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RESEARCH PAPER

Drug Target Prediction Using Elementary Mode Analysis in *Ascaris lumbricoides* Energy Metabolism

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Abstract Ascariasis, an intestinal worm infection is caused by the parasite *Ascaris lumbricoides* and a report by World Health Organization (WHO) on soil transmitted helminths suggests that over one billion people are affected by Ascariasis. This disease is prevalent in developing countries, and in places of poor sanitation and unhygienic conditions. Even though anthelminthic drugs are available for the treatment of ascariasis, it is considered as a neglected tropical disease (NTD). Resistance of the parasite to the existing drugs necessitates a detailed study of its energy metabolism for identification of new drug targets. The catabolic pathway of the parasite is an evolved design well suited for parasitic life and obtains constant input from its host. Its energy metabolism is predominantly anaerobic. The parasite mitochondrion plays a key role as it lacks the functional tricarboxylic acid cycle (TCA cycle) and cytochrome oxidase activity. In adult ascarid mitochondrion, there is no external final electron acceptor and endogenously produced fumarate and 2-methyl branched- chain enoyl – CoAs function as the terminal electron acceptors instead of oxygen. In this study, elementary flux mode analysis (EFM), a metabolic pathway analysis tool has been applied to model energy metabolism of the parasite *A. lumbricoides*. This study identifies a set of enzymes that have been suggested to be essential for the survival of the parasite; the inhibition of these enzymes paralyzes the parasite. The key enzymes of glycolysis and the phosphoenolpyruvate carboxykinase-succinate pathway are identified as drug targets since the knock-out of any of these enzymes results in zero flux value for all EFM that have been identified.

Keywords: *Ascaris lumbricoides*, carbohydrate catabolism, elementary flux mode, control effective flux, anaerobic mitochondria

1. Introduction

Ascaris is a gastrointestinal parasite affecting human and livestock. The roundworm *A. lumbricoides* infects the digestive tracts of humans, causing ascariasis, and is estimated to infect approximately 1.4 billion persons on a global basis. Ascariasis is prevalent in under-developed countries and unhygienic places [1]. The effective means of controlling ascariasis is regular administration of anthelminthic drugs to both animals and humans, many of which target nervous system of the helminths. The cholinergic anthelminthics levamisole, pyrantel and oxantel act as potent agonists at acetylcholine receptors on muscle bag membranes of the parasite [2]. Benzimidazoles *viz*. mebendazole and albendazole are the drugs of choice for treatment of ascariasis. The drug binds to the β-subunit of tubilin, preventing microtubule formation and also affects other parasitic functions, such as glucose transport and glycogen depletion [3]. The action of Avermectin B_{1a}, an antiparasitic agent, is not related to the action of acetylcholine but paralyses the parasite by disruption of the signaling between interneuron and motor neurons [4]. Many helminth enzymes have been proposed as targets for anthelminthic discovery. Even though medication is currently available, the ever increasing parasite resistance to the existing drugs drives towards new drugs against novel targets.

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Ascaris suum is a major parasite of the pig and the pig-parasite model is extensively used for the study of the interaction between human malnutrition, helminth infection, and congenital helminth infection. The latter is considered as a closely related model of the human parasite *A. lumbricoides* [5]. Currently, it is thought that *A. lumbricoides* and *A. suum* are different species; there has been much debate over years that these two parasites were originally the same, but over time evolved into two separate species. The adult parasites are morphologically distinguishable, but there is no procedure to distinguish the eggs of *A. lumbricoides* and *A. suum*. A study on the genetic structure and epidemiology of *Ascaris* populations concluded that the mitochondrial genome (mtDNA) sequence obtained fell into 2 different clusters, namely cluster-A from pigs and cluster-B from humans and they were found to be only 3 ~ 4% divergent and within each clusters the sequences differed by less than 1%. All *Ascaris* samples of human collected from different geographical locations have been found to differ by six nucleotides (1.3%) in the first internal transcribed spacer (ITS-1) when compared to pigs [6,7]. The complete mitochondrial genome sequence of *A. lumbricoides* revealed that the mitochondrial genome of *A. lumbricoides* and *A. suum* differed by only 1.9% [8]. These findings indicate that the two species are closely related at the phylogenetic level. In addition, a comparative study of electrophoretic banding protein extracts from the reproductive organ and body wall of *A. lumbricoides* and *A. suum* has revealed the close genetic relationship between these two species and that there is a high probability of cross infection between the two species [9]. The genetic and enzymatic data available for *A. suum* are considered in this study when the relevant data are not available for *A. lumbricoides,* as they are closely related on a phylogenetic basis.

The helminth parasite *A. lumbricoides* has evolved from the environment where oxygen is present in low concentration or completely absent [10]. In course of their evolution many pathways are abandoned and their metabolic functions were lost due to their adaptation to life within their host [11]. In this paper metabolic pathway analysis, a computational systems biology tool based on mathematical framework is used to identify potential drug target enzymes in the energy metabolism of the parasite *A. lumbricoides*. One of the major challenges in new drug development is to identify an efficient drug target that will incur minimal side effects to the patient. A potential application of EFM is in drug design to reduce drug effects and identify drug targets. The existence of an elementary mode is a sure event with probability one or it does not exist with probability zero. The new metabolic route formed after knocking out an enzyme can be found by elementary mode analysis. The knock-out of an enzyme indicates that the flux of the corresponding metabolic reaction is declared as zero. The key enzymes that participate in all the elementary modes are identified, the knock-out of those enzymes result in no elementary modes and alter the energy generation of the parasites.

2. Materials and Methods

2.1. Carbohydrate and energy metabolism

The carbohydrate dissimilation metabolic network of *A. lumbricoides* has been constructed by combining information from knowledge based annotation approach based on BRENDA [12], METACYC [13], ExPASy [14] and from available biochemical and physiological literatures [15-18]. Enzyme commission (EC) numbers were assigned to the reactions using BRENDA [12] and KEGG [19] pathway database. The regular glycolytic conversion of glucose to lactate is present in the larval stage of the parasite; whereas, the adult stage of the parasite possesses novel anaerobic mitochondrial energy generating pathways. The glycolytic design of this parasite is referred to as *Ascaris* type metabolism which fixes CO_2 and is composed of bits and pieces from other pathways such as glyconeogenesis, glycolysis, TCA cycle, and propionate metabolism. The *Ascaris* type metabolism is an evolutionary design well-suited for host-parasite interaction.

Adult *Ascaris* have active glycolytic pathways and a partial reverse TCA cycle, which is in contrast to mammals having active TCA cycle and electron transport systems. A conventional type of respiratory chain is absent in *Ascaris* and they do not use oxygen as final electron acceptor; *Ascaris* must have a fermentative metabolism instead and excrete organic substances as end products. CO_2 fixation is a characteristic of these intestinal parasites and it uses malate dismutation to maintain redox balance. *Ascaris* has a normal glycolytic sequence till phosphoenolpyruvate (PEP), the activity of pyruvate kinase (PK) is rarely noticed; phosphoenolpyruvate carboxykinase (PEPCK) competes with PK, instead of forming cytoplasmic pyruvate, CO_2 fixation takes place and PEP is converted to oxaloacetate by the enzyme PEPCK [20]. The oxaloacetate formed is reduced by NADH to malate thereby regenerating glycolytic NAD. The malate formed in the cytoplasm is transported to mitochondria for further anaerobic metabolism. Mitochondria utilize malate as a main substrate for two other linked pathways, called malate dismutation; malate is reduced in a series of reactions and oxidized in another series of reactions to maintain redox balance. The adult stages of most helminth parasites survive in an anaerobic environment by using the malate dismutation pathway. In the reduction route, two-thirds of malate is converted to fumarate by the

activity of fumarase and then to succinate by an NADH-linked fumarate reductase. While in the oxidation route, one- third of the malate is decarboxylated oxidatively to pyruvate *via* an NAD+ linked malic enzyme [21]. The flavin linked fumarate reductase reaction results in site I electron transport phosphorylation of ADP to yield ATP. In *Ascaris* type metabolism, the initial end products of carbohydrate catabolism are succinate and pyruvate; these are further metabolized to acetate, propionate, 2-methylvalerate and 2-methylbutyrate. The pyruvate formed is subsequently decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex (PDC) and it is converted to acetate by acetate:succinate-CoA transferase (ASCT). The conversion of succinate to propionate is not an ATP requiring process and is formed by a reversal of the pathway operating in mammalian mitochondria. Acetate and propionate are further metabolized to 2-methylbutyrate and 2-methylvalerate through a pathway similar to the reversal of β-oxidation by the condensation of propionyl-CoA and acetyl-CoA or by the condensation of 2 molecules of propionyl-CoA [22,23]

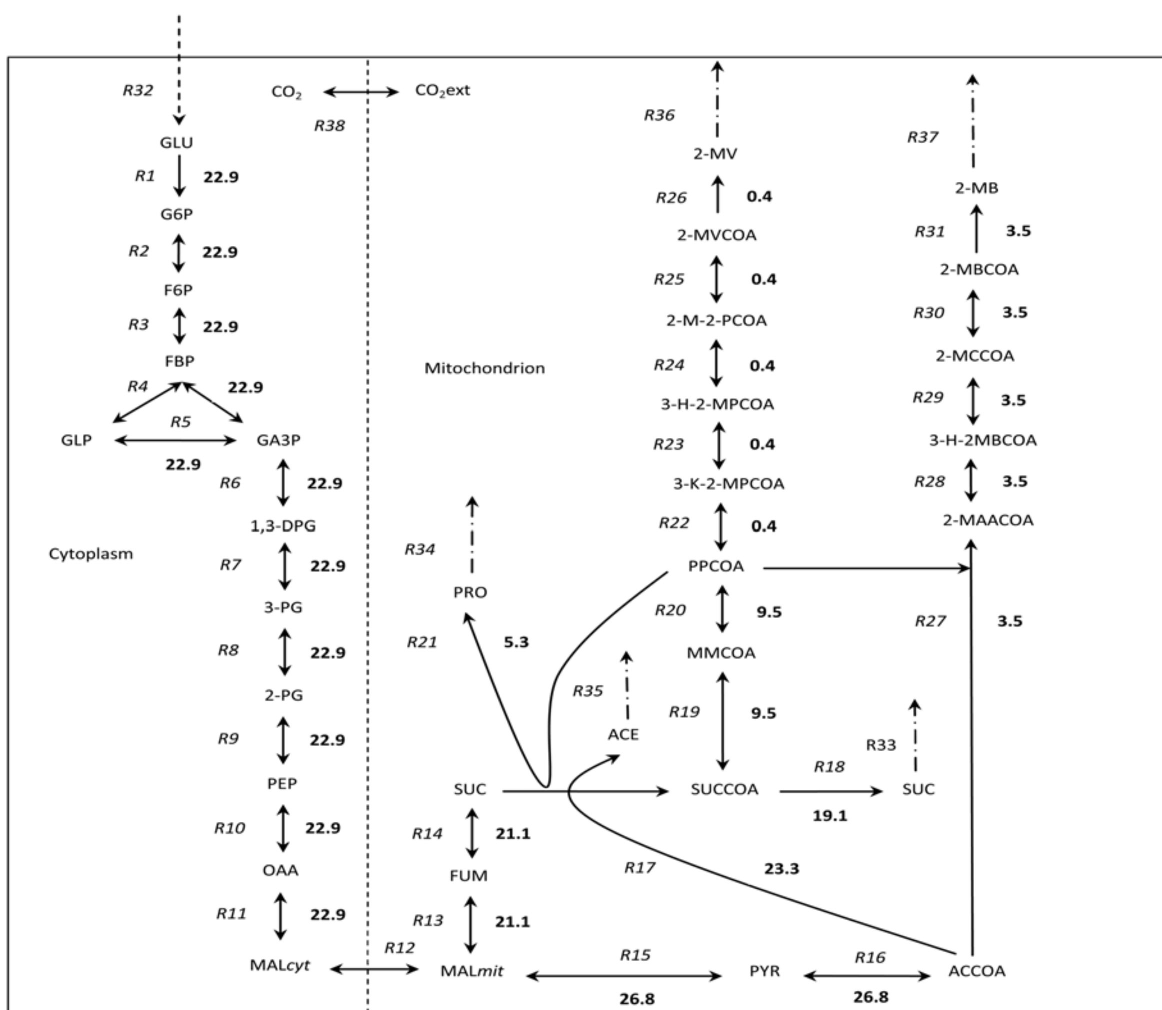

Fig. 1. Reaction network of carbohydrate catabolism in *Ascaris lumbricoides*. The numerical values are the flux optimization values given along the arrows. Single headed arrows indicate irreversible reactions and double headed arrows indicate reversible reactions. The dashed arrows indicate external metabolites. Abbreviations of metabolites: GLU: glucose, G6P: glucose 6-phosphate, F6P: fructose 6-phosphate, FBP: fructose 1,6-bisphosphate, GLP: glycerone phosphate, GA3P: glyceraldehyde 3-phosphate, 1,3-DPG: 1,3-diphosphoglyceric acid, 3-PG: 3-phosphoglyceric acid, 2-PG: 2-phosphoglyceric acid, PEP: phosphoenolpyruvate, OAA: oxaloacetate, MALcyt: cytoplasmic malate, MALmit: mitochondrial malate, FUM: fumarate, SUC: succinate, PYR: pyruvate, ACCOA: acetyl-CoA, ACE: acetate, SUCCOA: succinyl-CoA, MMCOA: methylmalonyl-CoA, PPCOA: propionyl-CoA, 3-K-2-MPCOA: 3-keto-2-methyl pentanoyl-CoA, 3-H-2-MPCOA: 3-hydroxy-2-methyl pentanoyl-CoA, 2-M-2-PCOA: 2-methyl-2-pentanoyl-CoA, 2-MVCOA: 2-methylvaleryl-CoA, 2-MV: 2-methylvalerate, 2-MAACOA: 2-methylacetoacetyl-CoA, 3-H-2MBCOA: 3-hydroxy-2-methylbutyryl-CoA, 2-MCCOA: 2-methylcrotonyl-CoA, 2-MBCOA: 2-methylbutyryl-CoA, 2-MB: 2-methylbutyrate, CO_2: carbondioxide, CO_2ext: carbondioxide ext.

and the end products excreted by the parasite are a complete mixture of succinate, acetate, propionate, 2-methylbutyrate, and 2-methylvalerate. The reaction network analyzed in the present study is shown in Fig. 1. The reactions of *Ascaris* type metabolism are given in Table 1. The glycolytic pathway of the parasite differs from its mammalian host and it will be a promising strategy if the enzymes involved in the glycolysis of the parasite are identified as new drug targets.

Table 1. Reaction stoichiometries and enzymes used to construct the model of carbohydrate catabolism in *A. lumbricoides*

#	Reaction	Enzyme name	EC number
R1	glucose + ATP → glucose-6-phosphate + ADP	hexokinase	2.7.1.1
R2	glucose-6-phosphate ↔ fructose-6-phosphate	glucose-6-phosphate isomerase	5.3.1.9
R3	fructose-6-phosphate + ATP ↔ fructose 1,6-bisphosphate +ADP	6-phosphofructokinase	2.7.1.11
R4	fructose1,6-bisphosphate ↔ glycerone phosphate + glyceraldehyde-3-phosphate	fructose-bisphosphate aldolase	4.1.2.13
R5	glyceraldehyde-3-phosphate ↔ glycerone phosphate	triose phosphate isomerase	5.3.1.1
R6	2 glyceraldehyde-3-phosphate + 2 NAD^+ + 2 PI ↔ 2 1,3-diphosphoglyceric acid + 2 NADH + 2 H^+	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	1.2.1.12
R7	2 1,3-diphosphoglyceric acid + 2 ADP ↔ 2 3-phosphoglyceric acid + 2 ATP	phosphoglycerate kinase	2.7.2.3
R8	2 3-phosphoglyceric acid ↔ 2 2-phosphoglyceric acid	phosphoglyceromutase	5.4.2.1
R9	2 2-phosphoglyceric acid ↔ 2 phosphoenolpyruvate + 2 H_2O	enolase	4.2.1.11
R10	2 phosphoenolpyruvate + 2 IDP + 2 CO_2 ↔ 2 oxaloacetate + 2 ITP	phosphoenolpyruvate carboxykinase	4.1.1.32
R11	2 oxaloacetate + 2 NADH ↔ 2 malate_cyt + 2 NAD^+	malate dehydrogenase	1.1.1.37
R12	malate_cyt ↔ malate_mit	transport reaction	
R13	1.33 malate_mit ↔ fumarate + H_2O	fumarate hydratase	4.2.1.2
R14	Fumarate + NADH + H^+ ↔ succinate + NAD^+	fumarate reductase	1.3.1.6
R15	0.66 malate_mit + NAD+ ↔ pyruvate + NADH + CO_2	malate dehydrogenase (decarboxylating)	1.1.1.39
R16	pyruvate + CoA + NAD^+ ↔ acetyl-CoA + NADH + H^+ + CO_2	pyruvate dehydrogenase complex	1.2.4.1
R17	acetyl-CoA + succinate → acetate + succinyl-CoA	Acetate: succinate-CoA transferase	2.8.3.8
R18	Succinyl-CoA + ADP + PI = succinate + ATP + CoA	succinyl-CoA synthetase	6.2.1.5
R19	Succinyl-CoA ↔ methylmalonyl-CoA	methylmalonyl-CoA mutase	5.4.99.2
R20	Methylmalonyl-CoA + ADP + PI ↔ propionyl-CoA + ATP + CO_2	methylmalonyl -CoA decarboxylase	4.1.1.41
R21	Propionyl-CoA +succinate → succinyl-CoA + propionate	propionyl-CoA succinyl-CoA transferase	2.8.3-
R22	2 propionyl-CoA ↔ 3-keto-2-methyl pentanoyl-CoA + CoA	Propionyl-CoA Condensing enzyme	2.3.1.9
R23	3-keto-2-methyl pentanoyl-CoA + NADH + H^+ ↔ 3-hydroxy-2-methylpentanoyl-CoA + NAD^+	3-hydroxy-2-methylbutyryl-CoA dehydrogenase	1.1.1.178
R24	3-hydroxy-2-methylpentanoyl-CoA ↔ 2-methyl-2-pentanoyl-CoA + H_2O	enoyl-CoA hydratase	4.2.1.17
R25	2-methyl-2-pentanoyl-CoA + NADH + H^+ ↔ 2-methylvaleryl-CoA + NAD^+	2-methyl branched-chain acyl-CoA dehydrogenase	1.3.1.52
R26	2-methylvaleryl-CoA → 2methylvalerate + CoA	acyl CoA transferase	-
R27	Acetyl-CoA + propionyl-CoA ↔ 2-methylacetoacetyl-CoA + CoA	propionyl-CoA condensing enzyme	2.3.1.9
R28	2-methylacetoacetyl-CoA + NADH + H^+ ↔ 3-hydroxy-2-methylbutyryl-CoA + NAD^+	3-hydroxy-2-methylbutyryl-CoA dehydrogenase	1.1.1.178
R29	3-hydroxy-2-methylbutyryl-CoA ↔ 2-methylcrotonyl- CoA + H_2O	enoyl-CoA hydratase	4.2.1.17
R30	2-methylcrotonyl-CoA + NADH + H^+ ↔ 2-methylbutyryl- CoA + NAD^+	2-methyl branched chain acyl-CoA dehydrogenase	1.3.1.52
R31	2-methylbutyryl-CoA → 2-methylbutyrate + CoA	Acyl- CoA transferase	-
R32	glucose_ext → glucose	Transport reaction	-
R33	succinate → succinate_ext	Transport reaction	-
R34	propionate → Propionate_ext	Transport reaction	-
R35	acetate → acetate_ext	Transport reaction	-
R36	2-methylvalerate → 2-methylvalerate_ext	Transport reaction	-
R37	2-methylbutyrate → 2-methylbutyrate_ext	Transport reaction	-
R38	CO_2 ↔ CO_2_ext	Transport reaction	-

cyt indicates cytoplasm, mit indicates mitochondria, ext indicates external metabolite, → indicates irreversible reaction, ↔ indicates reversible reaction.

2.2. Framework of the model and computational methods

2.2.1. Elementary flux modes

In addition to experimental techniques, mathematical methods are developed and applied to interpret and extract information from a metabolic network. Metabolic pathway analysis is a mathematical tool that uses the stoichiometry of metabolic network for identification of potential drug targets. Stoichiometric model needs no information on the kinetics of individual reaction and requires information about reaction stoichiometry of a metabolic network under study and it is based on the first principle of mass conservation of internal metabolites within a system. Flux is a quantitative measure very much essential for the function of the cell and it is difficult to accurately measure fluxes. Intracellular fluxes are calculated using a stoichiometric model based on steady state assumption. On steady state conditions, the concentration of all metabolites are time independent and the flux balance equation is $\frac{dx_i}{dt} = \sum_j S_{ij} v_j = 0$, x_i is the concentration of metabolite i and v_j be the flux of the reaction j. Any flux vector that satisfies $v_j \geq 0$ and is a solution of S.v = 0 (matrix form of $\sum_j S_{ij} v_j = 0$) is a potential state of operation of the cell [24]. The flux optimization values of the system of linear constraints S.v = 0 has been found using cellnetanalyser which uses the linprog option of MATLAB.

EFM are a unique set of pathways that have their origin from convex analysis and the flux distributions in a cell and are defined as the non-negative linear combination of EFM. In the language of mathematics, EFM are the basis vectors of the null space to the stoichiometric matrix with non-negativity restriction for irreversible reactions. Biochemically, EFM are a minimal subset of enzymes in a metabolic network that can operate at steady state with all irreversible reactions proceeding in the right direction, which are thermodynamically feasible and enable the route of conversion of an external substrate to an external product when all internal reactions are stoichiometrically balanced [25]. The new metabolic route, after blocking an enzyme, can be identified using elementary mode analysis. Elementary mode analysis is one of the most powerful metabolic pathway analytical methods in which drug target selection is determined for the human parasite, *Trypanosoma brucei* [26].

2.2.2. Control effective fluxes

The importance of a reaction in an EFM is quantified by the calculation of control effective flux (CEF). In the calculation of CEF, an efficiency is assigned to each mode based on the total substrate uptake and the modes output. If S_k is the substrate uptake reaction and R_1 is the objective reaction, then efficiency is $E_i(S_k, R_1) = \frac{e_i^{R_1}}{\sum_l |e_i^l|}$ where $e_i^{R_1}$ is the flux of the reaction R_1 in the ith elementary mode, $\sum_l |e_i^l|$ is the sum of all the fluxes in the ith elementary mode of the lth reaction. Control effective fluxes $V_l(S_k)$ are obtained by averaged weighting of the product of reaction specific fluxes and mode specific efficiencies over the inventory of elementary modes using the substrate under consideration, $V_l(S_k) = \frac{1}{Y_{X/S_k}^{max}} \left(\frac{\sum_i (E_i(S_k, R_1).|e_i^l|)}{\sum_i E_i(S_k, R_1)} \right)$, Y_{X/S_k}^{max} denote optimal yields for objective reaction. It is the weighted average values of all the fluxes of that reaction observed in all the elementary flux modes [27]. EFM and CEF analysis are used as tools in identifying targets for drug design in the treatment of patients with enzymopathies [28] and in sphingolipid metabolism associated with cancer therapy [29]. The two parameters necessary for the calculation of CEF are the objective reactions and the substrate uptake reactions. As glucose is the main substrate for carbohydrate breakdown in *Ascaris* metabolism, R1 the first reaction in glycolysis catalyzed by hexokinase is taken as the substrate uptake reaction. The objective reactions are chosen based on flux optimization values. The flux optimization values are the measurement of internal fluxes of a metabolic network under steady state. For our model the flux optimization values are given in Fig. 1. Malate dismutation reaction (R13 and R15) is inevitable in the anaerobic mitochondrial metabolism of helminths, when the reaction flux of R13 and R15 are set as zero and flux optimization is performed, then all the internal fluxes become zero. Reactions R13 and R15 are taken as the first set of objective function. Succinate is the key metabolite involved in all elementary modes; in the second set of objective reactions, R14 catalyzed by fumarate reductase is also added to R13 and R15. The third set of objective reactions contain R13, R14, R15, and R18, as there is synthesis of succinate in R18 reaction catalyzed by succinyl-CoA synthetase. The CEF has been calculated for all the three set of objective functions and the priority of the first 15 reactions remained the same.

External metabolites are often located at the boundaries of the mode. Currency metabolites such as ATP, ADP, NAD^+, NADH, Pi, metabolites buffered by connection to reservoirs such as H_2O, CO_2, metabolites such as glucose, glycogen that serve as entry and exit metabolites are termed as external metabolites. Other than external metabolites all remaining intermediates are termed as internal metabolites, which are either consumed or produced and their concentrations change accordingly. In this study, glucose, succinate,

acetate, propionate, 2-methylvalerate, 2-methylbutyrate, CO_2, NADH, NAD^+, ATP, ADP, ITP, IDP, Pi, and H_2O are considered as external metabolites; all other metabolites are termed as internal since their total rate of production equals their total rate of consumption. Glucose is the only input substrate in our model system and the output products are succinate, acetate, propionate, 2-methylvalerate, and 2-methylbutyrate. If there is any ambiguity regarding the irreversibility of a reaction, it is considered as reversible since considering an irreversible reaction as reversible only increases the number of elementary modes [26]. The EFM and CEF values have been determined using CellNetAnalyser Version 6.3 [30].

In the present study, the protein sequences of the enzymes of the parasite were collected from National Center for Biotechnology information (NCBI) and were cross checked against the host (*Homo sapiens*) proteome to identify the target specificity.

3. Results and Discussion

The metabolic pathway analysis of our model described in Fig. 1 resulted in eleven EFM, all of which are energy-supplying reactions as there is synthesis of either ATP or ITP. The eleven EFM determined are shown in Table 2, from the external substrate glucose, the mixture of end products formed are succinate, propionate, acetate, 2-methylvalerate, 2-methylbutyrate and CO_2, and their net reactions are shown in Table 2. If an enzyme is deleted from a network, the number of elementary modes remaining after deletion is a subset of all elementary modes determined for the metabolic network that does not involve that enzyme. In the present study, there are eleven enzymes that participate in all the eleven EFM, and the deletion of any one of these enzymes from the metabolic network results in no elementary modes. In addition, our model suggests that succinate is the major metabolite associated with the formation of the predominant end products of the parasite and a decrease in the succinate content may alter the energy generation of the parasite; and hence, fumarate reductase (FRD) and ASCT and have been identified as a candidate drug target. CEF have been determined directly from the set of EFM excluding the transport reactions using the third set of objective reactions and are presented in Table 3. The reactions are ranked based on their CEF values. The enzymes catalyzing the reactions ranked as one and two are suggested as potential drug targets and this coincides with the results of EFM.

The eleven elementary modes that have been found for the metabolic network shown in Fig. 1 are explained in detail and their net stoichiometries are provided. EFM1 represents succinate synthesis in the reduction route of malate dismutation reaction by the Embden-Meyerhoff

Table 2. Net stoichiometries of the eleven EFM identified

EFM	Net reaction	Participating reactions	Number of participating enzymes
1	0.66 glucose + 1.33 $CO_2 \rightarrow$ succinate	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14	13
2	3 glucose → 2 acetate + 1.5 succinate + 2-methylvalerate	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14, R15, R16, R17, R19, R20, R22, R23, R24, R25, R26	23
3	1.325 glucose → acetate + 2-methylbutyrate + 2.35 CO_2	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14, R15, R16, R17, R19, R20, R27, R28, R29, R30, R31	23
4	1.99 glucose → 2 acetate + 2-methylvalerate + 2.01 CO_2	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14, R15, R16, R17, R19, R20, R22, R23, R24, R25, R26	23
5	0.33 glucose → acetate + 1.34 CO_2	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R15, R16, R17, R18	15
6	3.01 glucose → acetate + 4.02 propionate	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14, R15, R16, R17, R18, R19, R20, R21	20
7	Glucose → acetate + 1.0015 succinate	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14, R15, R16, R17, R18	17
8	6.02 glucose → 7.04 propionate + acetate + 2- methylbutyrate	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11,R13, R14, R15, R16, R17, R19, R20, R21, R27, R28, R29, R30, R31	24
9	6.02 glucose → 6.04 propionate + 2 acetate + 2-methylvalerate	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14, R15, R16, R17, R19, R20, R21, R22, R23, R24, R25, R26	24
10	2.0015 glucose + $CO_2 \rightarrow$ 3.003 propionate	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14, R19, R20, R21	16
11	2.5 glucose → acetate + 1.7607 succinate + 2-methylbutyrate	R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14, R15, R16, R17, R19, R20, R27, R28, R29, R30, R31	23

Transport reactions are not included in the participating reactions. The common reactions that participate in all the eleven EFM are from R1 to R11.

Table 3. Calculated CEF values for the internal fluxes excluding the transport reactions

Reactions	CEF values	Rank
R13	2.0719	1
R14	2.0719	1
R2	1.9930	2
R3	1.9930	2
R4	1.9930	2
R5	1.9930	2
R6	1.9930	2
R7	1.9930	2
R8	1.9930	2
R9	1.9930	2
R10	1.9930	2
R11	1.9930	2
R15	1.8547	3
R16	1.8547	3
R17	1.6684	4
R19	1.3144	5
R20	1.3144	5
R18	1.1836	6
R21	0.8296	7
R27	0.8296	8
R28	0.1863	8
R29	0.1863	8
R30	0.1863	8
R31	0.1837	8
R22	0.1492	9
R23	0.1492	9
R24	0.1492	9
R25	0.1492	9
R26	0.1479	9

The CEF values are ranked in descending order.

scheme of glycolysis followed by CO_2 fixation into pyruvate. The fermentation of glucose to succinate *via* the glycolytic mechanism is consistent with the experimental data suggesting that the glycolytic pathway is either the only mechanism or the major one, whereby succinate is formed [31]. The formation of acetate by the activity of ASCT that transfers the CoA moiety to succinate *via* a ping-pong mechanism has been identified in mode 5. The produced succinyl-CoA is subsequently recycled to succinate by SCS, which generates ATP by substrate level phosphorylation [32,33]. Mode 10, the propionate synthesis is essentially the decarboxylation of succinate by the reversal of the pathway operating in mammalian mitochondria. These 2 pathways are equivalent to the experimental results discussed by Van Vugt *et al.* [17]. Mode 6 is the superposition of modes 5 and 10. Malate is utilized by an oxidation reduction reaction in mode 7 resulting in the formation of succinate and acetate, the superposition of modes 1 and 5. The formation of the predominant end products 2-methylvalerate and 2-methylbutyrate occurs in three different modes 2, 4, 9, and 11, 3, 8, respectively. 2-methylvalerate is formed by the condensation of 2 propionyl-CoAs and with the subsequent reduction of the condensation products. 2-methylbutyrate is formed by the condensation of an acetyl-CoA and a propionyl-CoA with the subsequent reduction of the condensation products. The mixture of other end products formed in these modes includes succinate, acetate, propionate, and CO_2. The synthesis of 2-methylvalerate and 2-methylbutyrate in EFM are in line with the published results [22,23].

Out of eleven elementary modes obtained ASCT is associated with nine elementary modes; the end products formed without the activity of ASCT are succinate and propionate. It is evident that the formation of the predominant end products 2-methyl valerate and 2-methyl butyrate are dependent on the enzyme ASCT as there is no alternative route for their formation.

It is also observed that SCS enzyme is associated with the formation of only acetate, as end product. If SCS is deleted from the metabolic network, then mode 5, 6, and 7 do not exist and there are only 8 EFMs and in all EFMs succinate is synthesized by the activity of FRD. Deactivation of FRD leads to the deletion of all modes except mode 5, FRD is associated with site I electron transport phosphorylation of ADP giving rise to ATP, it is a key enzyme in the metabolic network and targeting FRD may be lethal to the parasite. The analysis is also strongly in line with the published results that nafuredin, atpenins, paecilaminol, flutonil and verticipyrone are inhibitors of NADH fumarate reductase [34-38]. Fumarate reductase catalyses succinate formation, decreasing succinate concentrations alters the formation of acetate and propionate which are essential for the volatile fatty acid synthesis. Elementary mode analysis suggests that decreasing the succinate production may paralyse the parasite and this is in good agreement with the existing result that the anthelminthic piperazine affects the succinate production in *Ascaris* causing myoneural block, and also causes hyper polarization of the *Ascaris* muscle rendering it unresponsive to acetylcholine [39,40]. ASCT participates in nine EFMs and deletion of this enzyme results in modes 1 and 10 with the formation of succinate and propionate respectively, ASCT is essential for the branched chain fatty acid synthesis in *Ascaris* mitochondrion. ASCT is a parasite specific enzyme and the mechanism of formation of acetate in the host is not *via* the same mechanism as that of the parasite, acetate formation is an attractive target for the development of novel anti parasitic drugs. Apart from these two enzymes identified, there are eleven reactions common to all EFMs. The eleven enzymes catalyzing these reactions are hexokinase, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose bis-phosphate aldolase, triose phosphate isomerase, glyceraldehyde-

3-phosphodehydrogenase (phosphorylating), phosphoglycerate kinase, phosphoglycerate mutase, enolase, PEPCK, and malate dehydrogenase. These enzymes are equally essential and *in silico* deletion of any one of these eleven enzymes results in no elementary mode which shows that these enzymes are biologically significant and they are the functional modules of the network. Our analysis suggests that all these cytoplasmic enzymes can be targeted for antiparasitic drugs. The calculated CEF values for the third set of objective reactions are given in Table 3 and are ranked according to the importance of each reaction. This analysis suggests that the enzymes catalyzing the reactions R13, R14, R2, R3, R4, R5, R6, R7, R8, R9, R10, and R11 can be targeted for anthelminthic drugs. The results of the study using EFM and CEF suggest that the enzymes catalyzing R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R13, R14, and R17 are potential drug targets.

Previous studies on the kinetic differences between the *A. suum* and vertebrate PEPCK lead to the hypothesis of selective inhibition mechanism on nematode PEPCK [41]. Interestingly the enzymes of glycolytic pathway of *Trypanosoma brucei* are reported to be potential targets for drug design [42]. Fumarase (Fumarate hydratase) from *Ascaris suum* is an excellent drug target in spite of its high sequence identity (77%) with the corresponding human homologue, since malic enzyme and fumarase share a common fumarate binding site as well as a malate site. These enzymes would provide additional benefits for chemotherapy [43].

An ideal target is one whose sequence identity with the human homologue is considerably less and for the 14 proteins identified as targets, the sequence analyses have been carried out between the host and parasite proteomes using EMBOSS matcher, except for ASCT and FRD, and those proteins whose identity are less than 60% are suggested as potential drug targets. The drug targets proposed in this study using non-redundant BLAST search are hexokinase, 6-phosphofructokinase, phosphoglyceromutase, triose phosphate isomerase, PEPCK, and malate dehydrogenase.

Selective toxicity is a key issue to be considered, while developing drugs for parasitic infections, a comparison of the parasite enzymes with its corresponding human counterpart has been discussed.

Hexokinase: In humans, 4 hexokinase isomers are identified of which isoenzymes I, II, and III are monomers with a molecular weight of 100 kDa and isomer IV called glucokinase is a monomer with a molecular weight of 50 kDa. In adult *Ascaris,* a single isomer of hexokinase has been identified with a molecular weight of 100 kDa and there are differences in the properties of mammalian hexokinase and ascarid hexokinase. The ascarid enzyme has a low affinity for glucose and is weakly inhibited by glucose-6-phosphate in contrast to the mammalian isoenzymes I, II, and III, which have a high affinity for glucose and are strongly inhibited by glucose-6-phosphate [16,44]. The amino acid sequence identity of the ascarid enzyme with that of the corresponding human homologue is considerably less, which is 40%. These facts suggest that ascarid hexokinase can be targeted for chemotherapy.

6-phosphofructokinase (PFK): PFK is a key regulatory enzyme in the control of glycolytic flux in both mammals and helminths and are activated by fructose 2-6 phosphate and AMP, and inhibited by ATP or citrate. Citrate inhibits the helminth enzyme only slightly at level of 1 mM, as there is no functional citric acid cycle in the adult *Ascaris*. Mammalian PFK is a tetramer with a molecular weight of about 330 kDa [45] and the molecular weight of ascarid enzyme ranges from 360 to 400 kDa [46,47]. Even though there is not much structural difference between the helminth PFK and mammalian PFK, the helminth phosphorylation of PFK results in activation of the enzyme, whereas in mammals phosphorylation of the enzyme results in inhibition to mild stimulation. Phosphorylation of PFK plays a significant role in regulating the energy metabolism of helminth parasites [48]. The percentage of amino acid identity between the two enzymes is approximately 55. These differences offer a chance of selecting PFK as a chemotherapeutic target.

PEPCK: The primary function of PEPCK is gluconeogenesis in the mammalian host, but it has been observed that in *Ascaris* it is an essential regulatory enzyme of glycolysis. The native molecular weight of the *Ascaris* enzyme is 83 kDa [49] and the human enzyme has a molecular weight of 68.5 kDa [50]. In humans, the cytosolic form of PEPCK converts oxaloacetate (OAA) to PEP and CO_2, however in adult *Ascaris* it fixes CO_2 with PEP and produces OAA as the terminal step in glycolysis. This functional difference is related to significant differences in the molecular properties of the parasite PEPCK and human PEPCK; in addition, the human enzyme shares an approximate 59% identity with the parasite enzyme. These facts indicate that parasite PEPCK can be targeted for chemotherapy.

Malate dehydrogenase (MDH): MDH is an enzyme in the PEPCK-succinate pathway of the parasite. In humans, MDH catalyzes the final reaction, the oxidation of malate to oxaloacetate of the TCA cycle. In both host and parasite MDH is present in the cytoplasm and mitochondria. A comparison of amino acid sequences of *Ascaris* and *Homo sapiens* shows a sequence identity of 35% approximately. Cytoplasmic malate dehydrogenase and mitochondrial malate dehydrogenase of *A. suum* are suggested as active sites of inhibition by mebendazole [51].

There are not many reports on the enzyme triosephosphate isomerase and phosphoglycero mutase of *Ascaris,* both are

glycolytic enzymes and they share a sequence identity of 58 and 35% with its corresponding human homologue, respectively.

ASCT is a parasite specific enzyme, NADH-dependent FRD is much significant in the anaerobic respiration of the nematodes, and the respiration in the host mammals is aerobic.

4. Conclusion

Our study using metabolic pathway analysis proposes some novel drug targets for the treatment of ascariasis. The mathematical method presented here reduce the complexity of identifying putative drug targets experimentally, indeed the accuracy of the computational method relies on the knowledge of the biochemical pathway modeled from available literature sources. As a concluding remark the enzymes catalyzing the reactions R1, R3, R5, R8, R10, R11, R14, and R17 are identified as drug targets using EFM and CEF. It can be seen that the metabolic pathway analysis result coincide with few already existing drug targets in literature. The potential targets identified in this work are hexokinase, PFK, phosphoglyceromutase, triose phosphate isomerase, PEPCK, MDH, FRD and ASCT. All the genes identified have very important role in the energy metabolism of the parasite; however validating the targets needs further laboratory work.

Abbrevations

ADP : Adenosine diphosphate
ASCT : Acetate succinate CoA transferase
ATP : Adenosine triphosphate
BRENDA : BRauschweig enzyme database
CEF : Control effective flux
CO_2 : Carbon dioxide
E.C. number : Enzyme commission number
EFM : Elementary flux mode
ExPASy : Expert protein analysis system
FRD : Fumarate reductase
IDP : Inosine diphosphate
ITP : Inosine triphosphate
ITS-1 : First internal transcribed spacer
KEGG : Kyoto encyclopedia for genes and genomes
MDH : Malate dehydrogenase
mtDNA : Mitochondrial genome
NAD+ : Nicotinamide adenine dinucleotide (oxidised)
NADH : Nicotinamide adenine dinucleotide (reduced)
NCBI : National center for biotechnology information
NTD : Neglected tropical disease
OAA : Oxaloacetate
PEP : Phosphoenolpyruvate
PEPCK : Phosphoenolpyruvate carboxykinase
PFK : 6-phosphofructokinase
Pi : Inorganic phosphate
PK : Pyruvate kinase
SCS : Succinyl-CoA synthase
TCA : Tricarboxylic acid
WHO : World health organization

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