Molecular BioSystems

# Essential Gene Identification and Drug Target Prioritization in *Leishmania* species



| | |
|---|---|
| Journal: | *Molecular BioSystems* |
| Manuscript ID: | MB-ART-10-2013-070440.R1 |
| Article Type: | Paper |
| Date Submitted by the Author: | 10-Jan-2014 |





| | |
|---|---|
| Complete List of Authors: | M Losery, Stanly Paul; National Institute of Pharmaceutical Education and Research,, Pharmacoinformatics<br>Kaur, Amandeep; National Institute of Pharmaceutical Education and Research, Pharmacoinformatics<br>Geete, Ankit; National Institute of Pharmaceutical Education and Research(NIPER), Pharmacoinformatics<br>Masilamani, Elizabeth Sobhia; National Institute of Pharmaceutical Education and Research, Pharmacoinformatics |

Molecular BioSystems RSC Publishing

PAPER

# Essential Gene Identification and Drug Target Prioritization in *Leishmania* species†

Cite this: DOI: 10.1039/x0xx00000x

M.L. Stanly Paul[a], Amandeep Kaur[a], Ankit Geete[a] and M. Elizabeth Sobhia[a]*,

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Leishmaniasis is one of the neglected tropical diseases (NTDs), mainly affecting impoverished communities and having varied ranges of pathogenicity according to the diverse spectrum of clinical manifestations. It is endemic in many countries and poses major challenges to healthcare systems in developing countries. Despite the fact that most of the current mono and combination therapies are found to be failures, clear perception of gene essentiality for parasite survival are now desideratum to identify potential biochemical targets through selection. Here we used metabolic network of *L. major*, we have performed a comprehensive set of *in silico* deletion mutants and systematically recognized a clearly defined set of essential proteins by combining several essentiality criteria. Here we summarize the efforts to prioritize potential drug targets up to a five-fold enrichment compared with a random selection.

## Introduction

The genomic strategy on many microbial pathogens has been studied to identify effective novel drugs and their targets; however such exertions have not yet reached full fruition in the opportunistic pathogen like *Leishmania* that can attack immune-compromised patient.[1] Leishmaniasis can be considered as endemic, sporadic or epidemic, with different clinical features according to genetic variability within the species.[2] The study of *Leishmania* metabolism has gained importance due to the widespread emergence of drug resistance to the current chemotherapeutics and the lack of new anti leishmanials to substitute.[3-5] To survive environmental hardship, *Leishmania* alter their metabolism to encompass the requirements of cellular proliferation.[6] This functional response facilitates the metabolic pathways to adapt to the changing genetic and environmental hardship.[7] Diverse studies on *Leishmania* have exhibited resembling metabolic perturbations.[8-10] The utility of systems approaches has become a key for other biological disciplines and predicting the naturally occurring genetic perturbations by growth conditions.[10, 11]

In the present study, we used genome-scale reconstructed *L. major* metabolic pathway of Chavali et al (2008). It consists of gene-protein-reaction association (GPRs) constructed through extensive survey of literature and databases.

[a] Department of Phamracoinformatics, National Institute of Pharmaceutical Education and Research (NIPER), Sector-67, S.A.S Nagar, Mohali, India -160062.

†Electronic supplementary information (ESI) available. See DOI: 10.1039/b000000x/.

The *L. major* model contained 1112 reactions, 1101 metabolites, and 560 genes.[61] Church and co-workers illustrated that Minimization Of Metabolic Adjustment (MOMA) accurately identifies some lethal gene deletions, which are not identified by FBA.[13, 51] We used MOMA approach to estimate metabolic phenotype by minimizing the Euclidean norm in flux space with respect to the wild type, calculated through quadratic programming (QP). This approach appears specifically appropriate for analyzing gene deletions and helps to understand metabolic networks for a wider range of perturbations.[14]

Fundamental investigation to understand metabolic networks, leads to valuable insights into their function.[15,16] Hence, we performed lethality fraction analysis when all reactions corresponding to the metabolite are removed. This analysis based on network functions suggested that even the least connected nodes in genome scale metabolic networks are just as likely to be critical to the overall network functions as the most highly connected nodes.[17,18] Additional outcome comprise the depiction of network robustness and potency of new drug targets in the vicinity of enzyme inhibitors.[19] This strategy of systems analysis not only make a provision for semantic data integration, simulation, analysis, visualization and hypothesis generation but also it stimulates the research to discover novel drug targets in infectious neglected diseases.[20] Here we have attempted to prioritize the drug targets by using several essentiality filters like druggability, assayablility, epistatic interactions, molecular weight, sequence and structural likeness with proteome and microbiome of human to arrange potential anti-leishmanial drug targets in the order of priority.

We also carried out comparative analysis of binding pockets of highly prioritized targets of *Leishmania* with human highly similitude proteins in order to reckon their

characteristic feature for target binding.[21] This may aid in the drug discovery process in analyzing the mutual dealings between distantly associated proteins.

## Results

The *L. major* model (iAC560) containing 560 genes, 1112 reactions, 1101 metabolites and 8 unique sub-cellular localizations were considered for the study. The present *in silico* gene knockdown studies included single and double gene knockouts, reaction deletions using MOMA. Each of the 560 genes in the core *L. major* amastigotes model was knocked out corresponding to 560 genes using MOMA, and the resulting relative growth rates were plotted in Fig. 1. Reaction deletion predictions in amastigotes obtained using MOMA are shown in the form of a bar plot in Fig. 2B. In order to quantify *L. major* prioritized drug targets, we encountered three primary challenges. The first involved the specific use of MOMA models. The second included the essentiality criteria and the third included the filtering targets through comparison with experimentally validated drug targets, sequence analysis, pocket volume and pocket depth analysis.

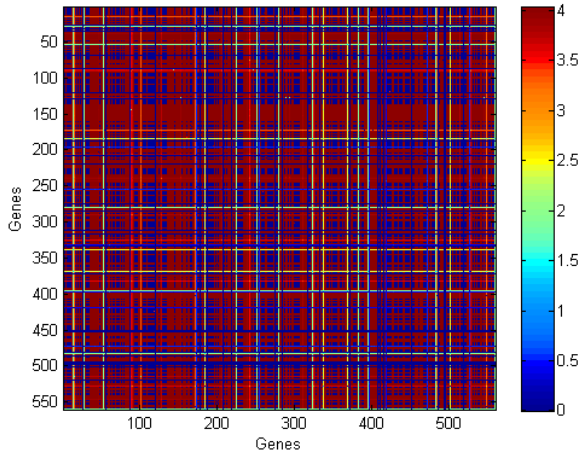


**Fig. 1** Gene knockout screen obtained using MOMA in double gene deletion analysis of amastigotes in *Leishmania* species. Color scale represents the biomass growth.

Previous studies were also reported to demonstrate that the *Leishmania* model under diverse conditions by using FBA, predicted deletion mutant variability with high accuracy.[25] However, in the present study, Genes in iAC560 *Leishmania* model were 'knocked-out' simultaneously through double gene knockout predictions using MOMA (Fig. 1) to explain the dynamics of the metabolic network of *Leishmania* (Fig. 2B).

Consequently, those having harmful effects on the organism's survival identified and divided as lethal, trivial lethal and non-lethal genes (Fig. 2C). The reaction deletion analysis helps in searching a metabolic network for reactions that are critical to the production of biomass. Each reaction can be classified as essential by substantially reducing flux through the biomass function. This was performed by removing each reaction in the network and measuring the predicted flux through the biomass function in case of single reaction deletion (Fig. 2B). The proteins are classified as lethal, trivial lethal (Fig. 2D) or non-lethal by observing the levels of biomass (Fig. 2C).

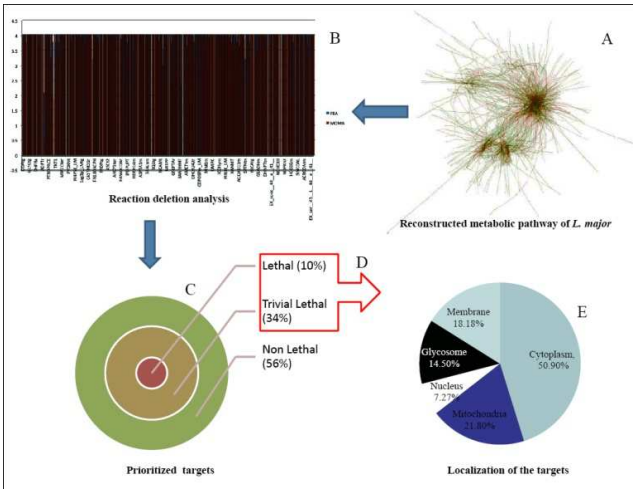


**Fig. 2** Workflow to define the localization of essential proteins in amastigote stage of *Leishmania* species. (A) Metabolic network of *L. major* consisting of 560 genes and their corresponding 1112 reactions and 1101 metabolites. (B) Reaction deletion obtained using MOMA. (C) Through MOMA analysis 10% of the reactions are found to be lethal, 34% as trivial lethal (represented in (D)) and 56% as non lethal. Lethal gene deletions can be divided into lethal, trivial lethal or non-trivial lethal. Trivial lethal means that at least one of the genes involved in a single deletion is lethal. In non-trivial, both genes involved in the double deletions are not lethal individually as single gene deletions but are lethal in combination. (E) Distribution of the target proteins in different cellular compartments. The maximum number of proteins are localized in the cytosol i.e 50.9%.

### Epistatic interactions in *Leishmania* species

*Leishmania* can adapt to dynamically changing needs. These systems can adapt to their environment, thereby creating an emergent behavior at different stages of *Leishmania* life cycle.

The effects of single and double gene deletions are quantified on two phenotypic traits. A comparative analysis demonstrates that the maximum phenotypic growth rate averaged over amastigotes and promastigotes of *Leishmania* lacking deletions for double mutants are highly effective than that of single mutants. This indicates the positive epistatic effect. This characteristic likelihood contributed by genes is appropriate to a diversity of functional classes for comparing MOMA and FBA epistasis predictions to experimental flux data for *Leishmania*. For this purpose, we used a standard MOMA model, which uses quantitative genetic theory for analyzing high order epistatic interactions for wild type and stage specific amastigotes of *L. major*. This model allowed us to verify the epistasis along with its different genetic components in *L. major*. Epistatic analysis also contributed meaningful support to the reaction deletion results. Our results suggest that the data on the rate of growth can be used as a substitution for the rate of total metabolism when we want to study the robust individual interactions or estimate the mean epistatic effect. It is essential to analyze both flux and growth in order to sense individual effects of epistasis.[26]Also, to figure out epistasis we used novel metric that is useful to give a clue how the two-mutation combine affects the growth rate. The results obtained where number of interactions, can be beneficial for the prediction of phenotype and for targeting interventions.

| *Leishmania* genes | Gene Description | Location | Modbase | Key info on gene | Essentiality | Druggability | Present in Humans | PDB structures | Phylogenetic distributio | Assayability | Pubmed papers | Epistasis | M wt. | % identity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LmjF05.0350 | Trypanothione metabolism | g | X | X | X | X | | X | X | X | X | | X | |
| LmjF12.0530 | Glycolysis/Gluconeogenesis | g | X | X | X | X | X | X | X | X | X | X | X | |
| LmjF36.1960 | Fructose and mannose metabolism | c | X | | X | X | X | X | X | X | X | | X | |
| LmjF35.1190 | Citrate Cycle (TCA) | m | X | | X | X | | | X | X | | | X | |
| LmjF01.0500 | Fatty acyl CoA synthetase 2 | c | X | X | X | X | X | | X | X | X | | X | X |
| LmjF32.1580 | Fructose and mannose metabolism | c | X | X | X | X | X | | X | X | X | | X | X |
| LmjF01.0470 | Fatty acyl CoA syntetase 1 | c | X | X | X | X | X | | X | X | X | | X | X |
| LmjF01.0490 | Fatty Acid Degradation | c | X | X | X | X | X | | X | X | X | | X | X |
| LmjF12.0280 | Urea Cycle | c | X | X | X | X | X | | X | X | X | | X | X |
| LmjF34.0110 | Purine Metabolism | c | X | | X | X | X | | X | X | X | X | X | |
| LmjF31.2460 | Glycerophospholipid metabolism | c | X | | X | | | | X | X | X | | X | |
| LmjF28.1970 | Pentose Phosphate Pathway | c | X | | X | | | | X | X | X | | X | |
| LmjF35.4410 | Amino acid permease | e | X | X | X | X | | | X | | | | X | |
| LmjF29.1960 | Citrate Cycle (TCA) | g | X | | X | | | | X | X | X | | X | |
| LmjF09.1040 | Glycerophospholipid metabolism | c | X | | X | | | | X | X | X | | X | |
| LmjF15.1140 | Tryptophan Metabolism | c | X | X | X | | | | X | X | | | X | |
| LmjF15.1040 | Tryparedoxin peroxidase | g | X | X | X | | | | X | X | | | X | |
| LmjF19.0710 | Citrate Cycle (TCA) | g | X | X | X | X | X | | X | X | X | | X | |
| LmjF32.2950 | Purine Metabolism | n | X | | X | X | X | | X | X | X | | X | |
| LmjF34.0080 | Pentose Phosphate Pathway | c | X | | X | X | X | | X | X | X | | X | |
| LmjF24.0320 | Citrate Cycle (TCA) | m | X | | X | | | | X | | | X | X | |
| LmjF30.3120 | Methionine Metabolism | c | X | | X | | | | X | | X | X | X | |
| LmjF35.3870 | Purine Metabolism | n | X | X | X | | X | | X | X | X | | X | X |
| LmjF35.3340 | Streptomycin Biosynthesis | c | X | | X | X | X | | X | | | | X | X |
| LmjF32.1960 | Glycerophospholipid metabolism | m | X | X | X | X | X | | X | | | | X | X |
| LmjF17.0360 | Pyrimidine Metabolism | c | X | | X | X | X | | X | | | | X | X |
| LmjF13.1620 | Steroid Biosynthesis | r | X | | X | X | X | | X | | | | X | X |
| LmjF36.2260 | Purine Metabolism | c | X | | X | X | X | | X | | | X | X | |
| LmjF31.2470 | Pyrimidine Metabolism | c | X | | X | | X | | X | | | X | X | X |
| LmjF34.1090 | Glycerophospholipid metabolism | g | X | X | X | X | X | | X | | X | | | X |
| LmjF06.0460 | Nicotinate and Nicotinamide Metabolism | c | X | | X | | X | | X | X | | | | X |
| LmjF07.0090 | Methionine Metabolism | c | X | | X | X | X | | X | | | X | | |
| LmjF30.3080 | Glycine, serine and threonine metabolism | c | X | | X | | | | X | | | | X | |
| LmjF36.0060 | Pentose and Glucuronate Interconversions | g | X | | X | | | | X | | | | X | |
| LmjF28.1970 | Pentose Phosphate Pathway | c | X | | X | | | | X | | X | | X | |
| LmjF34.2110 | Glycerophospholipid metabolism | m | X | X | X | | | | X | | | | X | |
| LmjF34.3780 | Fructose and mannose metabolism | g | X | | X | | | | X | | | | X | |
| LmjF26.2480 | Glycerophospholipid metabolism | c | X | | X | | | | X | | | | X | |
| LmjF07.0200 | Glycerophospholipid metabolism | m | X | X | X | | | | X | | | | X | |
| LmjF14.1360 | Streptomycin Biosynthesis | c | X | X | X | X | X | | X | | | | X | |
| LmjF35.1480 | Arginine and Proline Metabolism | g | X | | | | X | | X | X | X | | X | |
| LmjF33.2720 | Fatty Acid Biosynthesis | m | X | | X | X | X | | X | | X | | X | |
| LmjF28.0890 | Purine Metabolism | n | X | | X | X | X | | X | | X | | X | |
| LmjF04.0580 | Methionine Metabolism | c | X | X | X | X | X | | X | | X | | X | |
| LmjF26.2700 | Pentose Phosphate Pathway | c | X | X | X | | X | X | X | | | | X | X |
| LmjF26.1620 | Glycerophospholipid metabolism | c | X | | X | | X | | X | | | | X | X |
| LmjF30.3600 | Oxidative phosphorylation | m | X | | X | | X | | X | | X | | X | X |
| LmjF21.1770 | Oxidative phosphorylation | m | X | | X | | X | | X | | X | | X | X |
| LmjF14.1200 | Glycerophospholipid metabolism | c | X | X | X | | X | | X | | | | X | X |
| LmjF21.1340 | Oxidative phosphorylation | a | X | | X | | X | | X | | | | X | X |
| LmjF36.3010 | Glutathione Metabolism | c | X | | X | | X | | X | | X | | X | X |
| LmjF31.2970 | Fatty Acid Synthesis | m | X | X | X | X | X | | X | | | | | |
| LmjF32.2870 | Glycerophospholipid metabolism | c | X | | | X | X | | X | | | | X | X |
| LmjF35.1180 | Citrate Cycle (TCA) | g | X | | | | | | X | X | | | | |
| LmjF23.0110 | Fructose and mannose metabolism | c | X | X | X | | X | | X | | X | | X | |

184
185
186 **Table 1**. Prioritized essential targets of *Leishmania* Species. Gene deletion analysis provided essential targets for *Leishmania* Species.
187
188

It was found that epistasis relative to metabolic fluxes can be robustly detected independent of the metric used. Here we used multiplicative model and the conclusions drawn were tried out to be relative to various metrics.[27] These diverse classes of epistasis denote various ways in which the collective effect of two gene deletions may assume expectations and can indicate various types of fundamental issues that underlie functional relationships between genes.[24,28] The analysis of growth and flux phenotypes makes the classification of interactions more complex due to the fact that genetic perturbation can cause an increase or decrease in fluxes, while the growth rate only decreases.[29] This demonstrates that interactions related to metabolic flux phenotypes can lead to deep intuitive understanding of various features of the functional aspects between genes.

Metabolic systems approaches have provided various target genes and the proteins encoded by them. Some of these have been validated as potential targets against amastigotes of *Leishmania* as given in the literature, however, our study presumably provides a prioritized list of target genes and proteins for developing anti-leishmanial drugs (Table. 1).

The genes, enzymes, reactions related to this study have been provided in the (Supplementary information 1). These targets are subjected to prioritization by using various essentiality criteria's, which is helpful for the researchers in drug discovery process of neglected tropical diseases (Table. 1).

**Criteria to prioritize *L. major* -specific drug targets**

The drug targets obtained from the study were prioritized using the following essentiality criteria. These include (1) the targets should represent growth-essential genes and (2) they must possess experimental crystal structure information (3) the sequence of the targets should not be homologous to human proteins (4) there should be information on biological assays.

**Druggability, Assayablility, Essentiality, Molecular weight**

In addition to the above filters, we also considered the other features such as druggability range, assayablility, essentiality and molecular weight of the targets and the features were given different weights. The scores for parameters such as druggability and assayablility were set to an optimal value of +35. The criterion of essentiality was regarded significant in the study and therefore it was given a score of +50. The criterion of molecular weight bears the score of +20 depending on the weight of the polypeptide of the target gene.

**Presence of crystal structure**

The crystal structures assist in rational drug design by providing essential practical assets in high throughput screening for lead generation and optimization studies. Both, Protein Data Bank (PDB) (http://www.rcsb.org) and ModBase database (http://modbase.compbio.ucsf.edu) were manually searched for the presence of protein crystal structures of *Leishmania* as obtained from the above study. The scores for the availability of crystal structure were kept as high as +50 because it confers mainly pragmatic advantages, while identifying a new drug target.

**Probable binding site analysis for the 10 prioritized targets**

Binding sites and active sites are often associated with structural features of receptors/proteins. The study of proteins binding pocked volume and its electrochemical properties may aid in the rational design of novel compounds. In this study, we analyzed the size of the binding pockets of the all prioritized targets of *Leishmania* and human homologs. This provides a preliminary explanation for the large divergences in the size of binding pockets (Fig. 3). Some of these prioritized targets are present only in *Leishmania.* For example, Trypanothione reductase (TR) is found exclusively in *Leishmania* and can be targeted for obtaining anti-leishmanial (Table 1) agents.

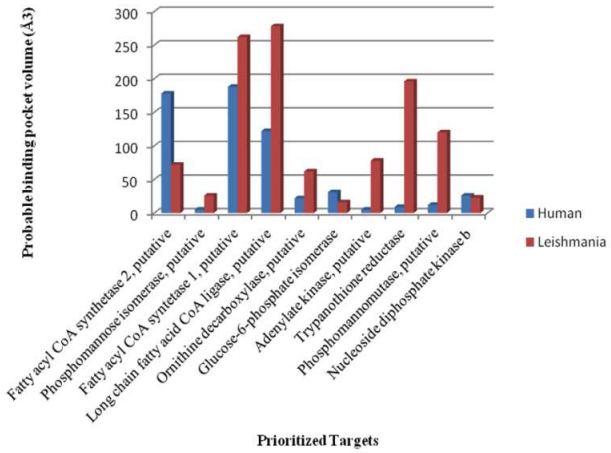


**Fig. 3** Comparative representation of probable binding pocket volume analysis for the ten targets.

**Structural information on the prioritized targets**

Applying all the essentiality criteria yielded four crystal structures out of the top ten prioritized proteins. The four crystal structures were obtained from Protein Data Bank (PDB) and were subjected to further analysis of probable binding pocket volume analysis. The four targets are Trypanothione reductase (TR), Nucleoside diphosphate kinase B (NDKb), Glucose-6-phosphate isomerase (G6PI) and Phosphomannomutase (PMM).

Further, the corresponding human proteins were obtained from the PDB and the targets from the both *Leishmania* and human were analyzed using comparative Molecular Electrostatic Potential (MEP) calculation and cavity-depth (CD) analysis.

**Trypanothione reductase(TR)**

Trypanothione reductase (TR), an NADPH-dependent disulfide oxidoreductase (Fig. 4) is identified as a unique viable chemotherapeutic drug target for *Leishmania.* Trypanothione reductase (TR) is acting as a main detoxificant of toxic radicals released during dexoyribonucleotide and hydroperoxide synthesis.[52-54] Various computational approaches were implemented to identify prospective ligands and High-throughput screening lead the identification of novel inhibitors for Trypanothione reductase.[55, 56]

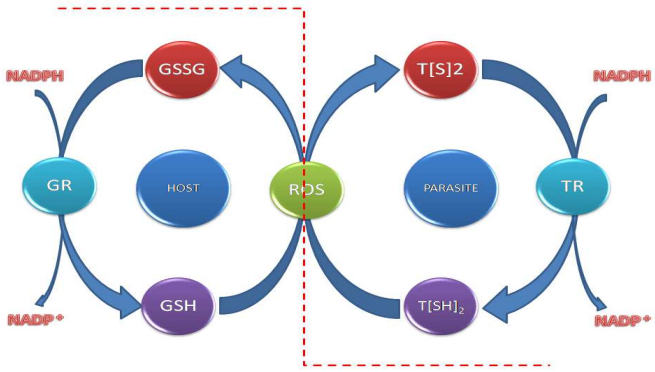


**Fig. 4** Trypanothione and glutathione redox system

Until now six crystal structures (PDB ID: 4APN, 4ADW, 2YAU, 2X50, 2JK6, 2W0H) are published for *Leishmania infantum* trypanothione reductase.

BioLip[61] indicated that residues viz., SER14, LEU17, GLU18, TRP21, VAL53, VAL58, SER109, TRY110, MET113, SER394, SER395, PHE396, THR397, LEU399, HIS461 and GLU467 are presumably present in the active site.

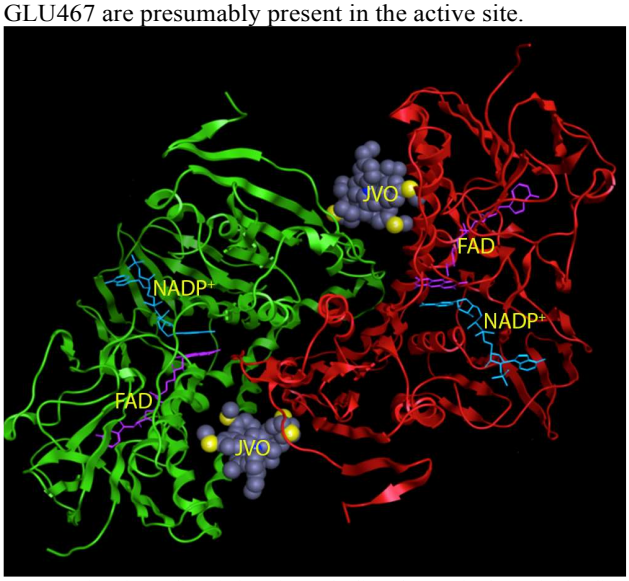


**Fig. 5** Active site at dimeric interface. The ligand (JVO)is shown in the dimeric interface.

Paola B et al also reported the binding of the ligand in the dimeric interface and showed that CYS52 and CYS57 are essential residues for the overall catalysis. Numerous gold containing compounds were also evaluated by Colotti G et al. Repurposing of anti-arthritic drug aurinofin, a gold containing ligand was clinically established for thiol and selenol groups of proteins like trypanothione reductase.[57-60] A comparative analysis of trypanothione reductase TR was also carried out with glutathione reductase, a well known target which is present in human which is also a NADPH-dependent disulfide oxidoreductase but they are mutually exclusive in terms of its substrates glutathione and its analog trypanothione. Comparative representation of probable binding pocket volume analysis of trypanothione reductase and glutathione reductase showed huge difference between pocket volumes showing its rationality to define as a drug target (Fig. 3). The blast results also show there is 35% sequence identity with glutathione reductase, indicating that trypanothione reductase (TR) acts is specific target for *Leishmania*.

**Nucleoside diphosphate kinase B (NDKb)**

Nucleoside diphosphate kinase B (NDKb) was also used for detailed comparative study of the probable binding site as its crystal structure for both human and *Leishmania* are available. Nucleoside triphosphate is a precursor for DNA and RNA synthesis. NDKb enzyme is responsible for nucleoside triphosphates synthesis, which is used, in various cellular processes by *Leishmania* species. NDKb is mainly involved in transfer of γ phosphate from nucleoside triphosphates to nucleoside diphosphates. The whole reaction is depicted as follows:

$$NDK + N_1TP \leftrightarrow NDK - P + N_1DP \leftrightarrow NDK - P + N_2DP \leftrightarrow NDK + N_2TP$$

Its role in purine-salvage pathway makes this target more attractive for drug discovery because protozoa unable to synthesize purines by de novo mechanism and depend upon host for survival.

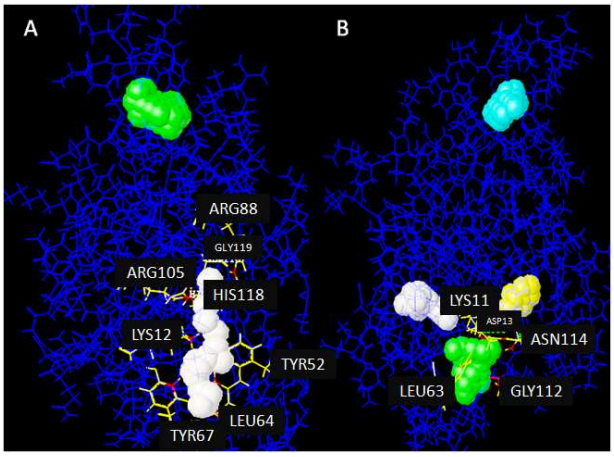


**Fig. 6** Nucleoside diphosphate kinase B (NDKb) binding site (A) Human Nucleoside diphosphate kinase B binding site. This figure indicates the probable binding site of NDKB with the important residues (B) *Leishmania* Nucleoside diphosphate kinase B binding site. Green and yellow pocket together form a binding pocket, which are accordance with experimental results.

Probable binding site analysis of NDKb reveals that the active site of human (with volume 18Å$^3$) is smaller than that of site of *Leishmania* with volume 30Å$^3$) (Fig. 6).[30] SiteID reported the presence of 8 residues (Tyr52, Leu64, Tyr67, Arg68, Arg105, Lys12, His118 and Gly119) in human NDKb (PDB ID: 1NUE) active site, in which the presence of His118 is in accordance with the experimental results of Morera *et al.*.[31]*Leishmania*NDKb (PDB ID: 3NGS) active site showed the presence of Asp13, Leu63, Lys11, Tyr66, Gly112, Asn114, Arg87, Thr93, and Arg104 in this presence of Lys11, Arg87, Thr93, Arg104, Leu63, Tyr66, Gly112 and Asn114 In the binding.[32]
Further MEP and cavity-depth analysis were performed on human and *Leishmania* NDKb to differentiate the active site. Superimposing both *Leishmania* NDKb and human NDKb provided RMSD deviation of 0.94 A. Cavity depth analysis

indicates that *Leishmania* NDKb binding site cavity is slightly deep and wider than the human NDKb showing the 15.90 Å

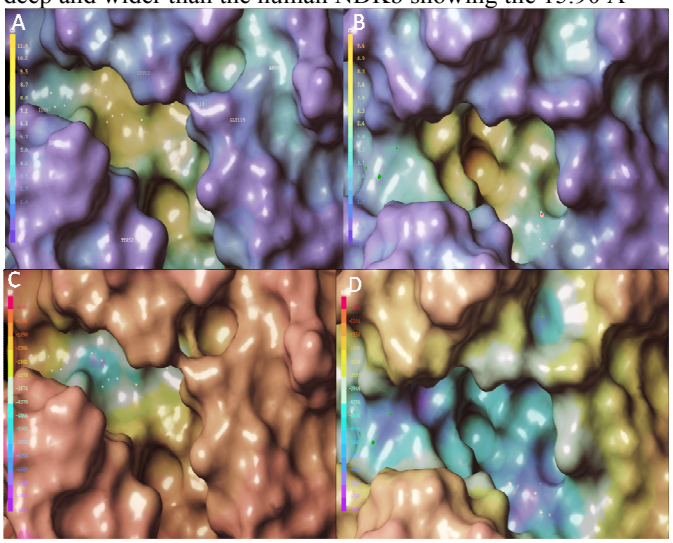


**Fig. 7** Nucleoside diphosphate kinase (NDKb) cavity depth (Fig. A and B) and MEP analysis (Fig. C and D) (A) Human Nucleoside diphosphate kinase cavity depth. Cavity is narrow and long than the *Leishmania* NDKb (B) Leishmania Nucleoside diphosphate kinase B cavity depth. Cavity is wider than human NDKb (C) Human Nucleoside diphosphate kinase B MEP analysis. Colour coding indicates that this has low electronegative potential than *Leishmania* NDKb (D) *Leishmania* Nucleoside diphosphate kinase MEP analysis. Deep purple colour indicates more electronegative potential of the *Leishmania* NDKb and difference from human NDKb cavity.

distance between LYS52 –GLY118 residues in *Leishmania* NDKb (Fig. 7A) and 14.00Å in between ILE52 - GLY118 in case of human NDKb as and (Fig. 7B). The MEP analysis report is shown corresponding (Fig. 7C and 7D).

These results show that human active site has less electronegative potential than that of *Leishmania*, and there are significant differences in these two structures proven as a valuable step toward the understanding of specific ligand design.

Glucose-6-phosphate isomerase (G6PI) and Phosphomannomutase (PMM) active site analysis details are given in (Supplementary information 2).

**Relation to a close homolog**

Targets are sorted out and ranked or prioritized on the basis of high-level sequence homology to its host or any other homolog. Eventually, intended pathogen specific drug target may be sought out which is having a remedial influence and without undesirable secondary effect on host.

One of our goals was to prioritize *L. major*-specific targets. Thus, positive high score of +25 was imputed to targets, which are absent in the human host and highly conserved across the *Leishmania* species in order to subside interactions in the host. Homologs to humans were penalized by because they may involve in undesirable interactions with proteins of which may be toxic in human.

## Discussion

The biggest factor resulting in the diversity of species and their genomes is natural selection, parasites that are better suited to their changing environment would have different characteristics and will favorably adapt to that change and as a result. Such changes are favored by the environment and will become more frequent due to the increased chances of the organisms survival. Out of the various unicellular eukaryotes known, *L. major* is unique in its evolutionary development and success rate of survival may be driven by a genetic abnormality.[12]

The laboratory conditions and experiments have induced various resistance mechanisms, which are increasingly being understood by DNA microarrays and proteomics studies.[33] The current challenge is to get a prioritized drug targets by systematically deleting genes and reactions involved with relative to the biomass (i.e. observable phenotypes) in a stage specific genome-scale metabolic model of *L. major* and this information can be useful in the endeavour for tracking resistance. The metabolic reactions distinctively expressed in stage specific amastigote of *L. major* are catalyzed by enolase, alcohol dehydrogenase, ATP synthase and hexokinase. We altered the boundary states of these reactions to alter the genome scale metabolic model in to amastigotes stage. Based on the computational algorithms such as MOMA and FBA, we obtained genes that affected the growth rate to the maximum extent in case of both single and double gene deletions. These genes are considered to be essential for the survival of the organism as observed by the knock out studies and thus these can be potential targets against the infection. By performing FBA, we identified 10% of reaction knockouts in the network as lethal and 12% as trivial lethal, and proposed 78% as non lethal reactions. In case of MOMA the results were slightly different such as 10% were found as lethal, 34% as trivial lethal and 56% as non-lethal. In case of amastigotes the results were nearly similar to that obtained for wild type, with 10% genes as lethal and 12% as trivial lethal, and proposed 79% as non-lethal using FBA. Whereas, MOMA provided 10% as lethal, 34% as trivial lethal and 56% as non lethal in amastigotes showing that the metabolic pathway has undergone some adjustment by taking another path for completion and thus providing other essential targets. These essential targets are further subjected to different protein essentiality criteria with scoring. The study of subcellular localization of proteins is crucial for genome annotation, protein function prediction and drug discovery.

The knowledge of protein localization helps in characterizing the cellular function of hypothetical and newly discovered proteins. Subcellular localization determines the environments in which proteins operate and influences protein function by regulating access to and availability of all types of molecular interaction partners. We observed that the proteins were present in different organelles of the parasite. Some were present integral to membrane or in mitochondria, cytosol, glycosome, nucleus, flagellum, or other complexes. The (Fig. 2E) shows the percentage wise distribution of various proteins in different cellular compartments with maximum number of proteins present in the cytosol.

These highly prioritized targets are subjected to BLAST against the entire human proteome of NCBI to get homology information and to filter out the proteins with high similarity (Fig.8D).

We performed active site analysis on top 10 prioritized targets. This reveals the similarity between the *Leishmania* and human proteins. This study reveals that there are small

differences between the homologues but if we consider cavity depth and electrostatic potential of active site it can give clues for better design drugs.

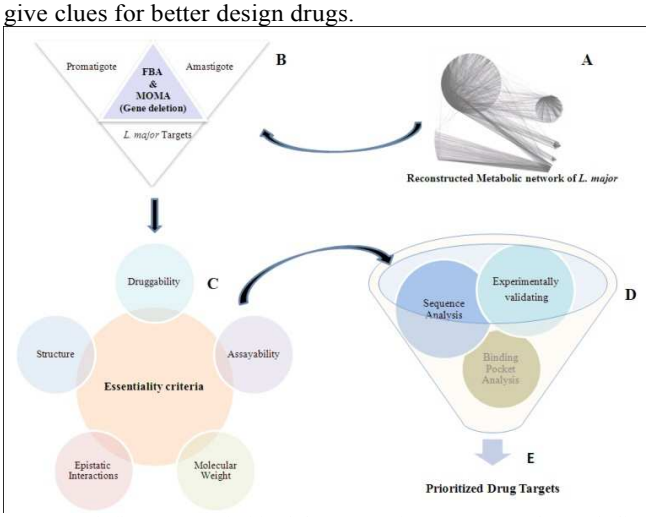


**Fig. 8** Work flow to get prioritized drug target from genome scale metabolic network. (A) Metabolic network of *L.major* consisting of 560 genes and their corresponding 1112 reactions and 1101 metabolites. (B) Analyzing gene deletions and their corresponding reactions through MOMA and FBA (C) Filtering through several essentiality criteria (D) Sequence and structure level analysis (E) Prioritized drug targets for *L.major*.

All these promising drug targets are compared with an experimentally testable hypothesis. The metabolic network was validated with experimental knockout data from related *Leishmania* and *Trypanosoma* species. The findings have enabled us to discover more about the organism's natural variation and genetic structure, which is vital for developing effective treatments. These findings have important implications and help in understanding the parasitic variations and the genetic basis of disease. Dispensable genes upon various analysis might be important, but under conditions not yet examined in the laboratory.

## Materials and Methods

We used a metabolic model of *L. major*[12] in order to get essential drug targets. Quadratic programming was applied to first find a feasible flux distribution. For all predictions, Minimization Of Metabolic Adjustment (MOMA) was employed to simulate the behavior of gene knockouts and their corresponding reactions that are essential for the production of biomass in the proliferating cells. During the knockdown process few genes reduced the maximal biomass production rate in >1 and those genes were considered to be growth supporting. The majority of the knockouts (95%) results in either no reduction of growth on its deletion were identified in the obtained outcome. The commercial CPLEX solver through the TOMLAB® optimization environment was utilized for resolving linear programming (LP), quadratic programming (QP), and other problems on an Intel Pentium 4 processor operating on Windows 7.

**FBA** It maximizes an objective function under different constraints using LP. In this study, we seek for a steady state flux distribution (v) that gives rise to the optimization of the phenotype under mass balance, flux capacity and thermodynamic constraints. [34-37] The LP problem is exemplified as follows:

$$\max f^T v,$$

$$S.v = 0,$$

$$v_{\min} \le v \le v_{\max}$$

Where $S$ is an n × m sparce matrix which could incorporate experimental data including number of metabolites denoted by "n", and number of reactions denoted by "m", f is an objective function maximization of biomass in the metabolic networking which is indicated by a reaction that deplete biomass constituents. Thermodynamic constraints ($T$) that restrain the flow of direction and capacity constraints are obligated by setting $v_{min}$ and $v_{max}$. Whereas $v_{min}$ is denoted as lower bound and $v_{max}$ is denoted as lower bound and $v_{max}$ as upper bound on flux distribution.

**MOMA** It uses quadratic programming to minimize the sum of squares difference between wild type and mutant flux distribution. It solves by minimizing Euclidean distance metric from the wild type flux distribution commonly not obtained by FBA. [13] MOMA tests the hypothesis that a gene deletion causes minimal flux redistribution with respect to the wild type metabolism.
MOMA problem is exemplified as follows

$$min(v-w)^T(v-w),$$

$$S.v = 0,$$

$$v_{min} \le v \le v_{max,}$$

$$vj = 0,$$

$$j \in A$$

Whereas $w$ denoted as wild type flux distribution, and set of reactions related with the knock out genes are denoted as A. MOMA searches for a point in the null space of a mutant metabolic network with the shortest distance from the initial flux vector of a wild type metabolic network. This objective function can be formalized as a standard quadratic programming (QP) problem under a set of linear constraints. If the null space of a mutant network is not empty, a solution that minimizes the distance will always exist. MOMA can be used for effectively calculating the significant phenotypic behavior of several metabolic networks after a gene deletion[13, 38] providing an alternative perspective for understanding metabolic systems under various perturbations.

## Gene deletion analysis

We have performed gene deletion mutants for all dispensable genes of the *L. major* (iAC560) and analysis was carried out using COBRA Toolbox 2.0.4. The gene was assumed to be essential, if the corresponding gene has highest effect (>90% effect on biomass) on the growth. The results obtained from the *in silico* experiments were compared with previously published experimental results. Single and Double gene deletions were helpful in the identification of genes that are essential for growth of *L. major*. Knockdown experiment was performed for individual genes in the metabolic model and by filtering the obtained

outcome the genes were classified into lethal or essential with respect to amastigotes of *Leishmania*.

We used MOMA to observe single gene and double gene deletion on the basis of biomass components; and the results were compared. Transport and exchange reactions reported in the literature or in databases were included in the model.[39] The biomass calculation provides a range of metabolites that are essential to support growth of the metabolic system.[36,40,41]

**Epistasis**

Epistasis happens when the growth rate is being affected due to mutation at one point of a locus determined by the occurrence of mutations in other loci of the metabolic network.[42]Since, epistasis illustrates genome wide genetic interactions using gene knockouts.[43] It provides additional insights into the significant portion of antibiotic resistant mutations that are research hotspots. Epistasis in the usual sense defines the concept of statistical interaction.[13, 44]Precisely; the quantitative genetic concept of epistasis may be epitomized for two loci by the linear model.

$$y = \mu + a_1x_1 + d_1z_1 + a_2x_2 + d_2x_2 + i_{aa}x_1x_2 + i_{ad}x_1x_2 + i_{da}x_1x_2 + i_{dd}z_1z_2$$

Where $x_i$ and $z_i$ are dummy variables and $y$ is a quantitative phenotype related to the underlying stage specific genotype at locus i. The coefficients $\mu$, $a_1$, $d_1$, $a_2$ and $d_2$ denote genetic parameters that may be estimated corresponding to the mean effect and additive and dominance effects at the two loci; $i_{aa}$, $i_{da}$, $i_{da}$ and $i_{dd}$correspond to epistatic interaction effects. Lack of epistasis in this model implies that all interaction coefficients are zero.

**Drug Target Identification**

**Weight selection for metabolic and *L.major*-specific target lists**

**Essentiality data**

In this study, simulations were carried out using COBRA Toolbox 2.0.4. The genes were assumed to be essential, if the corresponding gene has highest effect (>90% effect on biomass) on the growth. The results obtained from the *in silico* experiments were compared with previously published experimental results. We used MOMA to observe gene knockout on the basis of biomass components.[39] The biomass calculation provides a range of metabolites that are essential to support growth of the metabolic system.[36, 40, 41] Genome wide gene knockout or knockdown studies can be used to predict genes that are essential for growth. This criterion has been considered as vital in our studies and weighed heavily (+50 if essential).

**Druggability**

In this work for estimating druggability we used DoGSite[45], a structure-based technique to predict druggability and protein active sites based on a Difference of Gaussian (DoG) approach which originates from image processing. For each query a druggability score between zero and one is returned. The higher the score the more druggable the pocket is estimated to be. Proteins that scored >0.8 was considered as significant. This criterion allows users to search for pathogen genes that may be considered as drug targets. A heavy weight, +35, was chosen for the "druggablity" feature in the metabolic list. This weight was chosen because the chances of finding a known "druggable" domain to be much higher in proteins was expected, which could be mapped to known metabolic pathways. In the *L. major* specific list, quiet a large number of the prioritized targets were in the anticipated druggability limits.

**Assayability**

To select targets that are most amenable to high throughput screeningfor a suitable lead compound, biochemical and/or cellular assays for growth inhibition or lethality, binding and function are required. Target is considered assayable if an enzyme is included in Sigma-Aldrich's collection of assays, or if it has been assayed according to the BRENDA database. Establishing a meaningful assay depends on target class and the information on the target and it should be more heavily weighted relative to the other measures. Here we present +35 for new target tracking.

**Epistasis**

The percentage of modulated genes and their interactions should be considered significant to understand the regulation of gene expression in *Leishmania* occuring at different levels, which could contribute to disease tropism.[46] Comparative stage specific epistatic analysis in *L. major* is the first to date and may lead to understand genome-wide studies of genetic interactions using gene knockouts. Through this network approach we may uncover hidden variation in genome-wide association and thus helping to understand the disease mechanisms. Additionally, the proposed metabolic approach holds a promise for characterizing the gene-gene interaction landscape broadly in epistatic studies. We provided +25 for epistatic interactions.

**Structure**

Crystal structures mainly considered of practical applications that would not be the primary consideration to determine a new drug target. The weights for these features were kept low at +7 for have a crystal structure from PDB (http://www.rcsb.org)) and +5 for having PBD structure from ModBase database (http://modbase.compbio.ucsf.edu).

**Molecular weight**

Often in a drug discovery program, low molecular weight protein targets are considered as emerging targets for the design of novel therapeutic agents in drug discovery and this molecular weight parameter may in part explain oral bioavailability. It is generally regarded as too difficult to be targeted. We provided +20 for the molecular weight of <100 kilo Dalton.

**Sequence homology with human proteome and human gut microbiota**

The complete human proteome from NCBI and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to filter out the proteins, which hold homology of Hits accompanied with the identity of ≥35%, e-value of ≤0.0001 with ≥75% sequence coverage. Here we have chosen a heavy weight, +25 for the filtered out proteins. Microbes in human gut and oral flora are deeming to be influencing the immunological and physicochemical properties of the host and have a significant role in its development. To compare the predicted proteins, a word size of 3 with CD-HIT similarity of 60% was used against the complete human gut microbiota.

### Binding site similarity analysis

Out of the filtered 54 proteins, 33 proteins possess considerable extent of structural similarity at the binding site level with human proteome, while on the contrary 21 proteins are not having identical binding site with human proteome. Analysis of binding site identity with human proteome, given us interesting points to note that several targets that we listed are already experimentally validated drug targets.

### Binding site analysis

Identification of the pocket characteristics like occluded cavities and surface pockets is helpful in comparative analyses of protein binding sites thereby use in target assessment and validation. Normally, 1-2 mouth openings are observed at binding sites. These pockets differ broadly in size, the majority within the range of 102-103 Å. Most commonly, the biggest pocket/cavity in the protein is the active site; however there are a numerous edifying exceptions.[47] For this study, structures of highly scored from previous studies were collected from Protein Data Bank (PDB) and/or modbase.[48,49] To identify the difference between the active site of highly prioritized drug targets in human and *Leishmania*, we performed probable binding sites, Molecular Electrostatic Potential (MEP) and Cavity-Depth (CD) analysis using SiteID and MOLCAD module of SYBYL7.1[50] on a Silicon Graphics tezro workstation. SiteID module is used to determine the size and location of the probable binding pockets in proteins. It identifies the pockets using two methods: Grid Method and Solvation Method. Grid method uses flood-fill algorithm, which generates numerous water spheres to form clusters that can be the probable active site inside the protein. This method was used to determine the probable binding sites of proteins that are present in *Leishmania* and human. Cavity depth analysis indicates the depth of the cavity from the surface. Molecular surface of all protein structures were generated by MOLCAD module of SYBYL7.1 and cavity depth analysis was performed on highly scored proteins.[48]
Molecular electrostatic potential of protein surface can guide to identify the interaction site of receptor with ligand. Gasteiger-Hückel charges were applied to the proteins. The targets that are well scored from previous studies proteins were subjected to Molecular Electrostatic Potential (MEP) analysis and cavity depth analysis to get highly prioritized drug targets for *Leishmania* species[48] (Supplementary information 2).

### Literature dependent target validation

The list or predicted drug targets are substantiated based on underlying functional evidence obtained from the review of literature. By using manual curation technique and data mining made us to identify the literature based validated drug targets in *L. major*. Furthermore with literature survey we validated the proteins are accounted to be essential or non-essential in terms of *Leishmania* growth and survival.

## Conclusions

The clinical manifestations of leishmaniasis can be caused by several species of parasites belonging to the genus *Leishmania*. Worldwide, there are 12 million people currently infected and 350 million people at risk of leishmaniasis, but the existing treatment options mostly rely on ancient pentavalentantimonials, which have many limitations including systemic toxicity in humans and drug resistance in parasites. Usually during the infectious life cycle, *Leishmania* interchange among the insect promastigote stage and the vertebrate aflagellateamastigote stage that proliferates within infected host macrophages provoking the pathology of the disease. Our research focuses on discovering new stage specific drug targets, which is significant for contemporary drug discovery of neglected tropical diseases, like leishmaniasis. We applied several essentiality criteria to prioritize the drug target lists for *L. major,* the causative agent of leishmaniasis. With our results, researchers are be able to use the lists of highly prioritized drug targets, those can be further carry out experimentally.

## Acknowledgments

We are grateful to Haokip for excellent technical assistance.

## Author Contributions

Conceived and designed the experiments: MES, MLSP, AK. Performed the experiments: MLSP, MES, AK. Analyzed the data: MES, MLSP. Contributed reagents/materials/analysis tools: MES, MLSP. Wrote the paper: MLSP, MES, AK, and AG.

### Conflict of Interest

The authors declare that they have no conflict of interest.

## References

1 A. F. Cowman, B. S. Crabb,*Trends Parasitol.*, 2003,**19**, 538-543.
2 P. D. Marsden,*N. Engl. J. Med.*, 1979,**300**, 350-352.
3 S. L. Croft,*Trop. Med. Int. Health*, 2001, **6**, 899-905.
4 S. L. Croft, S. Sundar, A. H. Fairlamb,*Clin.Microbiol. Rev.,* 2006, **19**, 111-126.
5 S. Sundar, *Trop. Med. Int. Health*, 2001,**6,** 849-854.
6 A. G. M. Tielens, J. J. V. Hellemond,*Trends Parasitol.,*2009,**25**, 482-490.
7 A. Wagner, *Genome Res.*,2002, **12**, 309-315.
8 J. J. V. Hellemond, A. G. M. Tielens,*FEBS Letters*, 2006,**580**, 5552-5558.
9 C. Guimond, N. Trudel, C. Brochu, N. Marquis, A. El Fadili, R. Peytavi, G. Briand, D. Richard, N. Messier, B. Papadopoulou, J. Corbeil, M.G. Bergeron, D. Légaré, M. Ouellette,*Nucl. Acids Res.*, 2003,**31**, 5886-5896.
10 M.V. Rockman,*Nature*, 2008,**456**, 738-744.
11 J. Tegnér, M. K. S. Yeung, J. Hasty, J. J. Collins *Proc. Natl. Acad. Sci. USA,* **100**,2003, 5944-5949.
12 A.K. Chavali, J. D. Whittemore, J.A. Eddy, K.T. Williams, J.A. Papin,*Mol. Syst. Biol.*, 2008,**4**, 177.
13 D. Segrè, D. Vitkup, G.M. Church, *Proc. Natl. Acad. Sci. USA*, 2002,**99**, 15112-15117.
14 T. Shlomi, O. Berkman, E. Ruppin, *Proc. Natl. Acad. Sci. USA*, 2005,**102**, 7695-7700.
15 P. Kharchenko, G.M. Church, D. Vitkup, *Mol. Syst. Biol.*, 2005, **1**, 2005.0016.
16 R. Mahadevan, B. O. Palsson, *Biophys. J.*, 2005,**88**, L07-L09.
17 A. L. Barabasi, Z.N. Oltvai, *Nat. Rev. Genet.*, 2004,**5**, 101-113.
18 H. Jeong, S.P. Mason, A.L. Barabasi, Z.N. Oltvai, *Nature*, 2001, **411**, 41-42.
19 H. Kitano, *Nat. Rev. Drug.Discov.,*2007,**5**: 202-210.
20 A.R. Renslo, J.H. McKerrow,*Nat. Chem. Biol.*, 2006,**2**, 701-710.
21 L. Xie, L. Xie, P.E. Bourne, *Curr.Opin.Struct. Biol.*, 2011, **21(2)**, 189-99.

22 A.R. Brochado, S. Andrejev, C.D. Maranas, K.R. Patil,*PLoSComput. Biol.*, 2012,**8(11)**, e1002758.
23 L.M. Blank, L. Kuepfer, U. Sauer,*Genome Biol.*,2005,**6**, R49.
24 D. Segrè, A. Deluna, G.M. Church, R. Kishony,*Nature Genet.*, 2005,**37**, 77-83.
25 R. Harrison, B. Papp,C. Pál, S.G. Oliver, D. Delneri,*Proc. Natl. Acad. Sci. USA*, 2007,**104**, 2307-2312.
26 R. Mani, R.P. St. Onge, J.L. Hartman, G. Giaever, F.P. Roth,*Proc. Natl. Acad. Sci. USA*, 2008,**105**, 3461-3466.
27 L.D. Hurst,*Heredity*, 2000,**85**, 625-626.
28 L. Guarente, *Trends Genet.*, 1993, **9**, 362-366.
29 E.S. Snitkin, D. Segrè,*PLoS Genet.*, 2011,**7**: e1001294.
30 J. Janin, C. Dumas, S. Moréra, Y. Xu, P. Meyer, M. Chiadmi, J. Cherfils,*J.Bioenerg.Biomembr.*, 2000,**32(3)**, 215-25.
31 S. Moréra, I. Lascu, C. Dumas, G. LeBras, P. Briozzo, M. Véron, J. Janin, *Biochemistry*, 1994,**33(2)**, 459-467.
32 T. A. Souza, D. M. Trindade, C.C. Tonoli, C.R. Santos, R.J. Ward, R.K. Arni, A. H. Oliveira, M. T. Murakami,*Mol.Biosyst.*, 2011,**7(7)**, 2189-2195.
33 P.J. Myler, N. Fasel,*Caister Academic Press, Norfolk, UK,* 2008.
34 D. A. Fell, J.R. Small, *Biochem. J.,* 1986,**238**, 781-786.
35 K.J. Kauffman, P. Prakash, J.S. Edwards,*Curr.Opin.Biotechnol.*, 2003,**14**, 491-496.
36 A. Varma, B. O. Palsson, *Nat.Biotechnol.*, 1994,**12**, 994-998.
37 M. R. Waston, *Comput. Appl.Biosci.*, 1986,**2**, 23-27.
38 B. Papp, C. Pal, L. D. Hurst,*Nature*, 2004,**429**, 661-664.
39 G. Plata, T.L. Hsiao, K.L. Olszewski, M. Llinás, D. Vitkup D, *Mol. Syst. Biol*., 2010,**6,** 408.
40 J. S. Edwards, B. O. Palsson, *Proc. Natl. Acad. Sci. USA.*, 2000,**97**, 5528-5533.
41 A. Varma, B. O. Palsson, *J.Theor. Biol.*, 1993,**165**, 503-522.
42 F. Roth, H. Lipshitz, B. Andrews, *J. Biol.,* 2009,**8**, 35.
43 P.C. Phillips,*Nat. Rev. Genet.*, 2008,**9**, 855-867.
44 E.S. Snitkin, D. Segrè, *Genome Inform.*, 2008,**20**, 123-134
45 A. Volkamer, D. Kuhn, T. Grombacher, F. Rippmann, M. Rarey,*J. Chem. Inf. Model.*, 2012,**52(2)**, 360-372.
46 M.J. Herrgård, S. S. Fong, B. Ø. Palsson, *PLoSComput. Biol.*, 2006,**2(7)**, e72.
47 C. Mattos, B. Rasmussen, X. Ding, G. A. Petsko, D. Ringe,*Nat.Struct. Biol.*, 1994,**1(1)**, 55-58.
48 H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig H, I. N. Shindyalov, P. E. Bourne,*Nucl. Acids Res.*,2000,**28**, 235 - 242.
49 U. Pieper, N. Eswar, B. M. Webb, D. Eramian, L. Kelly, D. T. Barkan, H. Carter, P. Mankoo, R. Karchin, M. A. Marti-Renom, F. P. Davis, A. Sali,*Nucl. Acids Res.*, 2010,**37**, 1–10.
50 Tripos Associates Inc., *SYBYL Molecular Modelling Package*, Tripos Associates Inc., St. Louis, Mo, USA, 2001.
51 Segrè D, Zucker J, Katz J, Lin X, D'haeseleer P, Rindone WP, KharchenkoP,Nguyen DH, Wright MA, Church GM.*OMICS.*2003, **7,** 301-16.
52 Khan MO. *Drug Target Insights*.2007, **2**, 129-46.
53 Singh N, Kumar M, Singh RK. *Asian Pac J Trop Med*. 2012, **5**, 485-97.
54 Rai S, Dwivedi UN, Goyal N. *BiochimBiophysActa.* 2009, **1794**, 1474-84.
55 Perez-Pineiro R, Burgos A, Jones DC, Andrew LC, Rodriguez H, Suarez M, Fairlamb AH, Wishart DS. *J Med Chem.*2009,**52**, 1670-80.
56 Holloway GA, Charman WN, Fairlamb AH, Brun R, Kaiser M, Kostewicz E, Novello PM, Parisot JP, Richardson J, Street IP, Watson KG, Baell JB. *Antimicrob Agents Chemother*. 2009, **53**, 2824-33.
57 Sannella AR, Casini A, Gabbiani C, Messori L, Bilia AR, Vincieri FF, MajoriG,Severini C. *FEBS Lett*. 2008, **582**, 844-7.
58 Baiocco P, Colotti G, Franceschini S, Ilari A. *J Med Chem*. 2009, **52**, 2603-12.
59 Colotti G, Ilari A. *Amino Acids*. 2011, **40**, 269-85.
60 Ilari A, Baiocco P, Messori L, Fiorillo A, Boffi A, Gramiccia M, Di Muccio T, Colotti G. *Amino Acids*. 2012, **42**, 803-11.
61 Yang J, Roy A, Zhang Y. Nucleic Acids Res. 2013, 41: 1096-103.Cottret L, Wildridge D, Vinson F, Barrett MP, Charles H, Sagot MF, Jourdan F. Nucleic Acids Res. 2010 ,**38**, 132-7.