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In Silico Identification of *L. donovani* Excretory-secretory Proteins Interacting with Human SLC11 A1

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Abstract: ***Leishmania donovani*** **is a causative agent of neglected tropical disease Visceral Leishmaniasis.** ***Leishmania*** **have evolved several elegant escape mechanisms to subvert the primordial defense system of host macrophages. It resides in the hostile nutria-deprive phagolysosomes of host macrophages. Present study is focused on one of the survival strategies of** ***Leishmania,*** **based on the hypothesis that leishmanial proteins may interacts with host natural resistance associated macrophage proteins (NRAMP1) now referred as SLC11A1 to alter it functional activity. Inhibition of SLC11A1 divalent cations efflux pump activity may result in the acquisition of nutrient inside phagolysosomes of host macrophages for itssurvival, growth and proliferation. Thus,** ***in silico*** **identification of amastigotes excretory secretory (ES) proteins interaction with SLC11A1 was performed. Protein sequences were retrieved from NCBI, multiple sequence alignment of ES proteins with SLC11A1 was done. Five ES proteins randomly selected with good MSA score were structure- modeled and validated by Ramachandran plot and used for protein-protein docking study. We have identified 2 proteins, proteasome regulatory non ATPase subunit and biotin/lipoate protein ligase like protein interaction with SLC11A1 with Z Dock score of 19.04 and 17.82 with Z Rank score -158.381 and -141.296, respectively by using Z DOCK protein docking protocol of Discovery Studio Version 3.0. These identified proteins can act as candidate virulence factor.**

Index Terms: **Excretory,** ***in silico,*** ***Leishmania,*** **secretory, SLC11A1.**

I. INTRODUCTION

Visceral Leishmaniasis (VL) is a potentially fatal human disease caused by the intracellular protozoan parasites Leishmania donovani. The annual global prevalence of all forms of Leishmaniasis is nearly 10 million and approximately 350 million people are at risk (Desjeux, 2004; Mathers et al., 2007). An approximated global burden of VL is about 0.2 to 0.4 million and CL is approximately 0.7 to 1.2 million each year (Alvar et al., 2012) VL is responsible for significant morbidity and mortality in the developing world, particularly in India, Sudan, Nepal, Bangladesh, and Brazil (WHO, 2004). Moreover, the incidence of the leishmaniasis has been on the rise because of multiple factors including the AIDS epidemic, increased international travel, lack of effective vaccines, difficulty in controlling vectors, and the development of resistance to chemotherapy (Singh et al., 2012). Leishmania live as either extracellular, flagellated promastigotes in the digestive tracts of their sand fly vectors or as nonflagellated amastigotes within macrophages, where they survive and replicate within phagolysosomes. The metacyclic promastigotes of Leishmania parasite are inoculated in host by the bite of sand fly, the vector. Macrophages as part of both the innate and acquired immune systems are programmed to ingest and destroy intracellular pathogens. Thus, after reaching into blood these promastigotes are phagocytosed by macrophages followed by phagosome formation (Kamhawi, 2006). Onset and progression of disease depends on the successful survival and replication of parasite inside phagosomes and eventually transformation of promastigotes into resistant amastigote form. As soon as parasite is phagocytosed, a host transport protein natural resistance associated macrophage protein1 (NRAMP1) now referred as solute carrier protein family 11 member A1 (SLC11A1), recruited to phagosomal membrane. SLC11A1 is a transmembrane transporter present on LAMP+ lysosome and endosomes of macrophage, which acts as proton dependent divalent cation efflux pump, and has been found to be a link between iron transport and innate immunity (Barton et al., 1999; Huynh&Andrews, 2008). The purpose of this recruitment is to make the phagolysosomes (amastigotes residence) iron deprived ensuring the least availability of iron for the parasites from the host cell. Iron is essentially required for parasite replication, virulency as well as for protection from oxidative damage

(Appelberg, 2006). In addition, several lines of evidence have also converged to establish the role for the host macrophage SLC11A1 in the pathogenesis of Leishmania (Forbes& Gros,2001; Fritsche et al., 2007;Gruenheid et al., 1997). Hence, the mechanisms used by Leishmania to evade elimination by macrophages are important issues. One of the major hurdles for developing effective prophylactic or therapeutic vaccines as well as safer and more effective drugs has been a limited understanding of the VL pathogenesis. Two second line drugs i.e. amphotericin B, and miltefosine are being used to treat but they are also significantly associated with toxicity and resistance development (Singh et al., 2012). Hence due to limited drug regimen and unavailability of vaccine, identification of novel drug targets and virulence factors for development of true antileishmanial drugs are essentially required.

SLC11A1 confers resistance against intracellular pathogens through stimulation of several antimicrobial effectors pathways including NADPH oxidase activity and NO formation, which is known to be controlled by intracellular iron availability (Paramchuket al., 1997; Weiss et al., 1994). Furthermore, perturbations of intracellular iron availability are known to influence IFN-γ dependent macrophage effectors function. In addition, SLC11A1 functionality is associated with an enhanced activity of pro-inflammatory immune pathways, via transcriptional stimulation of inducible nitric oxide synthase (iNOS) expression (Fritsche et al., 2003). SLC11A1 expression promotes MHCII expression on dendritic cell, required for antigen presentation and further generation of antigen specific immune cells (Stober et al., 2007). It also promotes Th1 cytokine production, which are required for the protection from Leishmania infection, as in VL protective immune response is achieved when Th1 response dominated over Th2 response (Bacellar et al., 2002; Singh et al., 2012). Thus for mounting a protective immune response, proper SLC11A1 expression and functioning is essentially required.In our earlier studies, we found that L. donovani secretory peroxidoxin is significantly associated in the down regulation of SLC11A1 expression and function, and subsequent inhibition of macrophage functions that enable parasitic survival in host cells (Singh et al., 2013).

Hence, we hypothesized that leishmanial proteins may also be involved in the functional regulation of SLC11A1 enable parasite survival in host macrophages. Thus in present study, we screened in silico, L. donovani specific excretory or secretory proteins that interacted to host SLC11A1 leading to its functional impairment during infection. The role of most of amastigotes excretory secretory is also unknown and hence this study is also helpful in the functional exploration of ES proteins of Leishmania. Findings of this may direct drug and vaccine development studies into next level, where iron metabolism of host macrophages is also taken under consideration for achieving protection against Leishmania.

II. Materials And Methods

A. *Selection of ES proteins from L. donovani and identification of their functional motifs/ secondary structures*

Secretary proteins from *L. donovani* were taken from NCBI database based on the reported article by Silverman et al.(2008). FASTA protein sequences from *L donovani* genome were retrieved from NCBI using BLAST search tool. A total of 58 proteins with sequence homology more than 90% with *L donovani* were selected for further study. The functional motifs/secondary structure analysis was done using web servers viz. Pfam (pfam.sanger.ac.uk), interproscan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and Prosite (prosite.expasy.org).

B. *Multiple sequence alignment of ES proteins with human SLC11A1*

Multiple sequence alignment of identified ES proteins with host (*Homo sapience*) SLC11A1 sequence was performed by using clustal W (*www.genome.jp/tools/clustalw/*). Multiple sequence alignment of these secretary proteins with SLC11A1 provided similarity score, which is a predicative of similar secondary elements/motifs in the target and candidate proteins that may be involved in the protein-protein interaction.

C. *Tertiary structure prediction of L. donovani secretory proteins and validation*

Tertiary structures of ES proteins (PDB ID) were searched in Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). However, none of the ES proteins tertiary structures were available in Protein Data Bank. Thus, 3D structure prediction was performed, using Swiss model workspace (http://swissmodel.expasy.org/workspace/) (Arnold et al., 2006). Further, predicted 3D structures were checked with RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) server for their steriochemical validation (Lovell et al., 2002). The structure with <3.5% residues (amino acids) found in the outlier regions was selected as protein model.

The 3D structure (pdb file) of SLC11A1 was requested from Dr. Mathew. F. Celleir and all three conformation i.e. IF (inward facing), OF (offward facing), Occ (occulated) state were used for docking studies (Cellier, 2012).

D. *Protein-Protein Docking*

Protein–Protein interactions (ES and SLC11A1) were performed by discovery studio version 3.0, using default parameters with Z-DOCK protocol of Dock protein for receptor and protein interaction. Z-DOCK is a grid-based global search algorithm utilizing a Fast Fourier Transform (FFT) technique and a novel shape complementarity scoring function. The default parameters of Z DOCK protocol i.e 2000 protein poses, angular step size 15 were used. The Z Rank was kept at “True” and parallel processing was kept at “False” and finally Z- DOCK protocol was run to start Z DOCK protocol.

III. RESULTS

A. Identification of secretory proteins functional motif/ secondary structures

Total 58 proteins of *L. donovani* secretome were selected for analysis. Their secondary functional motifs were initially identified to check the maximum docking probability with SLC11A1. However, none of the ES protein showed functional motifs similar to that of SLC11A1 (data not shown).

B. Multiple sequence alignment of ES proteins with SLC11A1 and tertiary structure prediction

Multiple sequence alignment of ES proteins with SLC11A1 provided a matching score, which is a predicative of similar sequence/motif in the target and candidate proteins that may be involved in protein-protein interaction. Initially protein with highest MSA score [endoribonuclease-L-PSP (pb5); aligned score: 16.5644, accession no.-XP003860923.1] as candidate interactor but we didn't observe any docking score with SLC11A1. Thus, we randomly selected 4 more proteins viz. uracil phosphoribosyltransferase (accession no-XP003864273.1 and aligned score-12.3967), proteasome regulatory non ATPase subunit (accession no.-XP003862430.1, aligned. score-11.6364, proteasome regulatory non ATPase subunit11 (accession no.-XP003864232.1, aligned. score-12.6214, biotin/lipoate protein ligase like protein (accession no.- XP003863199.1, aligned score: 11.7871)with a good MSA score more than 11.5 for docking studies. The tertiary structures of all proteins are depicted in figures 1a, 2a, 3a, 4a and 5a.

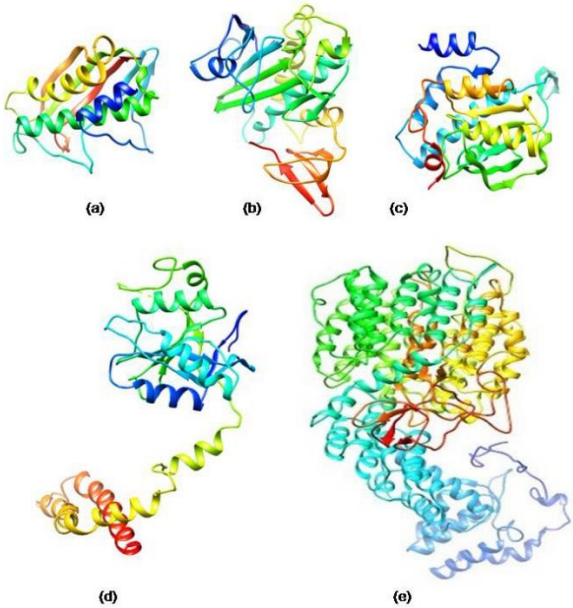

Fig. 1. (a) Tertiary structure (solid ribbon) model of endoribonuclease-L-PSP (pb5) (accession no. XP 003860923.1), (b) biotin/lipoate protein ligase like protein(accession no.XP003863199.1, (c) uracil phosphoribosyltransferase (accession no. XP003864273.1) (d) proteasome regulatory non ATPase subunit11 (accession no. XP003864232.1), (e) proteasome regulatory non ATPase subunit (accession no. XP003862430.1),)

Out of 5 proteins, only biotin/lipoate protein ligase like protein and proteasome regulatory non ATPase subunit was found to be interacting with either of SLC11A1 conformation viz. inward facing, outward facing or occulated state. In inward facing SLC11A1 conformation (Z Dock Score 17.82 and Z Rank Score: -141.296) the amino acid residues Ala^{71}- Leu^{82}, Glu^{142}-Asn^{189}, Gly^{191}-Leu^{192}, Lys^{194} -Leu^{195}, $Phe^{198-199}$, Leu^{202}-Ile^{203}, Ile^{205}-Met^{206}, Tyr^{212}, Phe^{310}, Lys^{313}, His^{328}- , Ile^{333} -Thr^{340}, Val^{343}-Thr^{380}, $Leu^{402-403}$, Arg^{405}-Ser^{406}, Ala^{408}-Asp^{420}, Arg^{422}-Asp^{423}, Ser^{425}-Gly^{426},Asn^{428}-Asp^{429} of SLC11A1 were involved in interaction with Pro^{6}, Thr^{18}-Met^{19}, Ala^{35}-Thr^{42}, Trp^{52}-Phe^{63}, Val^{81}-Gly^{133}, Thr^{135}-Ile^{137}, Ile^{146}-Phe^{193}, ASP^{195}-Ile^{196}, Ser^{199}-Pro^{200}, Val^{207}-Lys^{223}, Val^{235}-Leu^{237} amino acid residues of biotin/lipoate protein ligase like protein (Fig. 2).

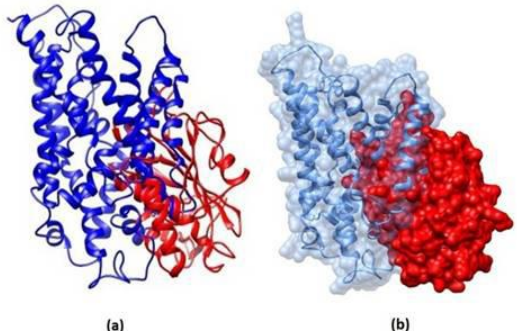

Fig. 2. Interaction ofbiotin/lipoate protein ligase like protein with SLC11A1 (a) Solid ribbon (b) Solid surface representation of both receptor (SLC11A1, IF) and ligand (biotin-lipoate protein ligase like protein)

This protein also interacted with occulated state of SLC11A1 (Z dock score 14.2 and Z Rank Score: -141.542). The amino acid residues Gln^{8}-Arg^{9}, Ser^{54}-Lys^{57}, Phe^{61}-Thr^{62}, Phe^{66}, His^{126}, Arg^{134}, Met^{282}-Ala^{291}, Ser^{293}, Ile^{297-98} of SLC11A1 were involved in interaction with Phe^{11}, Glu^{13}, Ala^{39}, Thr^{42}, Thr^{51}, Thr^{53}, Pro^{55}-Lys^{56}, Ala^{153}- Arg^{163}, Glu^{173}, Phe^{175} amino acidresidues of this protein (Fig. 3). However the interaction was weaker as compared to inward facing SLC11A1 conformation.

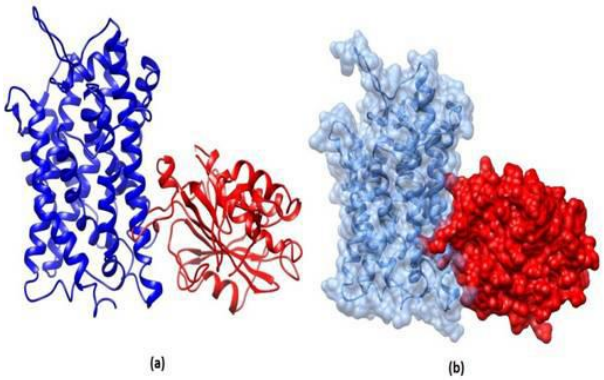

Fig. 3. Interaction ofbiotin/lipoate protein ligase like protein with SLC11A1 (a) Solid ribbon (b) Solid surface representation of both receptor (SLC11A1, occulated) and ligand (Biotin/lipoate ligase like protein)

The proteasome regulatory non ATPase subunit interacted only with inward facing SLC11A1 conformation (Z Dock Score 19.04 and Z Rank Score: -158.381).The residues involved in the interactions were Val^{146}, Asp^{149}-Phe^{186}, Val^{343}-Ile^{345}, Ser^{396}-Leu^{414}, Ala^{416}-Phe^{418}, Arg^{422} and Ala^{332}-Thr^{335}, Glu$^{337-338}$, Glu^{661}, Lys^{664}, Ile^{668}, Leu^{671}, Arg^{686}, Pro^{690}, Tyr^{693}-Ala^{694}, Ser^{697}, Asn^{702}-Leu^{718}, Thr^{720}-Ala^{721}, Asn^{723}-Gly^{729}, Val^{731}, Gly^{734}-Tyr^{750}, Gln^{752}, Ile^{755}-Phe^{756}, Ala^{759}-Thr^{760}, Val^{763}, Ala^{766}, Cys^{770}, Leu^{857}-Gly^{859} of SLC11A1and proteasome regulatory non ATPase subunit, respectively (Fig. 4).

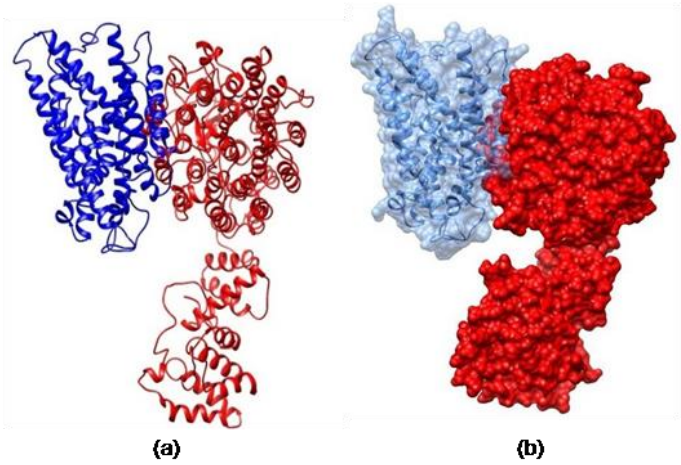

Fig. 4. Interaction ofproteasome regulatory non ATPase subunit with SLC11A1 (a) Solid ribbon (b) Solid surface representation of both receptor (SLC11A1, IF) and ligand protein proteasome regulatory non ATPase subunit.

IV. Discussion And Conclusion

Leishmania follows a characteristic mode to survive in the vertebrate blood stream and flourish within the hostile condition of the macropahegs phagolysosomes. After phagocytosis, they manipulate macrophage functions through inhibition of hydrolytic enzymes, toxic metabolic products, cell signaling, proinflammatory cytokines production and other events (Sacks&Sher, 2002). These strategies allow *Leishmania* to successfully undermine the host innate and acquired immune response and promote parasite survivia l (Kima, 2007). However, very little is known about the macrophages suppressive mechanism of *Leishmania.*

The role of SLC11A1 pathogenesis and resistance is quite established in many pathogenic diseases. The SLC11A1 function is known to regulate macrophage activation that confers resistance to intracellular pathogens including *Leishmania*. Although, SLC11A1 primarily acts as a proton dependent transporter for divalent cations such as iron and manganese, it also exerts pleiotropic effects on innate immune functions. SLC11A1 modulates the production of chemokines and cytokines such as macrophage inflammatory protein 1 α (Mip-1α), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and the formation of reactive oxygen and nitrogen species (ROS and RNS), as well as antigen processing and presentation (Blackwellet al., 2003; Fritscheet al., 2007; Kuhnet al., 1999; Nevo&Nelson, 2006; Valdez et al., 2008). Thus, to meets its iron requirement and to escape from macrophages innate immune response *Leishmania* has also developed other regulatory mechanism such as; it suppressed the redox activation of host macrophages via secretory peroxidoxin, leading to declined expression of SLC11A1, thus get access of phagosomal iron (Singh et al., 2013).

Leishmania secretes more than 151 proteins via classical and non-classical secretory pathways (Silverman et al., 2008). Most of these secretory proteins are cytosolic in nature however; their role in *L. donovani* survival remains elusive. The ES proteins of *Leishmania* are known for both, to suppress and activate macrophages. ES proteins activate macrophages via increasing their tyrosine phosphatase activity that regulate their activation status and suppress SLC11A1 expression (Fritscheet al., 2007). We have also observed that ES proteins may also be effectively used to activate macrophage effector functions and SLC11A1 regulation by redox status (Gour et al., 20122; Singh et al., 2013).However, the physical interaction of ES proteins and SLC11A1 is not yet reported.

In the present study, we observed that proteasome regulatory non ATPase subunit and biotin/lipoate protein ligase like protein docked with 3 different conformation of SLC11A1, which may modulate its functional activity i.e. iron efflux pumps. The finding of this study also indicated that it's not only the sequence similarity but some other regulatory elements/factors; also regulate protein-protein interaction. Since MSA score of biotin/lipoate protein ligase like protein was lower as compared to few ES proteins used in docking study. The biotin/lipoate protein ligase like protein interacted with both inward facing & occulated conformation of SLC11A1. However, proteasome regulatory non ATPase subunit interacted with only inward facing SLC11A1 conformation. Since, none of the protein interacts with outward side, which is a cytosolic side of SLC11A1 where divalent cations are effluxed out. It may be probably due to the binding of ES proteins with SLC11A1 from inward facing side that may resulted in some conformation alteration leading to blockage of divalent cation efflux pump function. These findings are quit supportive to our proposed mechanism of inhibition of NRAMP 1 efflux pump activity since acid resistant amastigote forms have developed several strategies including expression of ferrous ion transporter, LIT1 to uptake iron in starving condition of host, macrophages (Huynh et al., 2006; Jacques et al., 2010). In our previous study, we have observed that it's an efflux pump, and the present study also supports its efflux pump activity, as *in silico* study reported that ES protein bind dominantly from inward facing sides (Singh

et al., 2013).So, it may be possible that on binding with ES protein, SLC11A1 is not able to transport iron possibly due to conformational changes. Hence further *in vitro* validation of these interaction and modulation of NRAMP 1 efflux pump activity may direct the future course of drug or vaccine development.

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