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An *in silico* approach in identification of drug targets in *Leishmania*: A subtractive genomic and metabolic simulation analysis

Rohan J. Meshram[a,*], Mayuri B. Goundge[a,1], Baban S. Kolte[a,b], Rajesh N. Gacche[b]

[a] Bioinformatics Centre, Savitribai Phule Pune University, Pune 411007, India
[b] Department of Biotechnology, Savitribai Phule Pune University, Pune 411007, India

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ABSTRACT

Leishmaniasis is one of the major health issue in developing countries. The current therapeutic regimen for this disease is less effective with lot of adverse effects thereby warranting an urgent need to develop not only new and selective drug candidates but also identification of effective drug targets. Here we present subtractive genomics procedure for identification of putative drug targets in *Leishmania*. Comprehensive druggability analysis has been carried out in the current work for identified metabolic pathways and drug targets. We also demonstrate effective metabolic simulation methodology to pinpoint putative drug targets in threonine biosynthesis pathway. Metabolic simulation data from the current study indicate that decreasing flux through homoserine kinase reaction can be considered as a good therapeutic opportunity. The data from current study is expected to show new avenue for designing experimental strategies in search of anti-leishmanial agents.

1. Introduction

Leishmaniasis is a vector-borne disease caused by parasite *Leishmania*. This disease is responsible for high rates of mortality and morbidity, especially in tropical regions of the world including India. Leishmaniasis manifests different clinical symptoms including cutaneous, mucosal, and visceral forms [1]. Both the cutaneous and mucosal forms can cause severe deformities to patients, including ulcerative skin lesions and the destruction of mucous membranes, in some cases leading to permanent disfigurement. Visceral leishmaniasis is the most severe form of *Leishmania* infections that results in death of an individual and causes blackening of skin and hence called 'Kala Azar' or Black Death. This disease is found to be prevalent in > 88 countries on the globe and 0.7 to 1.2 million cases of cutaneous Leishmaniasis, while 0.2 to 0.4 million incidences for Visceral Leishmaniasis are reported annually [2]. This disease represents the greatest threat to human health, with symptoms ranging from fever, weight loss, swelling, edema, paleness to hepatosplenomegaly, leading to death in untreated cases [3].

The term *Metabolome* refers to entire metabolic content of an organism; while, *Comparative Metabolomics* refers to an approach for identification of differences in host and parasite metabolomes that leads to recognition of unique parasite enzyme which can act as effective drug targets. A "choke point reaction" is a biochemical reaction in which either a specific substrate is uniquely consumed or a specific product is uniquely produced in the metabolic network [4]. Targeting choke point enzymes from parasites can result in pathogen fatality, since it leads to either depletion or results in toxicity due to accumulation of certain metabolites in a pathway [5,6]. The norm of vitality plays an important role for an enzyme to be considered as target, because unless it holds key position in pathogen physiology, its inactivation will have no effect. The essentiality test on the Database of Essential Genes (DEG) resource is usually conducted in order to find out if the identified unique and choke point enzyme performs vital function in pathogen [7]. Deducing non homology of identified parasite enzyme with human genome ensures the fact that any homolog of identified target protein in human shall not be curtailed by drug identified to inhibit pathogen enzyme. Since this analysis starts out comparing metabolomes of two organisms on genomic scale, and subsequently the number of enzymes are trimmed at each step; therefore, such methodology is generally referred to as subtractive genomic approaches [8]. Prior attempts to implement subtractive genomic approach in target identification in *Leishmania* suffers limitations in terms of comprehensive druggability analysis as well as fewer tests were executed for shortlisting the drug targets [9]. Comparatively, the current study applies more rigorous shortlisting measures and discuss in detail the druggability issue to ensure identification of more potent drug targets in *Leishmania major*.

* Corresponding author.
E-mail address: rohan_meshram@rediffmail.com (R.J. Meshram).
[1] First co-author.

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Systems Biology based simulation of network models is becoming an important technique in identification of drug targets in prokaryotic as well as eukaryotic pathogens [10]. This method, relies on the calculation of metabolic flux values of a well-defined set of stoichiometric reactions in a metabolic network [11]. Individual metabolites participating in the network are mass-balanced. In addition, the metabolic system is presumed to be functional in the pseudo-steady state [12,13]. The biggest advantage of such constraint based flux analysis is that it allows analysis of structure of metabolic networks to identify weak points in it that can be effectively used as potential drug targets. The current study focuses on identification of putative drug targets implementing the subtractive genomics approach. Furthermore, metabolic simulation of identified pathways supports the druggability of identified drug targets.

2. Materials and methods

2.1. Subtractive genomic methodology

A hypergraphs based tool Rahnuma was utilized in comparison of entire metabolic networks of *Leishmania* and Human [14]. All available Kyoto Encyclopedia of Genes and genomes (KEGG) pathway maps of human and *Leishmania* were subject to standard comparative analysis using full network comparison mode on Rahnuma interface. MetExplore program was utilized for performing choke point analysis [15]. Choke point reactions were computed by selecting *Leishmania major* biosource. DEG (Database of essential gene) was searched to conduct the vitality test of identified target proteins [7]. BLAST-P was utilized for individual searching the chokepoint reactions (identified in previous step) in DEG database. The existence of an ortholog in DEG database indicates that the given protein is essential for survival of pathogen. If the query proteins exhibited e-value < 0.0001 [16,17] with similar function and 80% query coverage of the gene products in DEG, such enzymes were considered as orthologous to each other and likely to be essential gene products in *Leishmania major* [4]. Non homology analysis was carried out by performing a series of PSI-BLAST searches in human genome using default values for algorithm parameters. PSI-BLAST is a variant in BLAST algorithm that relies on profile based search methodology to identify distant homologs of the given query sequence [18]. It can be confirmed that a close homolog of any given protein is not present in given organism genome, if PSI-BLAST yields no significant new hits even after numerous iterations. Therefore, at the end of non-homology analysis, the list of targets was significantly narrowed down to only those enzyme that are confirmed to be non-homologous to human and represent final targets for our analysis.

2.2. Metabolic simulation method

2.2.1. Model construction

COmplex PAthway SImulator (Copasi) version 4.2.3 was utilized to build and simulate the kinetic model of the Threonine synthesis pathway [19]. The biochemical reactions of the pathway were obtained from BioCyc database [20] and crosschecked with KEGG database [21]. Initially, the compartments were defined followed by adding up the reactions involved in the Threonine synthesis pathway. The participating metabolites are abbreviated as per Supplementary Table 1. Reactions were assigned their respective rate laws as per Table 1 and their parameters were defined as per Table 2. Reactions with two substrates/two products have rate equations containing more than one parameters. These parameters were obtained from literature [22] and remaining parameters were obtained from BRENDA [23]. Initial concentrations of the metabolites were then defined.

2.2.2. Analysis protocol

The network was subject to steady state analysis followed by metabolic control analysis and flux balance analysis. Sensitivity analysis was performed and finally, time course simulations were conducted. Initially, time course simulation was performed to study the behaviour of model with respect to time. The effect of inhibition of two key enzymes homoserine kinase and threonine synthase in the threonine biosynthesis pathway were simulated by editing the equation of rate laws of respective enzymes and appending the theoretical expression of competitive inhibitor as shown in Eq. (A)

Eq. A: Expression for competitive inhibition term.

$$\frac{Vmax.\ [S]}{Km\left(1+\frac{I}{Ki}\right)+S} \tag{A}$$

where V_{max} is maximum velocity, S is substrate concentration, K_m is Michaelis menten constant, I concentration of inhibitor and K_i is inhibition constant. Term $\left(1+\frac{I}{Ki}\right)$ is considered as α.

For example, as per the literature [22], the rate law followed by homoserine kinase is shown in Eq. (B):

Eq. B: Expression for rate law of homoserine kinase.

$$\frac{\text{VHK. [hs]. [ATP]}}{\begin{array}{l}\{[\text{Khs}(1+[ATP]/\text{KiATP})(1+(Thr)/(\text{KiThr}))\\ +[hs]]*[\text{KATP}(1+[hs]/\text{Kihs})+[ATP]](1+[Lys]/\text{KiLys})\}\end{array}} \tag{B}$$

For simulating the effect of an inhibitor, the above mentioned equation was edited to include the term for a competitive inhibitor as illustrated in Eq. (C):

Eq. C: Expression for rate law of homoserine kinase appended with competitive inhibitor term.

$$\frac{\text{VHK. [hs]. [ATP]}}{\begin{array}{l}\{[\text{Khs}(1+[ATP]/\text{KiATP})(1+(Thr)/(\text{KiThr}))\\ +[hs]]*[\text{KATP}(1+[hs]/\text{Kihs})+[ATP]](1+[\text{Lys}]/\text{KiLys})\}\\ +\dfrac{Vmax.\ [S]}{Km\left(1+\frac{I}{Ki}\right)+S}\end{array}} \tag{C}$$

Similarly, the expression for threonine synthase was also edited to monitor the effect of its inhibition on the threonine biosynthesis pathway.

While simulating the inhibition, the value of α was initialized to zero and iterated up to ten in order to observe the changes in concentration of the product as well as steady state was observed at each iteration. The inhibition was performed in both, coupled and decoupled mode. In decoupled mode, the kinetic expression of one enzyme was edited at a time. While in coupled mode of inhibition, the inhibitory effect on both the enzymes were performed simultaneously.

3. Results

3.1. Comparative metabolomics

We have identified 1627 common reactions, 97 were found unique to pathogen metabolome, while 1036 were exclusive to humans and remaining 492 were common to both. These 97 unique reactions participate in 43 metabolic pathways encircling amino acid, lipid, carbohydrates, nucleotide, and cofactor metabolism, pathways that include glycolysis and Kreb's cycle and other metabolic conversions. Supplementary Fig. 1 depicts the statistics of results obtained in comparative metabolomics section. Our results indicate that leishmanial metabolome possess highest number of unique reactions that belongs to biosynthesis of secondary metabolites (15 reactions) followed by glutathione metabolism (12 reactions) and cysteine and methionine metabolism (11 reactions). Other unique reactions from remaining metabolic pathways detected in this study ranges from 1 to 6 reactions.

Table 1
Name of the reaction and rate law used during simulation.

Reaction name	Rate laws
AK	$\frac{VAK1\left([ASP].[ATP]-\left([aspp].\frac{[ADP]}{Keq}\right)\right)}{\left\{\left[KASP\left(\frac{1+\left(\frac{[Thr]}{KiThr}\right)hThr}{1+\left(\frac{[Thr]}{\alpha KiThr}\right)hThr}\right)+[aspp]((KASP)(Kaspp))+[Asp]\right]*\left[KATP\left(1+\left(\frac{[ADP]}{KADP}\right)\right)+[ATP]\right]\right\}}$
ASD	$\frac{VASD\left([aspp].[NADPH]-\frac{[ASA].[NADP+].[Pi]}{KeqASD}\right)}{\{[Kaspp(1+([ASA]/KASA))(1+([Pi]/KPi))+[aspp]][KNADPH(1+([NADP+]/KNADP+)+[NADPH]]\}}$
HDH	$\frac{VHDH\left([ASA].[NADPH]-\frac{[hs].[NADP+]}{Keq}\right)}{\{[(1+([Thr]/KiThr)h)/(1+([Thr]/\alpha KiThr)h)]][KASA+[hs](KASA/Khs)+[ASA]]*[KNADP(1+[NADP+]/(KNADP+))+[NADPH]]\}}$
HK	$\frac{VHK.[hs].[ATP]}{\{[Khs(1+[ATP]/KiATP)(1+(Thr)/(KiThr))+[hs]]*[KATP(1+[hs]/Kihs)+[ATP]](1+[Lys]/KiLys)\}}$
TS	$\frac{VTS.[hsp]}{Khsp+[hsp]}$

Table 2
Name of the reaction and kinetic parameters used during steady state and *in silico* inhibition of enzyme during simulations.

Enzyme	Kinetic parameters	Inhibition	Keq
AK	Aspartate → 0.97 ± 0.48 ATP → 0.98 ± 0.5 aspp → 0.017 ± 0.004 ADP → 0.25	KiThr 0.167 ± 0.003 mM hThr 4.09 ± 0.26 α 2.47 ± 0.17	$6.4*10^{-4}$
ASD	aspp → 0.022 ± 0.001 NADPH → 0.029 ± 0.002 ASA → 0.11 ± 0.008 NADP → 0.144 ± 0.02 Pi → 10.2 ± 1.4		$2.84*10^{5}$
HDH	ASA → 0.24 ± 0.03 NADPH → 0.037 ± 0.006 Homoserine 3.39 ± 0.33 NADP → 0.067 ± 0.006	KiThr 0.097 mM h 1.41 α 3.93	$1*10^{11}$
HK	Homoserine → 0.11 ATP → 0.072	KiThr 1.09 mM KLys 9.45 mM Kihomoserine 4.7 mM KiATP 4.35 mM	
TS	hsp 0.31 ± 0.03		

3.2. Choke point analysis

Metexplore utilizes BioCyc [24] metabolic resource for identification of choke point reactions while Rahnuma uses KEGG [21] for metabolic comparisons. Hence, utilizing both the major metabolic information resources had ensured comprehensive and optimal exploitation of available metabolic space in our analysis. Using choke point analysis, 62 choke point reactions were shortlisted. Out of 97 unique metabolic reactions identified in previous steps, only 11 reactions were found to be catalyzing choke point reactions. These reactions are catalyzed by enzymes including homoserine dehydrogenase (EC 1.1.1.3), L-gulonolactone oxidase (EC 1.1.3.8), ascorbate peroxidase (EC 1.11.1.11), serine *O*-acetyltransferase (EC 2.3.1.30), cysteine synthase (2.5.1.47), homoserine kinase (EC 2.7.1.39), β-fructofuranosidase (EC 3.2.1.26), indolepyruvate decarboxylase (EC 4.1.1.74), enoyl-CoA hydratase (EC 4.2.1.17), threonine synthase (4.2.1.17) and trypanothione synthetase (EC 6.3.1.9). Additionally Metexplore identified few of the unique choke point reactions that were missed while using Rahnuma server, these include sucrose-phosphate synthase (EC 2.4.1.14), aspartate-phenylpyruvate aminotransferase (EC 2.6.1.70), fructokinase (EC 2.7.1.4), methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) and UDP-galactopyranose mutase (EC 5.4.99.9). Some false positive reactions were identified as unique and chokepoint by Metexplore and interestingly, these reactions were identified to be present in both the organisms (*Leishmania* and Human) by Rahnuma server. These reactions include hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) and adenine phosphoribosyltransferase (2.4.2.7) that catalyze important reactions in salvage pathways of purines. Following reactions were also identified on MetExplore server that were missed on Rahnuma: transketolase (2.2.1.1), methylene-tetrahydrofolate dehydrogenase (EC 1.5.1.5), UDP-glucose 4 epimerase (EC 5.1.3.2), phosphomannomutase (EC 5.4.2.8), aspartate-ammonia ligase (EC 6.3.1.1), glutathionylspermidine synthetase (6.3.1.8) and enzymes from GPI anchor biosynthesis. Considering enormous therapeutic application of these enzymes, all the enzymes identified in this step were promoted for subsequent analysis. The number of chokepoint reactions identified and their respective metabolic pathways are presented in Supplementary Fig. 2. The highest number of choke point reactions was observed in fatty acid metabolism followed by ascorbate biosynthesis and glycoinositolphospholipid (GIPL) biosynthetic pathway.

3.3. Essentiality test on DEG database

Further essentiality test results indicate that 14 short listed enzymes (out of 73 choke point catalyzing reactions) are vital for pathogen survival. It is worth to note that most of the false positive reactions identified during choke-point analysis were eliminated in this step. Supplementary Table 2 summarizes the shortlisted vital enzymes from leishmanial metabolome.

3.4. PSI-BLAST results to ensure non-homology

As discussed in earlier sections, an ideal target enzyme should not have any homolog in human. Therefore, PSI-BLAST was utilized to find if homologs to these identified targets are present or not in human genome. Finally we obtained list of 6 enzymes that do not contain homolog in human genome. The enzymes known to be present in humans (for example hypoxanthine phosphoribosyl transferase, adenine phosphoribosyl transferase etc) were completely eliminated during this filter; thereby leaving the most potential therapeutic targets. These final six enzymes thus represent appropriate targets for *Leishmania*. Supplementary Table 3 summarize the set of enzymes that represent potential targets against leishmaniasis.

3.5. Metabolic simulation results

3.5.1. Steady state and time course analysis

It is very imperative to make sure that steady state is reached for the given system. Acquiring steady state insures that the amount of metabolites that are in-fluxed and out-fluxed are same. The plot of concentration of metabolites against time is generally analysed to infer if steady state is reached. As seen in the Fig. 1. The convergence of concentration values indicates reaching the steady state after initial dynamics.

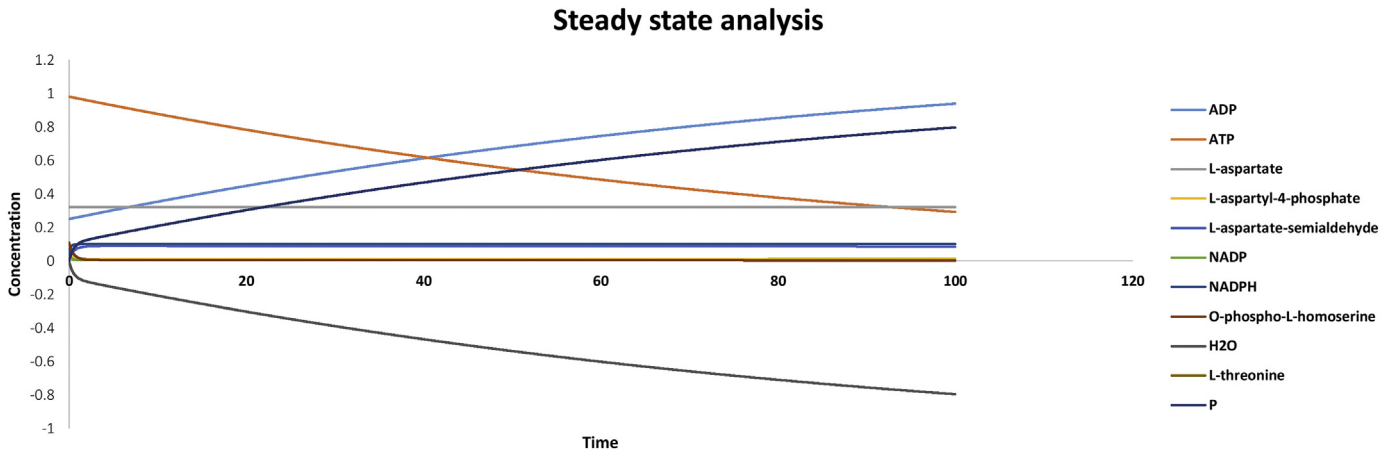

Fig. 1. Results from time course and steady state analysis.

The steady state fluxes and steady state concentrations of various metabolites through threonine synthesis pathway are provided in Supplementary Tables 4 and 5.

The main objective of performing steady state is to understand the dynamic behaviour of the system. It is generally conducted by performing linear stability analysis. The system is said to be well behaved if we demonstrate that steady state is stable. Stability of steady state is inferred from the convergence of concentration of metabolites in due course of simulation. Convergence indicate that system is well settled [25].

3.5.2. Sensitivity analysis

Sensitivity analysis is carried out to test the consequence of the parameters on the variables of the model like fluxes and metabolite concentrations [26]. During the simulation, the model outputs in the form of change in concentration of metabolites in the system. The ratio of this model output to the perturbation on parameters (which are expressed in terms of enzyme's Michaelis constant) refers to the sensitivity of the model on the given set of parameters [27,28].

Fig. 2A indicates that the reaction catalyzed by enzyme Aspartate Semialdehyde dehydrogenase is sensitive to the initial concentration L Aspartate Semialdehyde. While Fig. 2B represent sensitivity of all parameters of the model and their values. It can be clearly inferred that parameter KASA (i.e Michaelis constant for Aspartate semialdehyde in the rate law) shows highest sensitivity. Such observations can help initiate experimental studies to investigate these metabolic reactions in therapeutic perspective. These trends provide comprehensive details of dynamics involved in threonine synthesis pathway and demonstrate critical importance of this reaction in the pathway.

3.5.3. Metabolic control analysis

As per results obtained from metabolic control analysis (Fig. 3), the final enzyme of the pathway, threonine synthase shows highest flux control coefficient (8.6047). Other enzymes of the pathway had significantly lower flux control coefficient in comparison with this enzyme. Aspartate β semialdehyde dehydrogenase was observed to possess second highest flux control coefficient (5.8) followed by homoserine kinase (2) in the pathway. In metabolic point of view, two methods can be implemented for elimination of the pathogen. If the enzymes with high flux control coefficients are inhibited, it might decrease flux through the pathway to such a level that pathogen could not sustain [29]. Therefore, based on this data, wet lab experiments can be designed to target these enzymes to check if its inhibition restricts the pathogen growth or survival.

3.5.4. In silico enzyme inhibition

Based on results of subtractive genomics data (discussed in subsequent sections) and MCA analysis, we targeted threonine synthase and homoserine kinase for *in silico* enzyme inhibition.

The *in silico* inhibition of those enzymes performed sequentially, also their effect on steady state fluxes and concentration of metabolites was assessed. Both reactions in which HSK and TS are involved are reversible and are two substrate and two product reactions. Hence while inhibiting the enzymes, the expression of rate laws of

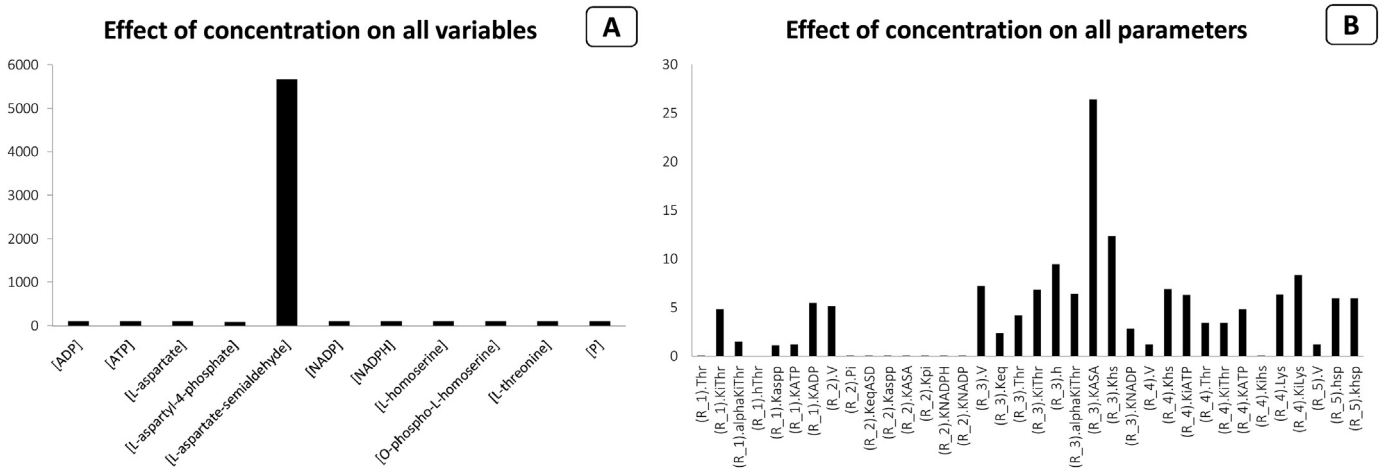

Fig. 2. Results obtained from sensitivity analysis (A) Effect of concentration on all variables of the model (B) Effect of concentration on all parameters of the model.

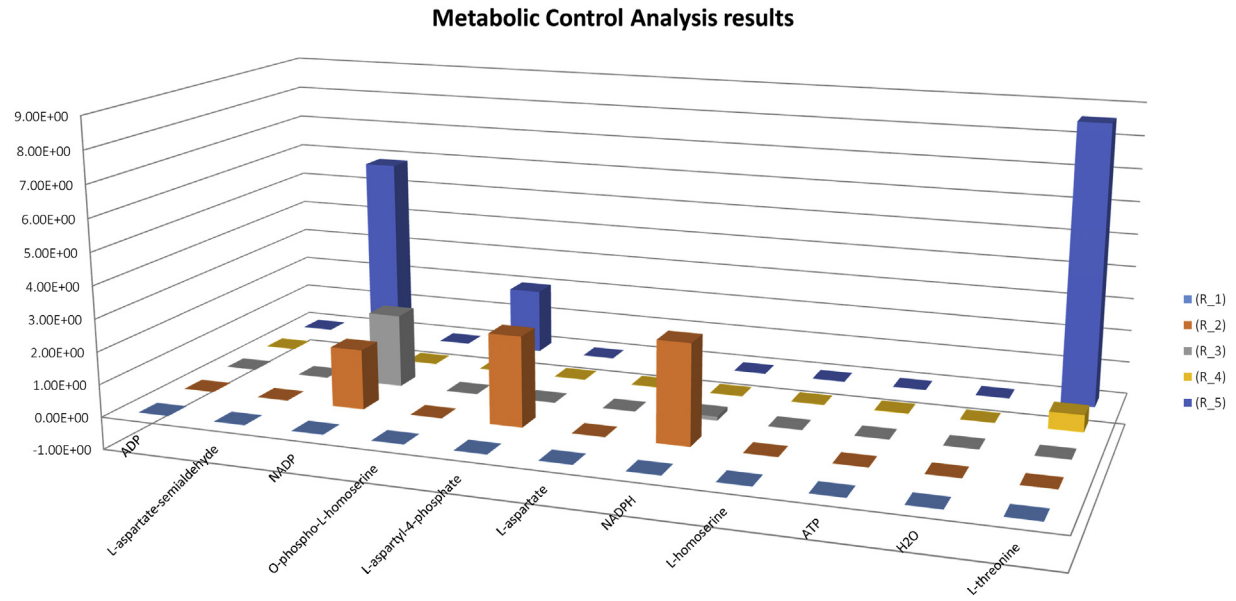

Fig. 3. Results obtained from MCA. Flux control coefficient are plotted against reaction and metabolites.

corresponding enzymes were edited to include the term for competitive inhibitor in order to observe the change in metabolic concentration as per the time changes. As mentioned earlier, changing the value of α changes the concentration of metabolites, the expression of competitive inhibitor included in the corresponding rate laws of both enzymes thereby demonstrates inhibitory effect.

To observe the effect of inhibition on overall network, the enzyme was inhibited separately and also in couple mode. Several model variants were constructed to explore the possibilities, reaction coupling due to HSK involved reaction acted as substrate for TS involved reaction. To observe the dependency of second reaction on first, coupled model was prepared while decoupled model was prepared to study behaviour of each reaction individually.

3.5.4.1. Inhibition of homoserine kinase. As mentioned above the initial concentration of the inhibitor was iterated from 0 to 10 to observe changes in the production of o-phospho homoserine and in result (Fig. 4) we observe high amount of decrease in concentration of o-phospho homoserine which can make it as effective drug target. While iterating the inhibitors concentration, we observed that at $(1 + I/Ki = 10)$, the percent decrement caused in concentration of homoserine through the pathway was about 95%, for competitive inhibition.

3.5.4.2. Inhibition of threonine synthase. As discussed earlier threonine is an important key enzyme for the survival of *Leishmania*. While simulating the inhibition of TS individually we observed similar amount of concentration of threonine in each iteration, all the iterative results shows initial decrement in the concentration followed by the subsequent increment in the concentration of threonine. Although these results appear to be contradicting, it can be attributed to facts like (1) High flux rate for this enzyme. (2) During the simulations, flux boundaries are usually applied over the enzyme. As a result of flux boundaries, the enzyme inhibition is supported only if the concentration exceeds the threshold. However, in due course of simulation when the flux continues and small amount of reactants are passed on to the enzyme in below threshold level, thereby increasing the threonine concentration. (3) In the model created in this study, the pathway terminates at the formation of threonine and do not account for further consumption of this metabolite in reactions that follows it. *In vivo*, the flux of threonine will be carried out towards next reaction and will not accumulate as it seems in this study.

3.5.4.3. Inhibition of both the enzymes in coupled mode. To observe the dependency of threonine synthesis reaction on the homoserine kinase based reaction, coupled model was prepared because intermediate of this coupled model *i.e.* o-phospho-homoserine who act as substrate for reaction 1 and product for reaction 2. The evidence for coupling comes

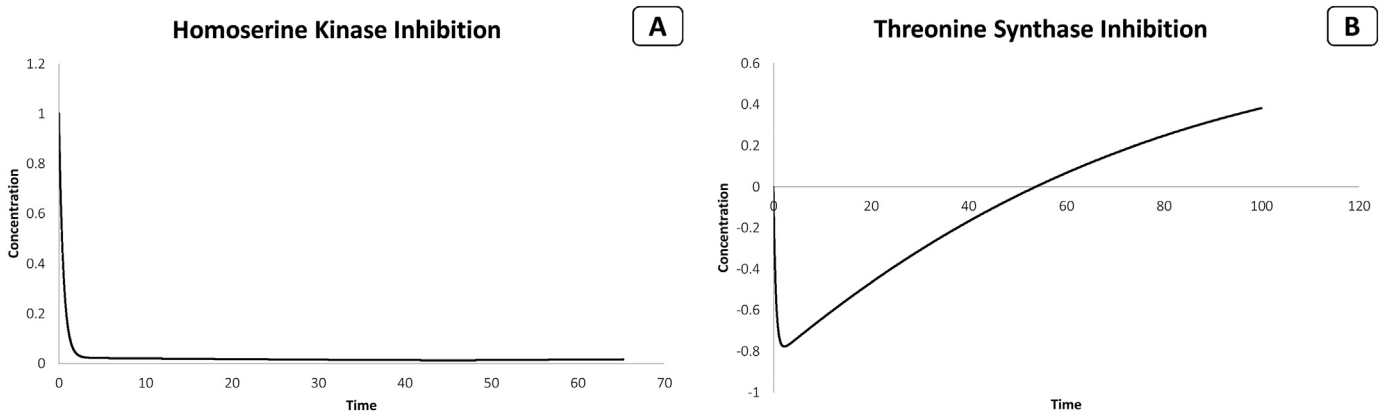

Fig. 4. *In silico* inhibition of homoserine kinase and threonine synthase in decoupled mode.

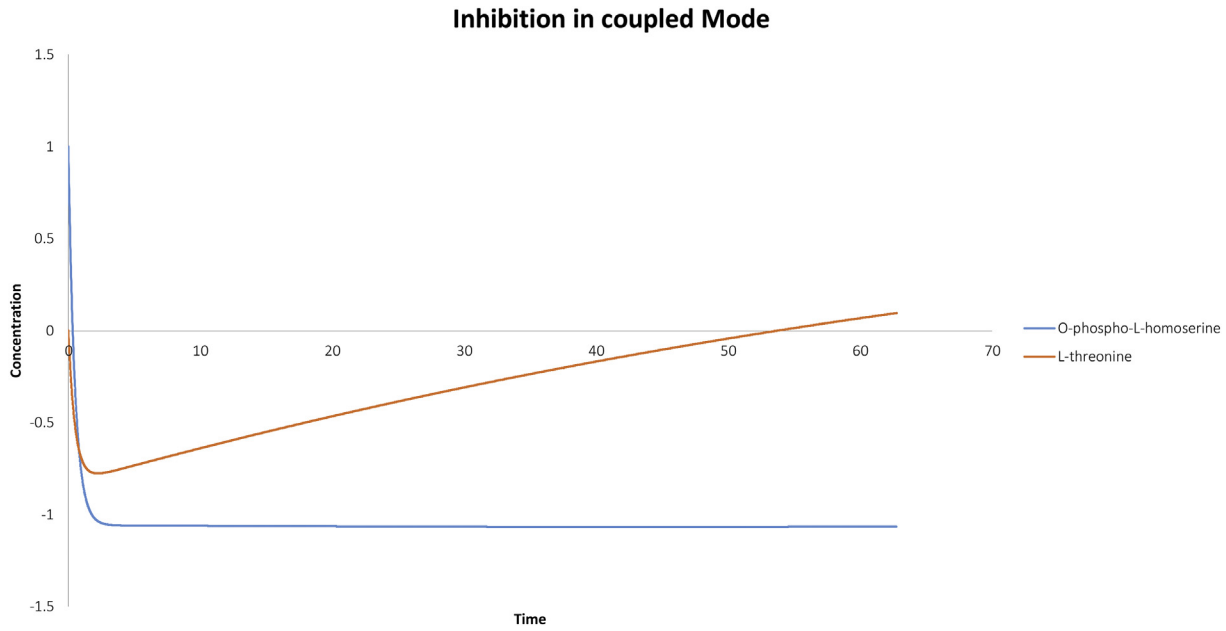

Fig. 5. *In silico* inhibition of homoserine kinase and threonine synthase in coupled mode.

from the fact that *Leishmania* does not have any other route for synthesizing o-phospho-homoserine except homoserine kinase based reaction 1.

The resulting graph (Fig. 5) demonstrates the changes occurred in the concentrations of homoserine & threonine in due course of time. Blue coloured line indicate concentration of ortho phospho homoserine which shows decrement in concentration while the orange coloured line indicates Threonine which shows increment in the concentrations can be credited to facts mentioned above. Based on *in silico* inhibition data, we can confidently assume homoserine kinase as potential drug target.

4. Discussion

4.1. Subtractive genomics

It is estimated that there are some 8272 protein-coding genes in leishmanial genome [30] and very recent report published that human genome is suspected to contain about 20,500 protein coding genes [31,32]. Therefore we began our analysis with comparing these genes and we keep on short listing the number of potential target on basis of facts like uniqueness, chokepoint, essentiality (or vitality) and occurrences of leishmanial homologs in human genome. In results obtained from comparative metabolomics analysis carried out in this study, we have identified total six enzymes from four metabolic pathways (Supplementary Table 3) as putative and suitable targets for therapeutic intervention. *Leishmania major* is a trypanosomatid that occupies a very unique position in tree of life. Trypanosomes are present at the very intersection that gave rise to plants, fungi and animals, therefore it has retained some prokaryotic metabolic pathways as well as evolved many functional metabolic pathways that reflect plant like, animal like and fungi like properties. Therefore, metabolic composition of *Leishmania* displays shades of biochemical variety from multiple kingdoms.

4.2. Choke point analysis

Advancements in biological applications of graph theory has recently accelerated pace of research in infection biology and metabolic analysis [33,34]. According to graph theory, a metabolic network can be represented in terms of nodes (metabolites) and connectors/edges that connects these nodes (reactions) [35]. Topological analysis of these networks has already demonstrated an impact on identification of therapeutic drug targets [36,37]. In graph theory context, metabolic pathways can be represented as a directed graph, which in turn is defined as an ordered triple expression:

$$G = (V, E, f)$$

where f corresponds to a mathematical function implemented to map every element in edge (E) to an ordered pair of vertices (V) [35]. These ordered pairs of vertices are also referred to as directed edges. Thus, a directed edge E can be formulated as

$$E = (i, j)$$

where directionality of E is considered from i to j [38]. As per these directives, a choke point can be defined in a directed network as a node connected with single outgoing edge or a solitary incoming edge. Therefore, an algorithm searching for choke points in a topology of metabolic network shall entail two aspects (1) searching for a particular Reaction X1 in such a way that only one reaction X1 generates metabolite M and at least single reaction should exits that consumes metabolite M (Fig. 6A). (2) searching reaction X2 in a network that is present in such a manner that only a single reaction X2 consumes metabolite M and a minimum one reaction exist that creates M (Fig. 6B). These algorithms are implemented provided that pathway is unidirectional and M do not correspond to a dead-end metabolite in a network. For example, in threonine biosynthesis pathway (Fig. 6C) identified in present study, reactions catalyzed by enzymes homoserine kinase and threonine synthase perfectly fit in the concept of chokepoint reactions discussed above.

4.3. Druggability assessment of the identified metabolic pathways

The following section discuss the significance of the identified metabolic pathways followed by therapeutic applications of inhibiting these enzymes.

4.3.1. Threonine biosynthesis in Leishmania

Generally, biosynthesis of threonine from aspartate begins with cofactor dependent phosphorylation event of aspartate to give 4-phospho-L-aspartate. This reaction is catalyzed by a dimeric multifunctional enzyme aspartate kinase (EC 2.7.2.4) [39]. Phosphorylated aspartate undergoes dehydrogenation reaction to produce aspartyl

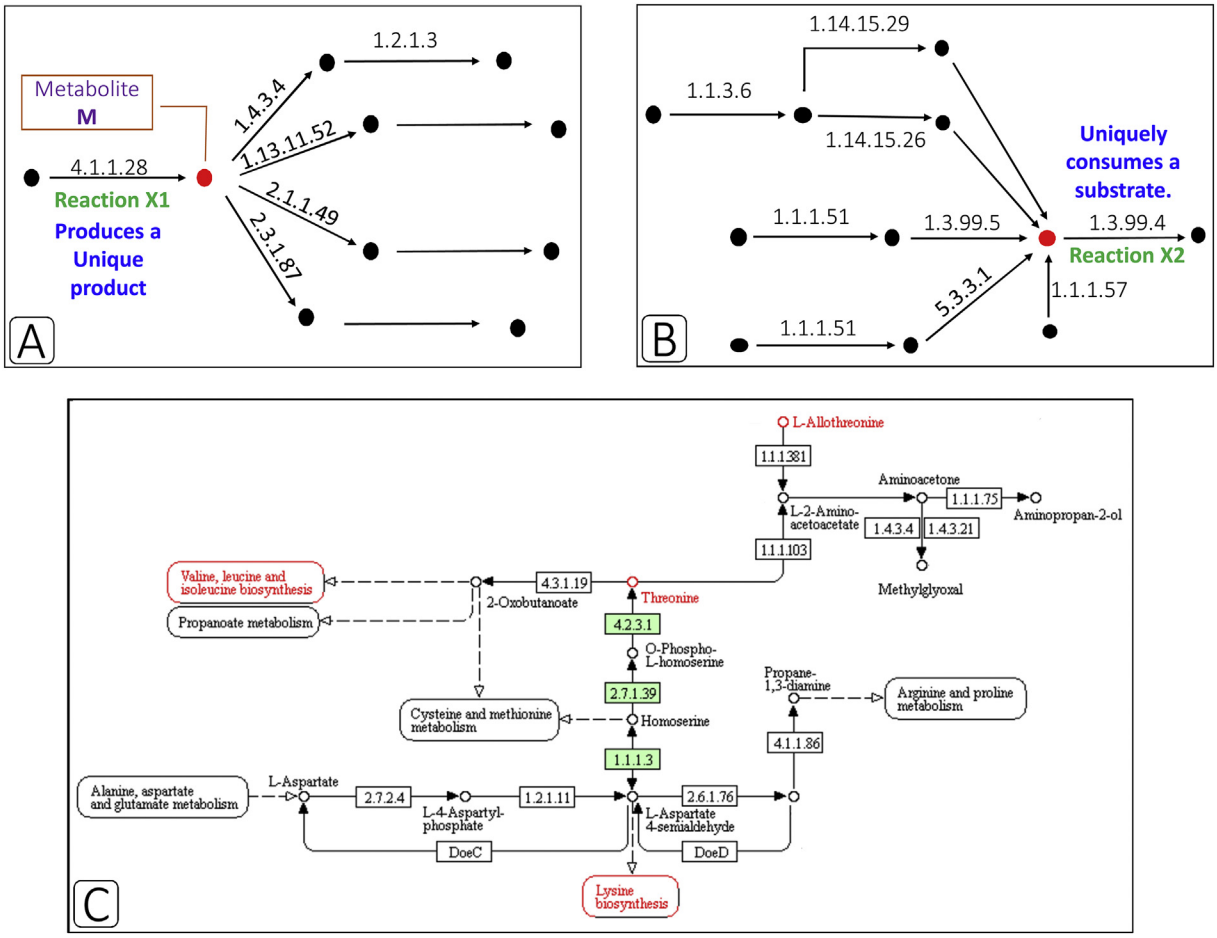

Fig. 6. Choke Point Analysis (A) and (B) represents general depiction of choke point reaction X1 and X2 described in text. (C) Illustration demonstrating chokepoint reactions identified in threonine biosynthesis pathway. Reactions catalyzed by enzymes with EC number 4.2.3.1 (threonine synthase) and 2.7.1.39 (homoserine kinase) corresponds to choke point reactions and are highlighted in green. It is to be noted that enzyme homoserine dehydrogenase (1.1.1.3) is also highlighted, but cannot be considered as chokepoint reaction since it is bidirectional. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

semialdehyde, which on another dehydrogenation yields L-homoserine. Aspartate beta-semialdehyde dehydrogenase (EC 1.2.1.11) is a key homo dimeric NADPH reliant enzyme in threonine biosynthetic pathway catalyzing the second reaction in the aspartate pathway [40]. This enzyme brings out transfer of the pro-S hydrogen from NADPH to produce positively charged aspartyl semialdehyde [41]. Homoserine dehydrogenase is a bi-functional NADH dependent oxido-reductase holoenzyme that catalyzes formation of homo-serine from aspartyl semialdehyde [39] (Fig. 7). After kinase activity on homo-serine yields phospho-homoserine, which upon hydration generates threonine. Homo-serine kinase is the fourth enzyme in threonine biosynthesis [42] that catalyzes cation dependent phosphorylation of homoserine following a random bi-bi mechanism of action [43]. The final reaction in *de novo* threonine biosynthesis is biochemically mediated by threonine synthase [44], a pyridoxal 5′-phosphate dependent [45] multimeric protein that eliminates phosphate group from beta position of phospho-homoserine forming threonine [46,47].

Biochemical data published by Opperdoes and Coombs supports the verity that threonine is in fact synthesized in L. *major* and involves enzymes HSK and TS [48]. In humans, threonine is considered as an essential amino acid which is obtained from external dietary sources. Therefore, these enzymes are completely absent in humans and thus making them as attractive drug targets in *Leishmania*. HSK and TS catalyzes the final and committed steps in threonine biosynthesis and inactivation of HSK is already established to cause threonine auxotrophy in bacteria as well as eukaryotes [49]. Deactivation of these enzymes might result in threonine starvation in *Leishmania* hampering its fitness for survival leading to homoserine-mediated lethality and eventually establish as effective drug targets.

4.3.2. *Ascorbate metabolism*

The general reactions of ascorbate metabolism entail initial six biochemical reactions that generate gulono lactone starting from glucose 1 phosphate (Fig. 8). Gulono lactone on oxygenation creates (*erythro*) ascorbate that undergoes ascorbate peroxidase reaction to yield dehydro (erythro) ascorbate. Oxygenation of gulono lactone is a final reaction in ascorbate biosynthesis [50] and this reaction is enzymetically catalyzed by guluno lactone oxidase (EC number 1.1.3.8), a flavoprotein (FAD) that generates a transient and unstable intermediate compound xylo-hexulonolactone that spontaneously isomerize to ascorbate. Ascorbate peroxidase reaction is responsible for scavenging most of the free radicals that can potentially create oxidative stress. Ascorbate peroxidase is a heme containing protein that oxidizes ascorbate and low molecular weight aromatic substrates. The monodehydroascorbate radical produced is either directly reduced back to ascorbate by mono-dehydro-ascorbate reductase or undergoes non-enzymic disproportionation to ascorbate and dehydro-ascorbate. Ascorbate peroxidase enzyme is actively found in mitochondrial intermembrane space of *Leishmania* and thus utilized to tolerate the oxidative stress created by reactive oxygen species [51,52]. If dehydroascorbate is present in *Leishmania*, then logically, a molecule similar to ascorbate must exist; which again, must have been synthesized by

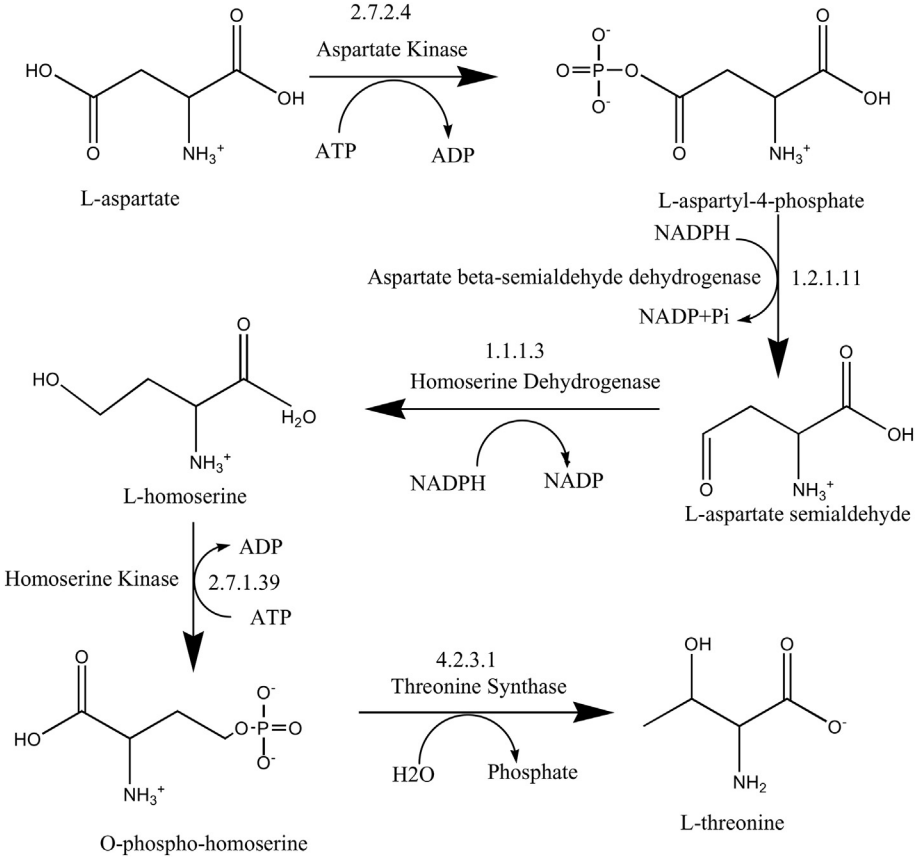

Fig. 7. Metabolic biosynthesis of threonine from aspartate.

gulono lactone oxidase like enzyme.

The presence of ascorbate-dependent peroxidase in *Leishmania* indicates the existence of ascorbic acid in trypanosomatids and this ascorbic acid might be used in protection from oxidative stress in host macrophages. Genome mining and *in vitro* investigational data recently published demonstrated that fungal type of ascorbate biosynthesis might be functional in the trypanosomatids with some missing enzymes [48]. No strong reports are published so far that demonstrate biochemical detection of ascorbate in *Leishmania* thus creating an uncertainty regarding the functionality of typical ascorbate biosynthetic pathway [48]. In contrast, Denton et al. reported another enzyme that might reduce dehydroascorbic acid using thiol dependent mechanism of action [53]. Collectively, these facts points out that some of the enzymes for ascorbate biosynthesis might have been lost during Leishmanial evolution. However, the remaining enzymes and their functional activities from ascorbate biosynthesis pathway seems to be well preserved, that might be repurposed by the organism (for example in hydrogen peroxide management). Hence, the possibility of existence of such a pathway cannot be entirely ruled out [48].

In a nutshell, humans have lost the final enzyme in ascorbate biosynthesis during evolution [54–56] and *Leishmania* has somehow retained it (however in an alternative form). Therefore, targeting the enzyme from ascorbate biosynthesis identified in this study might result in disturbances in hydrogen peroxide metabolism, thus leaving L. *major* susceptible to excess of oxidative damage due to reactive oxygen species and hence might act as an efficient drug target.

4.3.3. GPI biosynthesis

Leishmanial promastigotes are known to be enclosed in a thick glycocalyx canopy. *Leishmania* must express this coating owing to several motives. Some of them are discussed below. *Leishmania* infects human and other vertebrates in metacyclic stage. The first and the biggest challenge for this parasite is to defend itself against the hosts complement action after successful infection. The next task is attachment and gain successful entry into host macrophages. Major difficulties in this stage involve escaping host oxidant defence system and evade activity of hydrolytic enzymes. All these feats are to be accomplished without activating macrophages and simultaneously metamorphose into amastigote stage. Transformation to amastigote stage is essential for parasite in order to ensure its long-standing endurance and replication in acidic phagolysosome environment of macrophages. These facts are efficiently reviewed in [57]. Therefore glycocalyx plays a central role in survival of *Leishmania* in its host. Glycosylphosphatidylinositols (GPIs)/ GPI-anchors are glycolipids that are universally found in all eukaryotic organisms. Molecules attached to GPIs are identified as major components of glycocalyx [58]. Glycocalyx is by large composed by GPI-anchored molecules like proteophosphoglycans (PPGs) and gp63 (a leishmanial surface protease), GPI-anchored phosphoglycans like lipophosphoglycan (LPG) and short GPI-anchored glycosylinositolphospholipids (GIPLs) [59–61]. Parasitic protozoans are well established to use these cell surface proteins and glycans for attachment to hosts cell surfaces [62]. *Leishmania* is known to be rich in GPI-anchored cell surface macromolecules that play an imperative role in parasites infectivity and survival. Since GPI-anchored proteins serve to attach proteins to cell surface [63], therefore targeting GPI-anchor biosynthesis might act as an attractive therapeutic strategy that would restrict entry of *Leishmania* parasite into host cells.

The discovery of GPIs was driven by identification of covalently associated cell surface glycoprotein that was recognized to possess an important role in attaching proteins on cellular surfaces [64]. GPI anchors are initially synthesized and then a protein is covalently attached to it; this activity occurs in endoplasmic reticulum. Upon their synthesis these GPI-anchored proteins are transported to cell surface following normal secretory pathways.

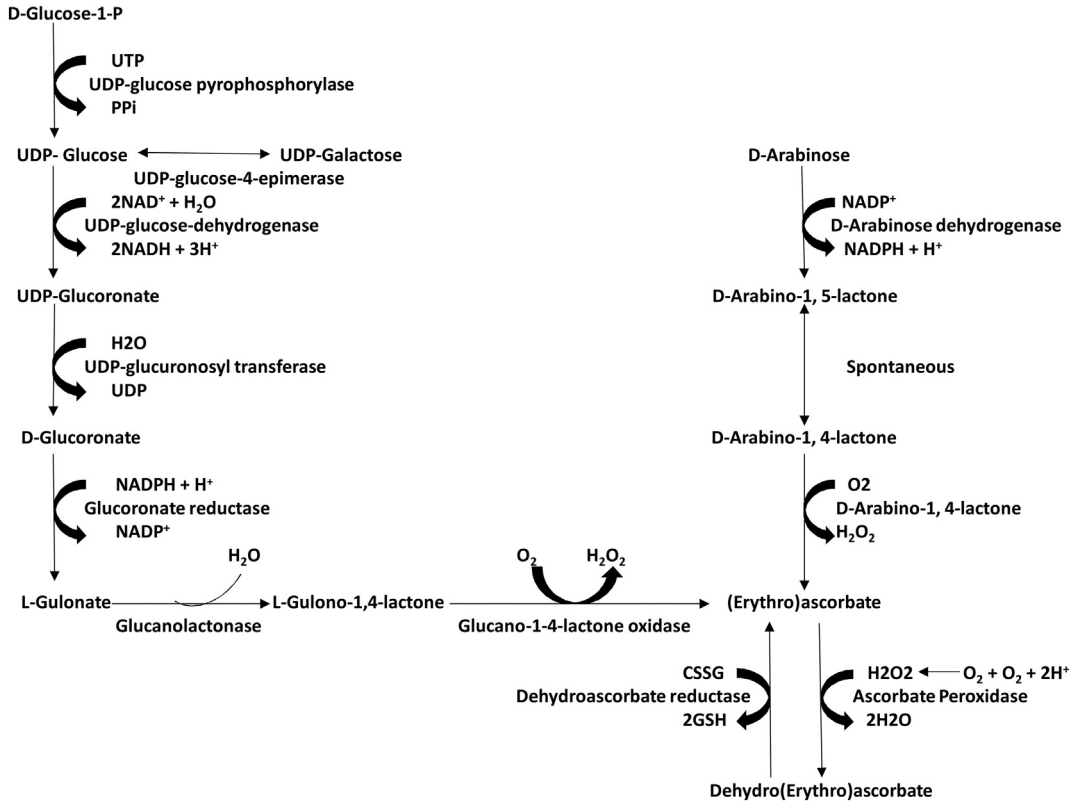

Fig. 8. Ascorbate biosynthesis in trypanosomes. The pathway is initiated on the cytoplasmic face of the ER and proceeds clockwise in the figure to yield the lumenally oriented mature GPI structure, *i.e.* the product of step 9 (mammalian cells synthesize another mature GPI which is identical to the product of step 9, except that it contains an additional EtNP residue linked to the second mannose). GPI is attached to ER-translocated proteins bearing a C-terminal, GPI-directing signal sequence. See text for other abbreviations.

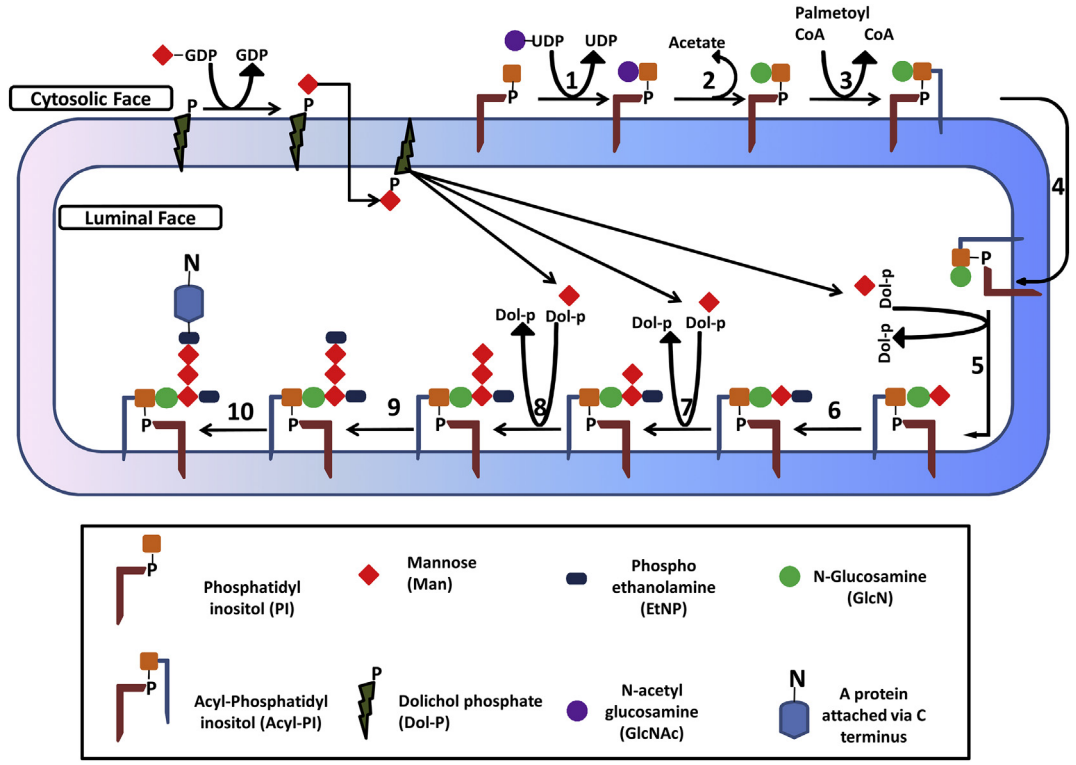

Fig. 9. GPI biosynthesis pathway in the endoplasmic reticulum. The pathway is initiated on the cytoplasmic face of the ER and proceeds clockwise in the figure to yield the lumenally oriented mature GPI structure, *i.e.* the product of step 9 (mammalian cells synthesize another mature GPI which is identical to the product of step 9, except that it contains an additional EtNP residue linked to the second mannose). GPI is attached to ER-translocated proteins bearing a C-terminal, GPI-directing signal sequence. See text for other abbreviations.

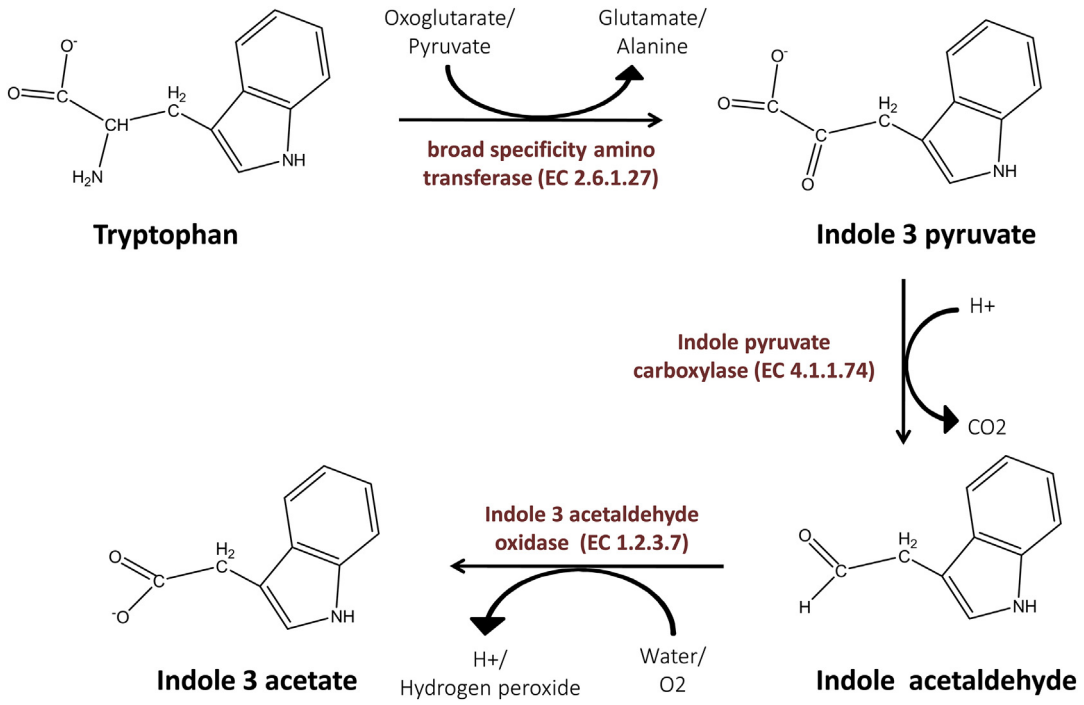

Fig. 10. Possible target pathway of tryptophan degradation.

Initiation of GPI biosynthesis initiates in ER on the cytoplasmic side. In the very first step a suger nucleotide called uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) transfers its *N*-acetylglucosamine moiety (GlcNAc) to membrane bound phosphatidylinositol (PI) to form *N*-acetylglucosamine phosphatidylinositol (GlcNAc-PI) (Fig. 9, step 1). A multi enzyme complex that catalyzes this step is known to involve six subunits that include PIG-A, PIG—C, PIG—H, GPI1, PIG—P, and DPM2. PIG-A is believed to be probable catalytic component. In second step, the GlcNAc-PI is subject to de acetylation yielding glucosamine phosphatidylinositol (GlcN-PI) (step 2). This reaction is biochemically mediated by enzyme GlcNAc-PI de-*N*-acetylase, a product of gene PIG-L. Third step of GPI biosynthesis involves acylation (palmitoylation to be specific) (step 3). In general, palmitoylation marks the fitness of precursor GPI complex for flipping across the membrane for subsequent mannosylation (step 4). Once on luminal side of ER, an enzyme GPI α l-4-mannosyltransferase encoded by gene PIG-M catalyzes first mannose addition reaction (step 5). Step 6 adds phosphoethanolamine (EtNP) at the second position of the first mannose residue (step 6) in an EtNP transferase catalyzed biochemical reaction. EtNP transferase is encoded by gene PIG—N. Subsequently, a second mannose residue is added by an unidentified enzyme that performs α l-6-mannosyltransferase activity (step 7). Third mannose residue is attached to GPI anchor by an enzyme with α l-6-mannosyltransferase function. This mannosyl transferase activity is performed by enzyme coded by gene PIG-B (step 8). Step 9 involves transfer of EtNP residue on third mannose catalyzed by a dimeric enzyme formed by PIG-O and PIG-F gene product. PIG-O is most probably the site of EtNP transfer. A protein is attached to GPI anchor on this terminal EtNP moiety *via* its C terminal end. The pathway discussed here represents a very basic scheme for GPI anchor biosynthesis [63].

This is the smallest GPI anchor known to attach to proteins. Furthermore, the GPI anchor may be embellished with subsequent EtNP and/or sugar fragments in a highly species specific manner [65]. For example, further glycolysation reactions are demonstrated to occur in trypanosomatids [66]. In case of different *Leishmania* species, up to nine mannose residues along with two glucosyl moieties are reported to be present on GPI anchor before transfer to a protein. In case of higher eukaryotes like humans, the Rft1 protein dependent translocation (or flipping) process of the dolichol-diphospho-glycan across the membrane occur only after addition of the fifth mannose residue [67]. This forms another contrasting point in GPI biosynthetic machinery between *Leishmania* and humans.

Since 1984, addition of sixth to ninth mannose residues was considered to be catalyzed on single enzyme with multiple functions with EC number 2.4.1.130. Later on in 2011, each of the four reactions were assigned a separate enzyme function and hence these reactions are now covered by enzymes with EC 2.4.1.258 (Dol-P-Man:Man5GlcNAc2-PP-Dol alpha-1,3-mannosyltransferase), EC 2.4.1.259 (Dol-P-Man:Man6GlcNAc2-PP-Dol alpha-1,2-mannosyltransferase), EC 2.4.1.260 (Dol-P-Man:Man7GlcNAc2-PP-Dol alpha-1,6-mannosyltransferase) and EC 2.4.1.261 (Dol-P-Man:Man8GlcNAc2-PP-Dol alpha-1,2-mannosyltransferase). Where Dol stands for dolichol, PP correspond to di-phosphates, Man symbolize mannose, GlcNAc represent N acetyl glucosamine. These four mannose additions are sequentially catalyzed by above mentioned enzymes.

It has been already established that mammalian and trypanosomatid GPI anchor biosynthetic mechanism differ significantly [62]. Following this notion, various therapeutic approaches have been designed for targeting GPI anchor biosynthesis. So far, reports are published to target the inhibition of first [65] and second [68] α-mannosyltransferase reaction in trypanosomatid parasites. However, our comparative metabolomic data identified fifth to eighth α-mannosyltransferase reaction to be specific in leishmanial parasite, thus representing novel approach to inhibit GPI anchor bioysnthetic machinery.

4.3.4. Tryptophan degradation

Tryptophan degradation in trypanosomatids is often reported to form indole containing end products (Fig. 10) [69]. The metabolic pathway for one of the possible fate of tryptophan generally begins with an amino transferase activity; where in, the amino group is transferred to either oxo glutarate or pyruvate to form glutamate or alanine respectively. This reaction is believed to be carried out by a broad specificity amino transferase to generate indole-3-pyruvate [70]. In subsequent step, carbon di oxide may be removed from indole-3-pyruvate to produce indole acetaldehyde. This decarboxylation reaction can

possibly catalyzed by a putative indole pyruvate decarboxylase. Finally, indole acetaldehyde might get oxygenated to form indole-3-acetate by indole-3-acetaldehyde oxidase type enzyme.

Most of these indole end products are also reported to be associated with pathogenicity of trypanosomatids [69]. Moreover, indole pyruvate decarboxylase reaction stands upstream to metabolic process that involves management of hydrogen peroxide which forms a major source of reactive oxygen species. Thus, inhibition of this reaction might result in ROS dependent oxidative stress in *Leishmania*. In humans, formation of indole acetaldehyde is carried out by entirely different route [71]. In case of humans, indole acetaldehyde is generated *via* formation of metabolite tryptamine in a tryptamine deaminase [72] catalyzed enzymatic reaction and not by forming indole 3 pyruvate intermediate (as in *Leishmania*) [73]. Therefore targeting enzyme indole pyruvate decarboxylase offers an exciting therapeutic opportunity.

5. Conclusions

To summarize, subtractive genomic and target identification procedure applied here successfully identified metabolic pathways that can be targeted in drug discovery campaigns. We report employing a reductionist approach in comparative metabolomics for identification of six metabolic enzymes that has enormous potential to act as new targets for Leishmaniasis. For example HSK and TS reactions from threonine biosynthesis pathway operate uniquely in *Leishmania* and hence forms attractive drug targets. Similarly, gulonolactone oxidase, an important enzyme in leishmanial ascorbate biosynthesis can be efficiently used in drug design procedure. Enzymes responsible for addition of mannose residues to GPI anchor has enormous potential to be used as putative drug targets. We have identified indole pyruvate decarboxylase enzyme from leishmanial tryptophan degradation pathway that can act as an effective therapeutic drug target. Steady state analysis of Threonine biosynthesis pathway indicate construction and analysis of a well settled threonine biosynthesis model. Sensitivity analysis demonstrates robustness of the metabolic model while concentration of Aspartate Semialdehyde and its Michaelis constant might play an imperative role in therapeutic inhibition of this enzyme. Metabolic control analysis indicate that along with homoserine kinase and threonine synthase; Aspartate β semialdehyde dehydrogenase, can also be considered as a good target. *In silico* enzyme inhibition data demonstrates homoserine kinase as putative drug target. Overall, data from metabolic simulation point out that Threonine synthesis pathway can act as a good source of drug targets.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2018.11.006.

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