Meglumine Antimoniate Resistant Isolates from Cutaneous Leishmaniasis Patients Using Molecular Dynamics Method

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Abstract

Background: In this study, mutations in the tripanothion reductase of *Leishmania tropica* isolated from Iran was investigated using sequencing and simulation of the enzyme by the molecular dynamic method.

Methods: Fifteen susceptible and 15 clinical resistant *L. tropica* specimens were collected from skin lesions from different regions of Iran in 2017. After DNA extraction, trypanothione reductase (*TRYR* or *TPR*), gene fragment was amplified using PCR and sequencing methods. In the case of structural mutations, the components were simulated by molecular dynamics using the GROMACS software.

Results: Some structural mutations were observed in 9 amino acids surrounding the active site of the *TRYR* gene of *L. tropica* with three-dimensional trypanothione reductase alteration.

Conclusion: Change in the active site of *TRYR* of *L. tropica*, could probably contribute to the development of resistant *L. tropica* to glucantime. Because of the likely occurrence of mutations in glucantime as well as the ease of development of *L. tropica* resistant populations, more samples are needed to demonstrate the relationship between mutations in this enzyme and clinical resistance to glucantime. On the other hand, it is recommended that enzymatic studies be performed to confirm the role of mutation in the function and expression of trypanothione reductase in glucantime resistant and susceptible populations.

Introduction

Pentavalent antimonial drugs, i.e. meglumine antimony (glucantime) and sodium stibogluconate (pentostam) have been known as the first line for the treatment of leishmaniasis in Iran (1-3). In the recent years, resistance to glucantime have been reported in Iran (1-3). In drug resistance, a significant reduction in drug sensitivity among a particular microbial population was observed due to the natural selection of a strain, which is genetically distinct from the wild strain (4-6). If there is no sign of recovery in the lesion, after 2 weeks of completion of local or systemic treatment with glucantime, it is considered the failure of treatment. If the systemic treatment failed or relapsed up to twice, it is considered as a case of clinical resistance (1-3). In the acquired resistance to glucantime in *Leishmania*, the ease of obtaining resistance to drugs and the extension of the drug resistant strains has great important role (6). It is possible that *Leishmania* resistant strains against glucantime caused by new mutations in the structure of the *Leishmania* gene (7).

For the mechanism of drug resistance to meglumine antimonate, it is assumed that the drug is an inactive pentavalent compound fed into the body (8) and reacts with thiols to form a non-enzymatic mechanism in the host macrophage or in the parasite itself. (9, 10). The Thiol-dependent reductase (TDR.1) and antimoniate reductase (ACR2) enzymatic mechanisms reduced it from pentavalent to trivalent form Sb(III) (11, 12), which in the host macrophage cannot be sufficiently reduced, and intracellular mechanisms, whether enzymatic or non-enzymatic are necessary. Glucantime has a high affinity to react with thiol groups in the pentavalent form. It can be seen in the levels of enzymes in the form of cysteine amino acid or in the form of TSH2 or GSH, which leads to the formation of stable complexes T-Sb and Sb (GS). Reaction with thiol-containing groups lead to the release of thioles from the cell or to prevent their entry into the cells involved in drug resistance (8, 13-17). In each form, if the drug is injected into the cell, it can react with the compounds of the zinc finger domain, which causes the zinc ion to escape and induces apoptotic death by the drug, or inhibited formation of more glutathione-spermine conjugate complexes, trypanothione (T(SH)2), by reduction of trypanothione reductase enzyme (8, 18).

In the *Leishmania* a protein called trypanothione reductase is associated with the action of the glucantime drug (13). In enzymology, this protein acts on the basis of thiol metabolism. It can donate the electron from NADPH to trypanothione sulfide T(S) 2 and reduce it to trypanothione T(SH)2, based on its enzymatic property (13). Trypanothione in the depressed state is essential for parasite life; it prevents the production of oxidative stress in the parasite. A mutation in the gene structure of this enzyme is likely to play an important role in the development of drug resistance (8, 13-17).

Mutations in the tripanothion reductase of *L.tropica* isolated from cutaneous leishmaniasis patients in different parts of Iran was investigated using sequencing and simulation of the enzyme by the molecular dynamic method.

Materials & Methods

Ethics approval

The study protocol was reviewed and approved by Medical Ethic Committee of Tarbiat Modares University, Tehran, Iran (IR.TMU.REC.1394.868). Informed consent was taken from all participants.

Sampling, DNA extraction

Totally, 15 clinical samples sensitive to glucantime and 15 clinical samples resistant to

glucantime were collected from the skin biopsies of the ACL patients, in the form of stained smears from different geographical locations in Iran such as Mashhad, Kerman, Esfahan and Ilam in 2017. The stain of the smear was removed in alcohol 96%; then, they were collected from the slides using the distilled water. Then, 200 µl of each sample were put in a 1.5 ml tube. Finally, the DNA genetic material was extracted using the GeneAll® Exgene TMCell SV kit (GeneAll Biotechnology Co. Ltd., Seoul, South Korea).

Primers Designing

To design degenerate primers, Fasta files of the desired gene part were extracted from the NCBI. Then, it was blasted and similar sequences of this piece were saved in fasta form with highly overlapping, and they were aligned with the Mega 6 program with clustal-W and the 4 primers were designed based on the similarity in degenerate form. The total length of the fragment is 1580bp, the length of the upstream fragment was 743bp and the length of the downstream fragment was 958bp, which are overlapping in 121 bp. The forward and the reverse primers of upstream fragment were 5'-CCRGCYRTCAAAGACKMGCTCTTGC-3' and 5'-CTTCGTCAGGCTCTTGCGCACCTC–3' and the forward and the reverse primers of downstream fragment were 5'-TTGAGTTTGCCGGCATCTTCAACG–3' and 5'-GCATGCCTGCTGGTCTGTGATACC-3' respectively. Thermal cyclic conditions for priming PCR was initially denaturalized at 95 °C for 5 min; then, 40 cycles with a denaturing temperature of 95 °C for 30 sec, an annealing temperature of 61 °C for 20 sec, an extensional temperature of 72 °C for 60 sec and finally the extension at a temperature of 72 °C for 10 min were conducted.

Sequencing

Five uL of PCR product was electrophoresed on a 1% agarose gel in Tris-Acetic acid-EDTA (TAE) and visualized the band using ultraviolet illuminator. Then, all PCR products were sequenced by the ABI3730XL sequence analyzer (Macrogen, Korea). The species of *Leishmania* are confirmed by DNA sequencing.

Computational studies

Modeling, modification and molecular dynamics

DNA sequences were aligned for glucantime sensitive and resistant isolates of *L. tropica* and translated into protein sequences using the MEGA 6 software. Protein sequences obtained from the glucantime-sensitive and resistant *Leishmania* samples were processed using the SWISS MODEL software, which compared the target protein structure with three-dimensional homologous proteins known in their PDB file. The software modeled and presented several models with the highest overlapping with the existing structures as PDB. The trypanothione reductase PDB file of the glucantime-sensitive and resistant isolates was used to initiate molecular dynamics in GROMACS software. To re-evaluate the molecular movement, the structure obtained as PDB was simulated using the GROMACS software in the water solvent, so that the stability of the model structure was evaluated in the natural environment of protein through a more precise modelling the co-reactions between proteins and water molecules. Before starting the simulation, the energy minimization process was performed to eliminate the structure of the Van der Waals interactions that are inadequate in terms of energy, and allow the structure and environment to establish suitable interactions such as electrostatic bonding.

In the next step, simulations were initially performed in NVT conditions (number of particles, constant volume and temperature) for 5 ns of NVT temperature equilibration to 300 K. Then in order to achieve equilibrium pressure, the simulation was carried out in NPT conditions (particle number, constant

pressure and temperature) for 5 ns, and the components of the system were placed under a constant pressure of 1 bar. Finally, to verify the stability of the model and to ensure its equilibrium in the solution, the Root Mean Square Deviation RMSD charts were plotted and compared to alpha carbon at 20 ns. The RMSD is the most commonly used quantitative measure of the similarity between two superimposed atomic coordinates.

Determination of the active site and geometric distances

Based on the crystallographic structure of antimony ion of trypanothione reductase protein related to the *L. infantum*, the active site of antimony ion of the target protein sequences of both sensitive and resistant isolates of *L. tropica* were simulated by YASARA software. The software measures the distance between the alpha carbon (CA) from the residues surrounding antimony ion in modulated trypanothione reductase protein in sensitive and resistant isolates of *L. tropica* to glucantime.

Investigate the radius of gyration in the active site

To calculate the radial distribution around a bunch of atoms or the mass center of a bunch of atoms, the gyration radius of the active site and whole protein was determined in both of sensitive and resistant *L. infantum*. For this purpose, all of the residue was considered in both sensitive and resistant isolates in the 6 angstroms surrounding antimony (Fig. 1).

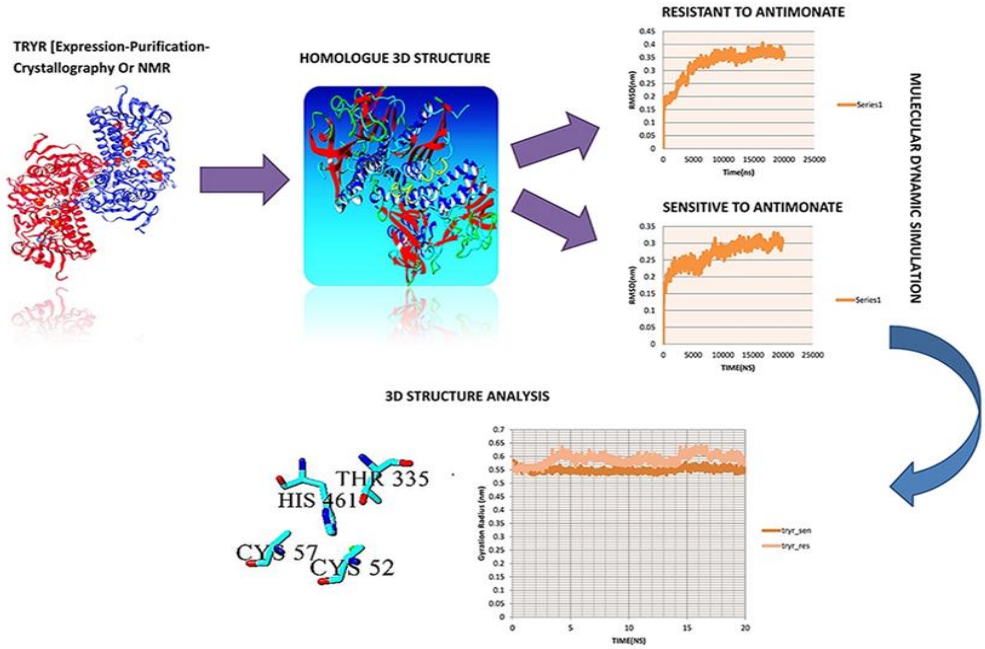

Fig. 1: The summary of different procedures of present study (Original figure)

Results

Detection of mutation result

Following sequencing of 15 glucantime-resistant and 15 glucantime-sensitive samples and translation into protein sequences related to the desired fragment of the *TRYR* gene with a length of 491 amino acids, their data were compared with the sequence of the protein associated with trypanothione reeducates *L. infantum* (Protein_ID=AAS73185.1) with a length of 491 amino acids.

Among 15 resistant isolates of *L. tropica* 4 isolates (26.67%) show structural mutations in

the TYPE protein. In Table 1 geographic location of glucantime-resistant samples with structural mutations is shown. In the resistant isolates, some structural changes were seen in 9 amino acids surrounding the active site of the *TRYR* protein of *L. tropica* (Table 2).

Table 1: Frequency of clinical glucantime-resistant samples with structural mutations collected from different cities in Iran

City	*Frequency*	*Percentage*
Mashhad	1	25
Esfahan	2	50
Kerman	1	25
Total	4	100

Table 2: Frequency of mutant amino acids in clinical cutaneous leishmaniasis resistant to glucantime samples

Amino Acid	*Frequency*	*Percentage*
Arginine (Arg)	4	16.66
Alanine (Ala)	4	16.66
Valine (Val)	4	16.66
Aspartic Acid (Asp)	4	16.66
Glycine (Gly)	4	16.66
Serine (Ser)	4	16.66
Total	24	100

The change is in the form of amino acid replacement. The replacements were conducted in resistance isolates compare to the sensitive isolates as follows: the amino acid of arginine (Arg) at position 28 instead of the amino acid of lysine (Lys); the amino acid of alanine (Ala) at the 36 position instead of the amino acid of valine (Val); Val instead of the amino acid of alanine (Ala) at the 38 position; the aspartic acid amino acid(Asp) instead of the amino acid of Glycine at the position 41; aspartic acid amino acid (Asp) replaces Gly at the position 105; Gly replaces the amino acid of the argentine in the place of 138; amino acid serin (Ser) replaced by Gly at the position 209; the amino acid aspartic acid (Asp) replacing the amino acid asparagine (Asn) at position 385 and histidine amino acid (H) at the position 472 replacing the ARG in sensitive *L. tropica* isolates (Fig. 2 and Table 3).

Table 3: Amino acid replacement in different positions of trypanothione reductase protein of glucantime-resistant *L. tropica* isolates compare to sensitive isolates.

Amino acids in sensitive isolates		*Amino acids in resistant isolates*		*Position*
Lysine	K	Arginine	R	28
Valine	V	Alanine	A	36
Alanine	A	Valine	V	38
Glycine	G	Aspartic Acid	D	41
Glycine	G	Aspartic Acid	D	105
Arginine	R	Glycine	G	138
Glycine	G	Serine	S	209
Asparagine	N	Aspartic Acid	D	385
Arginine	R	Histidine	H	472

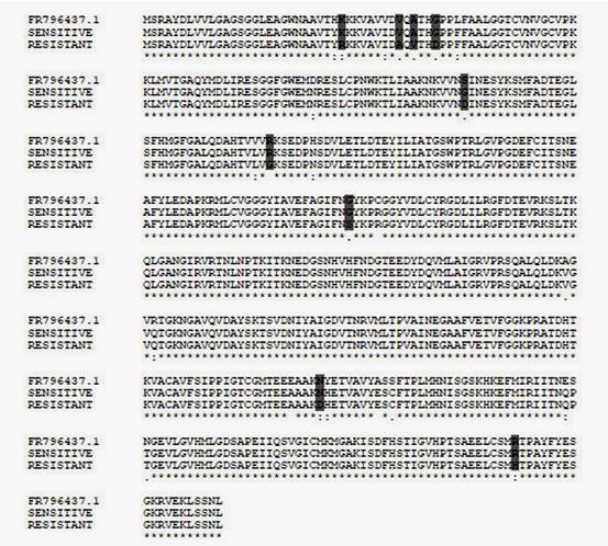

Fig. 2: Multiple alignment of protein sequences related to *TRYR* gene fragment from glucantime resistant and sensitive isolates of *L. tropica* compared with protein sequences related to *TRYR* gene fragment of *L. infantum* (FR796437)

Computational study result

Models made from the *TRYR* gene protein sequence, obtained from sensitive and resistant isolates to glucantime, were approximately 98% similar to the tripanotion reductase protein (Fig. 3).

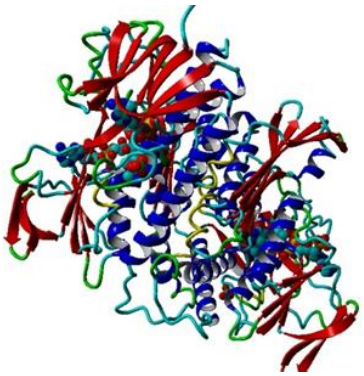

Fig. 3: Three dimensional structure of *Leishmania tropica* (PDB cod:2w0h)

Concerning the crystallographic structure, the antimony ion is geometrically located in the center of a tetrahedral quadrilateral geometry and the corners of this tetrahedral are atoms of SG Cys 52 (2.8Å), Cys 57 (3.0 Å), OG1 the Thr 335 (3.0 Å) amino acid from the first chain and the ND1 atom of the His 461 (3.2 Å) amino acid, which was related to *L. infantum*. The target proteins were introduced into YASSARA software in both sensitive and resistant isolates of *L. tropica* and determined the position of antimony ion included of CYS 52, CYS 57, THR 335 and HIS 461 (Fig. 4). The average distance between the alpha carbon of the CYS 52, CYS 57, THR 335, HIS 461, in glucantime-resistant isolates is 7.6 A, and among the sensitive isolates, the total average is 6.7 A; that is about 0.9 A wiser in active position.

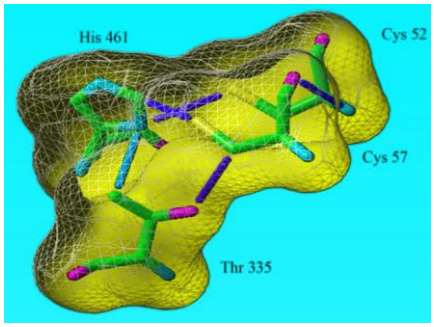

Fig. 4: Simulated active site of antimony ion in *L. tropica* isolate. The target proteins were introduced into YASARA software in both sensitive and resistant isolates of *L. tropica* and determined the position of antimony ion included of Cys 52, Cys 57, Thr 335 and His 461

After modelling with the Swiss Model Software, the basic simulation was conducted for 20 nanoseconds, in order to verify the stability of the model and to ensure its equilibrium in the solution. The RMSD charts were plotted for alpha carbon at 20 nanoseconds and compared (Fig. 5). The analysis of the radius of gyration also revealed a constant increase in the radius of gyration at about 0.9 A in all simulation time scales. Changes in the radius of gyration around the antimony ion were calculated for both resistant and sensitive isolates. For this purpose, all the residues found in 6 A around antimoan were considered. The changes in the around the antimony position were simulated for resistant isolates to glucantime with the increase of gyration radius and for sensitive isolates, almost without the radius change.

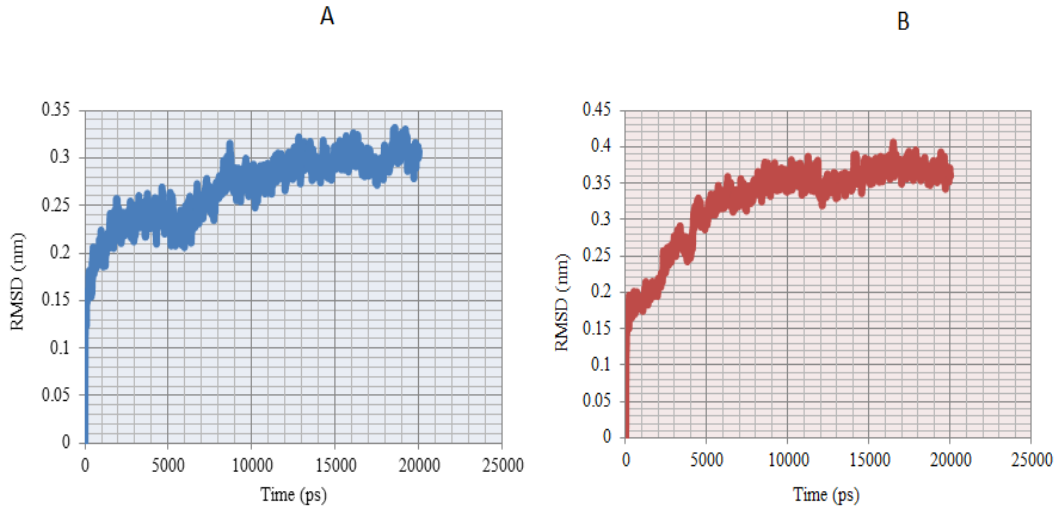

Fig. 5: Root Mean Square Deviation (RMSD) change of trypanothione reductase protein in glucantime-resistant *L. tropica* isolates during simulation (A). RMSD change of trypanothione reductase protein in glucantime-sensitive *L. tropica* isolates during simulation (B). To verify the stability of the model and to ensure its equilibrium in the solution, the RMSD charts were plotted and compared for alpha carbon at 20 ns

Discussion

Some studies in Iran have attempted to explore the genes involved in the resistance of *Leishmania* to glucantime (19-24). It has been shown that glutathione cysteine synthetase encodes genes to increase in arsenitis-resistant *L. tarentolae*, which expresses the role of thiols in drug resistance (11). Other studies have shown the expression increase of ACR2 gene of *L. major* proteins known as the reducing of pentavalent antimonial drug and arsenate in the E.coli expressive vectors lacking ArsC, or in the *Saccharomyces cerevisiae* species lacking the ScACR2 gene transferred to the parasite *L. infantum*, and its susceptibility to the pentostam drug increased. It has been assumed that a functional mutation in the enzyme can result in drug resistance (12). There is a direct relationship between the TDR1 enzyme, which is a thiol-dependent reductase, and reduction of pentavalent drug to trivalent drug (18). In another study, the crystallographic structure of trypanothion reductase, it has been shown that a trivalent antimony ion can be located in the active site of the enzyme and inhibit the production of T (SH) 2, or trypanothione (13).

In terms of molecular geometry, the trivalent antimony ion is located in the center of a tetrahedral geometry (13), whose corners are

occupied by SG atoms of Cys 52 (2.8Å) and Cys 57 (3. 0 Å) and the Og1 atom of Thr 335 (3.0 Å) of the first chain; and the ND1 atom of His 461 (3.2 Å) amino acid is from the similar second bundle or the second fold of this protein (13). The trypanothione T(SH)2 itself can also cause pentavalent (sbV) antimony recovery to trivalent (sbIII) under acidic conditions without enzymatic intervention at the 37 °C temperature. This may be done either in the host macrophage cytosol or in the parasite cytosol (4, 8, 14-17). Trivalent antimony has a high tendency to bond with thiol groups (SH) in trypanothione (8, 13-17). The metal-thiol conjugates either enter in the parasite through the carriers of ABC (MRPA) or excrete through the release of other types of ABC carriers (8, 13-17). In the glucantime-resistant isolates, the increase of trypthanitone has been observed with other agents such as glutathione, thiol, cysteine and spermidine in the parasite (8, 13-17). On the other hand, the release of thiol-metal conjugates out of the parasite through the pumps increases and their entry into the parasite decreases (8, 13-17). In another study, it was confirmed that the trypanothione reductase and glutathione peroxidase enzymes are inhibited by trivalent antimony (SbIII); therefore, an increase of the stress oxidative and thiol groups, can be considered as potential agents for drug resistance (25). The resistant genes were evaluated as diagnostic markers for *L. braziliensis* and *L. guinensis*, including *HSP70*, *AQP1*, *MRPA* and *TRYR*; the polymorphism in the genes was observed, except for *HSP70*, which had no significant relationship with the patients' response to treatment or treatment failure. As a result, these genes could not be used as diagnostic markers for the disease in clinical status (26).

The current study is the first molecular dynamics investigation on the role of mutation in the three-dimensional structure of tryptophan reductase. Out of 15 resistant and 15 clinically sensitive glucantime samples, only 4 (27%) of the drug-resistant population showed significant mutations with three-dimensional trypanothione reductase alteration. Because of the likely occurrence of mutations in Glucantime as well as the ease of development of *L. tropica* resistant populations, more samples are needed to demonstrate the relationship between mutations in this enzyme and clinical resistance to glucantime. On the other hand, it is recommended that enzymatic studies be performed to confirm the role of mutation in the function and expression of trypanothione reductase in glucantime resistant and susceptible populations.

According to the our results, some structural mutations were seen in 9 amino acids surrounding the active site of the *TRYR* gene of *L. tropica*, which increased the active genes' active site with an average of 0.9 A°. The value is likely to cause drug resistance.

Conclusion

Change in the active site of *TRYR* of *L. tropica*, could probably contribute to the development of *L. tropica* resistance to glucantime. For better understanding of the mechanism, further complementary study is required.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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