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Computational and experimental analysis identified 6-diazo-5-oxonorleucine as a potential agent for treating infection by *Plasmodium falciparum*

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ABSTRACT

Plasmodium falciparum (PF) is the most severe malaria parasite. It is developing resistance quickly to existing drugs making it indispensable to discover new drugs. Effective drugs have been discovered targeting metabolic enzymes of the parasite. In order to predict new drug targets, computational methods can be used employing database information of metabolism. Using this data, we performed recently a computational network analysis of metabolism of PF. We analyzed the topology of the network to find reactions which are sensitive against perturbations, i.e., when a single enzyme is blocked by drugs. We now used a refined network comprising also the host enzymes which led to a refined set of the five targets glutamyl–tRNA (gln) amidotransferase, hydroxyethylthiazole kinase, deoxyribose–phophate aldolase, pseudouridylate synthase, and deoxyhypusine synthase. It was shown elsewhere that glutamyl–tRNA (gln) amidotransferase of other microorganisms can be inhibited by 6-diazo-5-oxonorleucine. Performing a half maximal inhibitory concentration (IC_{50}) assay, we showed, that 6-diazo-5-oxonorleucine is also severely affecting viability of PF in blood plasma of the human host. We confirmed this by an *in vivo* study observing *Plasmodium berghei* infected mice.

1. Introduction

In the present, half of the world's population are at risk of malaria. According to WHO, there were about 3 billion people threatened by malaria in 109 countries and territories from South America to the Indian peninsula. Malaria kills about 3 million people each year, of which more than 1 million are children under the age of five. In addition, up to half a billion people suffer from the effects of malaria (Snow et al., 2001). Although there are several different treatments for malaria, the parasite is becoming increasingly resistant to conventional antimalarial drugs. This has contributed to increasing morbidity and mortality. Four species of the *Plasmodium* genus cause human malaria. Among these, *Plasmodium falciparum* (henceforth *P. falciparum* or PF) inflicts the most mortality and is responsible for about 90% of malaria deaths (Snow and Omumbo, 2006). The latest additions to the armoury of antimalarial drugs are artemisinins. These are remarkably potent against the asexual blood stage of the parasite's life cycle and have been shown to be effective against all stages (except the liver stages) of the parasite's lifecycle (Delves et al., 2012). Therefore, artemisinin-based combination therapies (ACTs) have now been adopted globally as the first line of treatment. It is to note that artemisinin monotherapy was withdrawn to protect the class against the emergence of resistance (WHO, 2005). Currently, ACTs with mosquito-control measures such as insecticide treated bed nets have driven down malaria rates across sub-Saharan Africa. But the fact

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remains that the supposed mechanisms of action of artemisinins are unknown or controversial (Schlitzer, 2008) and delayed rates of parasite clearance after administration of ACTs are already evident near the Thai–Cambodian border, a hotbed of multidrug resistance. So time is for alternative treatments. For the discovery and development of antimalarial drugs, the application of computational methods and predictions has shown beneficial impact. It is well known that the ability to discover drug or vaccine targets can be enhanced from our understanding how enzyme dis-function can affect metabolic pathways (Folger et al., 2011; Plaimas et al., 2010, 2008). Metabolism is the central cellular process and its malfunction is a major contributor to harm the parasite. The discovery of genomic information of a species allows now for a comprehensive genome-scale reconstruction of its metabolism. For PF, the necessary data for reconstruction can be retrieved from different databases, in particular BioCyc (Karp et al., 2005), The Malaria Parasite Metabolic Pathways (MPMP) (Ginsburg, 2006), The Kyoto Encyclopedia for Genes and Genomes (KEGG) (Kanehisa et al., 2008), and PlasmoDB (Aurrecoechea et al., 2009). The prospect of understanding the relationship between the genome and the physiology of an organism is an important incentive to reconstruct metabolic networks and to perform network based analyses of the parasite's metabolism to identify effective drug targets.

We performed recently a computational network analysis of metabolism of PF using these databases. The topology of the network was analyzed to observe the robustness of the parasite's metabolic network when single enzymes were blocked by mimicking *in silico* their inhibition by drugs. This analysis led to the identification of 22 potential drug targets (Fatumo et al., 2009). We now used a refined network including also enzymes of the human host to get less false–positives which function may be substituted by the human enzymes. Performing a computational chokepoint analysis, we yielded a list of five potential targets (glutamyl–tRNA (gln) amidotransferase, hydroxyethylthiazole kinase, deoxyribose–phophate aldolase, pseudouridylate synthase, and deoxyhypusine synthase). It was shown in *Chlamydomonas reinhardtii* that 6-diazo-5-oxonorleucine (DON) is an effective inhibitor of glutamyl–tRNA (gln) amidotransferase (Jahn et al., 1990). We performed an experimental viability assay (IC_{50} analysis) and could show that DON suits as a valid agent against PF (laboratory strain Dd2). This was confirmed by an *in vivo* study using *Plasmodium berghei* infected Swiss albino mice.

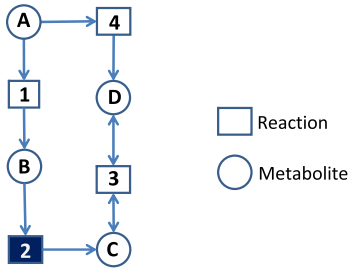

Fig. 1. An example to illustrate the computational essentiality feature we used. Rectangles represent reactions, circles represent metabolites and lines represent either consumption or production of metabolites by reactions. The filled rectangle (reaction 2) represents the observed reaction. Reaction 2 is a chokepoint reaction because it uniquely produces metabolite C and there is no deviation (or alternative pathways) from B to C. Therefore, blocking reaction 2 may severely harm the cell.

Table 1
List of potential drug targets from Fatumo et al. (2009) and our new analysis results.

EC	Reaction	Chokepoint
2.1.2.9	Methionyl–tRNA formyltransferase	Y*
2.4.1.119	Dolichyl–diphosphooligosaccharide–protein glycosyltransferase	
2.4.2.11	Nicotinate phosphoribosyltransferase	
2.4.2.30	NAD(+) ADP–ribosyltransferase	
6.3.5.7	**Glutamyl–tRNA(Gln) amidotransferase**	**Y**
2.5.1.46	**Deoxyhypusine synthase**	**Y**
2.7.1.1	Hexokinase	
2.7.1.35	Pyridoxal kinase	
2.7.1.50	**Hydroxyethylthiazole kinase**	**Y**
2.7.4.7	Phosphomethylpyrimidine kinase	
2.7.4.9	Thymidylate kinase	
2.7.7.2	FMN adenylyltransferase	
2.7.8.-	Cardiolipin synthetase	
2.7.8.11	CDP-diacylglycerol–inositol 3-phosphatidyltransferase	
3.1.2.6	Hydroxyacylglutathione hydrolase	
3.5.1.19	Nicotinamidase	
4.1.2.4	**Deoxyribose–phosphate aldolase**	**Y**
4.2.1.17	Enoyl–CoA hydratase	
4.2.1.60	3-Hydroxydecanoyl-[acyl-carrier protein] dehydratase	
4.2.1.70	**Pseudouridylate synthase**	**Y**
6.1.1.19	Arginine–tRNA ligase	
6.2.1.3	Long-chain-fatty-acid-CoA ligase	

* Y: essential according to the chokepoint analysis.

2. Results and discussion

2.1. A computational analysis identifies five potential drug targets interfering metabolism of PF

Twenty-two predicted drug targets (enzymes) for *P. falciparum* were extracted from our former computational analysis (Fatumo et al., 2009). We performed a network analysis using a reconstructed metabolic network which included PF and human enzymes (see Section 4, and as described previously (Fatumo et al., 2011)) to get an improved precision. We analyzed the topology of the network and used the established method of chokepoint analysis (Plaimas et al., 2008; Yeh et al., 2004). Briefly, the chokepoint analysis tests if after deletion of an enzyme, either one of its products cannot be produced (by any other enzyme) anymore, or if one of its substrates cannot be consumed any more. The enzyme is predicted to be essential (a chokepoint reaction) if such substrates or products are determined (see Fig. 1). With this analysis, we yielded five enzymes: glutamyl–tRNA (gln) amidotransferase (EC 6.3.5.7), hydroxyethylthiazole kinase (EC 2.7.1.50), deoxyribose–phophate aldolase (EC 4.1.2.4), pseudouridylate synthase (EC 4.2.1.70), and deoxyhypusine synthase (EC 2.5.1.46) (Table 1, marked as *Y*). We now wanted to use these predictions to identify small compounds tipping PF. For this, we searched for inhibitors that were effective to block an enzyme in other organisms. It was reported that DON successfully inhibited glutamyl–tRNA (Gln) amidotransferase of *C. reinhardtii* (Jahn et al., 1990). We did a homology analysis of the gene encoding glutamyl–tRNA (gln) amidotransferase in *C. reinhardtii* (gat1), in *P. falciparum*, and the human host. We found significant similarity between all three organisms (Fig. 2) making it for our investigations suitable, but note that homology to the human host may make the identification of specific drugs challenging (which was out of the scope of our study). Homologs of hydroxyethylthiazole kinase could not be found. Hydroxyethylthiazole kinase participates in the biosynthesis of thiamine (vitamin B1). It was reported that *Plasmodium* can obtain thiamine from the extracellular space (Wrenger et al., 2008) and the parasite may hence be independent of this reaction. For the other enzymes we could not find known inhibitors. Therefore we restricted our experimental analysis to DON. It is to note that DON can act on several glutamine metabolizing enzymes. A literature summary about other metabolic effects of DON will be given below.

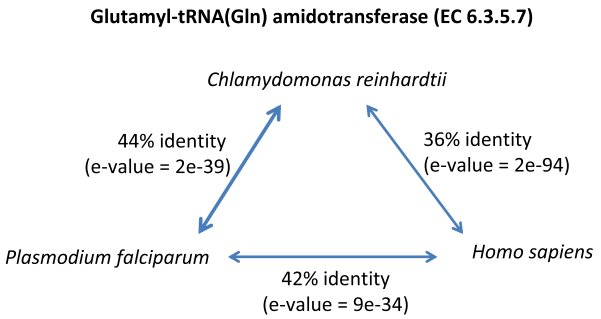

Fig. 2. Homology comparison of the coding genes for glutamyl–tRNA (gln) amidotransferase.

2.2. Inferring a new node for the network

Glu–tRNA (gln) amidotransferase (EC 6.3.5.7) is needed to synthesize glutamyl–tRNA–gln. Glutamyl–tRNA–gln serves as a building block for protein synthesis and this is essential for PF to proliferate. Glutamyl–tRNA–gln can be generated from tRNA and glutamine by two ways, either directly using glutaminyl–tRNA synthetase (EC 6.1.1.18), or by two steps using glutamyl–tRNA synthetase (EC 6.1.1.24) and glu–tRNA (gln) amidotransferase (EC 6.3.5.7). The two-step synthesis has been observed in mitochondria and bacilli (Ibba and Soll, 2000). Our network did not include glutaminyl–tRNA synthetase (EC 6.1.1.18) as we did not find it in the used databases. Still, glu–tRNA (gln) amidotransferase (EC 6.3.5.7) was found in Biocyc and MPMP for *P. falciparum*, and there exists a coding gene for glu–tRNA (gln) amidotransferase (PFD0780w). Glu–tRNA (gln) amidotransferase (EC 6.3.5.7) is only useful for PF if the two step pathway exists. We wanted to fill the gap for the two step pathway. To find putative genes coding for glutamyl–tRNA synthetase (EC 6.1.1.24), we performed an NCBI blast search using glutamyl–tRNA synthetase (EC 6.1.1.24) from *Bacillus subtillis* as query (WP_014475595.1) and the non-redundant protein sequence database of PF as the sequence database. We yielded XP_001350283.1 with good homology (*E*-value: 2E-91). XP_001350283.1 is annotated as putative glutamate–tRNA ligase. We finally added glutamyl–tRNA synthetase (EC 6.1.1.24) to our network (see Supplementary 1 list of reactions). Performing a chokepoint analysis with the new network yielded the same five reactions, and in addition glutamyl–tRNA synthetase (EC 6.1.1.24) (on which we did not follow up in this study), confirming glu–tRNA (gln) amidotransferase (EC 6.3.5.7) to be essential for PF.

2.3. DON successfully affected the parasite's viability in human blood cultures

Glutamyl–tRNA(Gln) amidotransferase catalyzes glutamyl–tRNA Gln into glutaminyl–tRNA (gln) which is used for protein synthesis. DON is a structural analog of glutamine and inhibits irreversibly glutamine amidotransferases of several organisms (Clark et al., 1987; Jahn et al., 1990). DON is a non proteinogenic amino acid. It was originally derived from *Streptomyces* (Dion et al., 1956) and showed anticancer effects in animal models (Yoshioka et al., 1992). We performed a half maximal inhibitory concentration (IC_{50}) study and found that DON showed excellent antimalarial activity *in vitro* when applied against *P. falciparum* Dd2 ($3–4 \times e^{-7}$ M, Fig. 3a). We performed positive control experiments

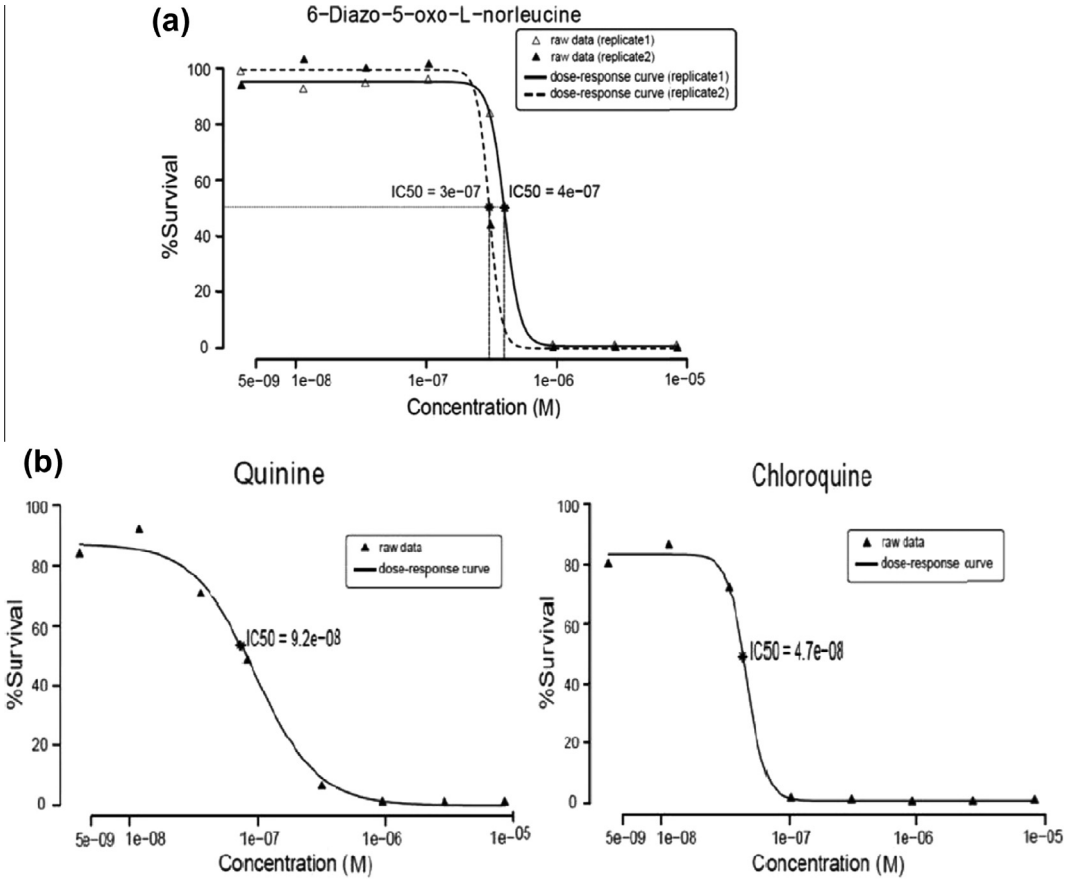

Fig. 3. The IC_{50} results of (a) DON and (b) quinine and chloroquine as positive controls.

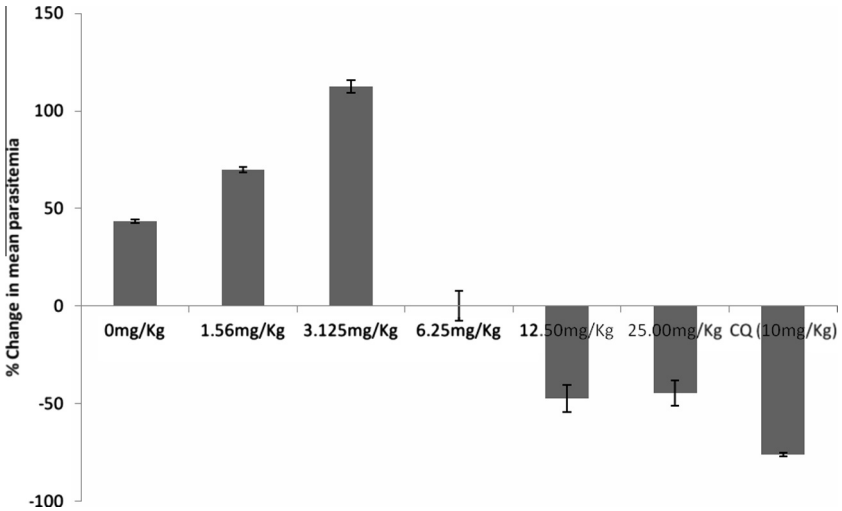

Fig. 4. Effects of DON on parasitemia in mice 24 h after treatment.

using quinine and chloroquine. Quinine and chloroquine showed a slightly better dose response of $9.2 \times e^{-08}$ and $4.7 \times e^{-08}$ M, respectively (Fig. 3b).

2.4. DON treatment reduced parasitemia in mice

To find out if DON suits for affecting parasitemia *in vivo*, we performed a mouse model study. *P. berghei* infected mice were injected intraperitoneally with DON dissolved in normal saline and parasitemia was observed 24 and 72 h after treatment. Fig. 4 shows the results for 24 h and Fig. S1 of Supplementary 2 the results for 72 h after treatment. 24 h after treatment, 50% reduction of *P. berghei* was observed when administering 12.5 mg/kg DON, and even 80% reduction 72 h after treatment. Interestingly, at very low concentrations (<6 mg/kg) we observed an increase of parasitemia after 24 h. DON did not severely harm our animal models as the treated animals survived until the time of writing (>6 weeks). Again chloroquine showed a better performance of 80% reduction of parasitemia (24 h after treatment) at comparable concentrations (10 mg/kg). 72 h after treatment, the effect of DON and chloroquine was comparable (80–90% reduction). None of the infected control animals survived 72 h.

2.5. DON can effect other glutamine metabolizing enzymes

We performed an elaborate literature study for DON to find evidence for DON effecting other enzymes besides glutamyl–tRNA (gln) amidotransferase. In several studies, glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme for hexosamine flux, was inhibited using DON (Goldberg et al., 2002; James et al., 2010, 2002; Lim and Chang, 2006). However, these studies used higher concentrations (James et al.: 10 μM, Lim et al.: 40–75 μM, Goldberg et al.: 20 μM, James, Tang et al.: 40 μM) when compared to our analysis (IC_{50} = 0.4 μM). Only very high concentrations of DON inhibited NADH-dependent glutamate synthase (NADH-GOGAT; EC 1.4.1.14): 1 mM resulted in 11% activity, and 0.5 mM showed no effect (Hirayama et al., 1998). Using 2 mM DON yielded 98% reduced CTP synthetase activity (Robertson, 1995). Interestingly, Hofer et al. reported to inhibit the proliferation of cultured trypanosomes using DON at low concentrations (25 mg DON/kg, i.e., twice as much as we used in our *in vivo* study) and treatment of *Trypanosoma brucei* infected mice with DON reduced the number of trypanosomes in the blood to undetectable levels. They found that DON mainly affected *de novo* purine biosynthesis in mammalian cells (Hofer et al., 2001). Alkylation of guanosine 5′-monophosphate (GMP) synthetase with DON inactivated GMP synthetase (concentration of DON: 16 μM) (Zalkin and Truitt, 1977). It was shown that DON is an inhibitor of glutaminase (Hartman and McGrath, 1973). Summing up, DON is an unspecific inhibitor and the *in vitro* and *in vivo* effects we obtained for malaria treatment need further investigation to better identify the molecular mechanisms of DON. In addition, we performed spectrophotometric enzyme assays for β-NAD synthetase and uracil phosphoribosyl transferase. β-NAD synthetase (EC 6.3.5.1) is part of the NAD biosynthesis pathway and metabolizes deamido-NAD^+ and L-glutamine to NAD^+ and L-glutamate. We wanted to observe β-NAD synthetase to find out if DON acts as its inhibitor (competing with its substrate glutamine). For higher concentrations (25 and 50 mg/kg), the activity was reduced (40 instead of 56 units/mg protein) in liver of DON treated mice (Supplementary 2, Fig. S2a, supplementary material). We therefore cannot exclude that DONs influence on β-NAD synthetase may have influenced the *in vitro* culture and *in vivo* parasitemia reduction. Interestingly, 72 h after treatment, the cells from the treated and untreated mice showed the same activity when regarding DON treatment of 25 mg/kg (our treatment for parasitemia: 12.5 mg/kg). Further analyses on other glutamine metabolizing enzymes will be necessary to shape out the concrete mode of action of DON. Further, we observed the activity of uracil phosphoribosyl transferase. Uracil phosphorybosyltransferase (UPRT, EC 2.4.2.9) plays an important role in the pyrimidine salvage pathway since UMP is a common precursor of pyrimidine nucleotides. The activity of UPRT was therefore assayed to observe a potential metabolic shift in pyrimidine metabolism. Interestingly, mice treated with DON showed higher activity (82 instead of 30 units/mg protein) of uracil phosphoribosyl transferase (Supplementary 2, Fig. S2b) hinting for increased pyrimidine metabolism. Unfortunately, we were not able to perform such activity measures for glutamyl–tRNA (gln) amidotransferase.

3. Conclusion

We employed a computational analysis to identify potential targets in the parasite-host metabolic network. We predicted glutamyl–tRNA (gln) amidotransferase, hydroxyethylthiazole kinase, deoxyribose–phophate aldolase, pseudouridylate synthase, and

deoxyhypusine synthase as potential drug targets against *P. falciparum*. Selecting DON as a potential inhibitor for glutamyl–tRNA (gln) amidotransferase, we observed a good dose response for DON when measuring its effectiveness on viability of *Plasmodium* in human blood culture by a half maximal inhibitory concentration (IC_{50}) assay and in mouse models. These are promising results hinting for DON to be used as an anti-malaria drug. However, DON can have severe site effects. DON has been observed to alter cell cycle and may increase mutagenesis, in particular during development. It was shown that DON prevented G2-M phase transition in oocytes of *Xenopus laevis* (Dehennaut et al., 2007). DON caused renal dysplasia of chicken and impaired their embryo formation (Spencer and Maizels, 1987). Therefore DON needs more investigation *in vivo*. It may only be used at low concentrations and in combination with other drugs such as artemisinins. In addition, follow up studies are needed to elucidate the concrete mechanism of DON. DON can act on a variety of glutamine metabolizing enzymes and this may be better estimated performing spectrophotometric assays for more potentially effected enzymes. Also studies estimating bond-tightness may improve concretizing effects of DON. Finally, further *in vivo* and culture based research should include also other strains of *Plasmodium* to determine a more general applicability of DON for treating malaria.

4. Materials and methods

4.1. Reconstructing the metabolic network and chokepoint analysis

The network was put up as described previously (Fatumo et al., 2011) using the databases of PlasmoCyc version 11.6 (Karp et al., 2005) and Malaria Parasite Metabolic Pathways (MPMP, see (Ginsburg, 2006)). All reactions were set as reversible to avoid false negatives if reactions were wrongly annotated to be irreversible. It is to note that considering all reactions to be reversible may yield additional false positives and may need more literature investigation of the results. The network comprised of reactions from *P. falciparum* (PlasmoCyC and MPMP) and human. Reactions were only selected if a coding gene was assigned to them. The human metabolism was extracted from the database HumanCyc version 11.6 (Romero et al., 2005). The MPMP database consisted of 4806 genes with a total of 1265 enzyme ids. Out of all genes in MPMP, 2033 genes mapped with their corresponding reactions in PlasmoCyc. The network was constructed as a bipartite graph consisting of alternating nodes of enzymes and metabolites. If a metabolite was a substrate of one reaction and a product of another reaction these reactions were linked via this metabolite. Hub compounds such as water, oxygen, ATP, ADP, and co-enzymes were discarded to avoid unspecific links. Finally, we yielded 723 (reversible) reactions (modeled as 1446 considering both directions). A list of all reactions is given in the supplementary material (Supplementary 1 List of reactions, including also reaction glutamyl–tRNA synthetase (EC 6.1.1.24), see Section 2). Table 2 shows the statistics for the network.

4.2. Chokepoint analysis

A reaction that uniquely consumed or produced a certain metabolite in the metabolic network was considered to be a chokepoint as described elsewhere (Rahman and Schomburg, 2006; Yeh et al., 2004; Plaimas et al., 2008). As each reaction was considered to be reversible, an in- or out-degree of 2 of a substrate or a product of the considered reaction hinted for the reaction to be a chokepoint reaction (taking into account the links to the considered reaction). Fig. 5 shows a flowchart of the algorithm and Fig. 1 illustrates the principle idea.

Table 2
Statistics of the reconstructed model.

Number of reactions	723
Number of metabolites	842
Number of links between reactions	8694
Density of the network	3.3%

4.3. Finding inhibitors

After potential target candidates were selected, their effective inhibitors were identified which were reported to block an enzyme in other organisms. The inhibitors were collected searching the Brenda Enzyme database (Schomburg et al., 2013), Drugbank (Wishart et al., 2008) and Sigma (http://www.sigma.com). DON was found bei Jahn and coworkers to be an effective inhibitor of glutamyl–tRNA(Gln) amidotransferase of *C. reinhardtii* (Jahn et al., 1990). This compound was further investigated and ordered from Sigma (http://www.sigma.com).

4.4. Parasite cultures for the in vitro anti-plasmodial assay

RPMI 1640 culture medium supplemented with L-glutamine, 35 mM HEPES and 23 mM $NaHCO_3$, stored at 4 °C (Invitrogen) *P. falciparum* Dd2 was used. The parasites were cultured in A^+ human erythrocytes at a hematocrit of approximately 5% in Petri dishes of either 10 cm diameter containing 15 ml of HEPES-buffered RPMI medium supplemented with 10% human serum type A^+, 20 μg/ml of gentamicin and 100 μM of hypoxanthine. The parasite cultures were incubated in an atmosphere of 3% CO_2, 5% O_2, 92% N_2, and 95% humidity at 37 °C. Every two to three days, the parasitemia determined by Giemsa-stained blood smears and medium was exchanged. By the time the parasitemia had reached 5–10%, the culture was split in order to avoid parasite death due to high levels of toxic metabolites in the medium. Drug activity *in vitro* was determined based on the inhibition of the incorporation of [^{3}H]-hypoxanthine into parasite nucleic acids according to the method of Desjardins et al. (1979). Briefly, one hundred-microliter parasite culture medium containing serially diluted drug were placed in the wells of 96-well microtiter plates. Then, one hundred-microliter

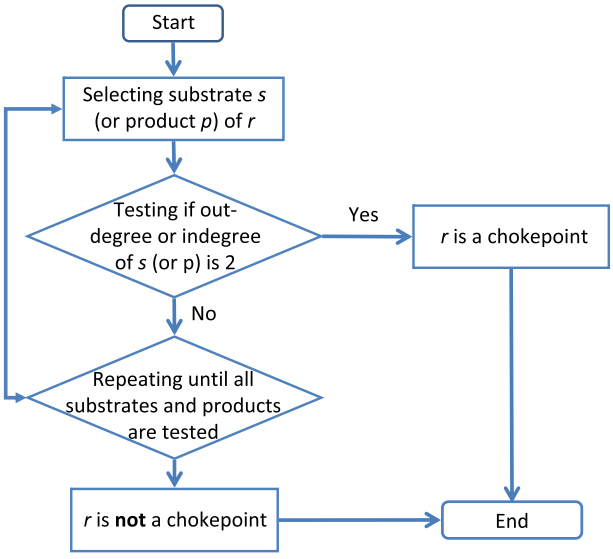

Fig. 5. Flowchart for the chokepoint procedure (to find out if reaction *r* is a chokepoint).

aliquots of a cell suspension containing highly synchronized rings (2 – 6 h post invasion) at a parasitemia of 0.5% and a hematocrit of 2.5% were added to the wells. [^{3}H]-hypoxanthine was added 48 h later to monitor parasite viability. Reactions were stopped 24 h later, and parasitemia were expressed as a percentage of the control (without drug).

4.5. DON treatment of P. berghei infected mice

Chemicals were obtained from Sigma–Aldrich, USA. *P. berghei* infected mice (8 weeks old Swiss albino mice) were injected intraperitoneally with DON dissolved in normal saline (saline concentration was 0.9% ($^{w}/_{v}$) NaCl solution). The treatment groups were as follows (each $n = 5$): group 1: control (mice administered with normal saline), group 2: 1.56 mg DON/kg body weight, group 3: 3.125 mg DON/kg body weight, group 4: 6.25 mg DON/kg body weight, group 5: 12.50 mg DON/kg body weight, group 6: 25.00 mg DON/kg body weight, and group 7: 10.00 mg Chloroquine/kg body weight. Dosages were chosen in accordance to early work on DON administered to mice to treat cancer (Barclay and Phillipps, 1966) and *T. brucei* (Hofer et al., 2001). To diagnose parasitemia, blood smear was prepared from the tail blood of the mice after 24 h of drug administration. Thick and thin films were fixed with methanol, air-dried and stained with Giemsa stain. Parasitemia, i.e., Parasite density per microliter of blood, was estimated from the thick film, taking the number leucocytes (WBC) per microliter of blood as 8000 and was expressed as follows: Parasite density/ μL of whole blood $= \frac{\text{Parasite count}}{\text{Number of WBC Counted}} \times 8000$ as described by Olasehinde et al. (2012).

4.6. Effect of DON on enzymes of nucleic acid metabolism

DON was dissolved in normal saline which was administered intraperitoneally to pathogen free mice that were grouped as follows ($n = 12$ of each group): group 1: control (mice were administered with normal saline), group 2: 25 mg DON/kg body weight, and group 3: 50 mg DON/kg body weight. Six mice from each group were sacrificed after 24 h of drug administration and six were sacrificed after 72 h. The sacrifice was done under light ether anesthetic. Liver was excised into ice cold saline and homogenized in ice cold 0.25 M sucrose, 1 mM EDTA, 10 mM HEPES-NaOH, pH 7.4 containing SIGMAFAST™ Protease Inhibitor. The homogenate was centrifuged at 800*g* for 15 min and the supernatant used for the activity assays of the enzymes. Spectrophotometric methods were used to assay the enzyme's activities as follows: Uracil phosphoribosyl transferase (Liu et al., 2000), and β-NAD synthetase (Yi and Dietrich, 1972). The Lowry protein assay method (Lowry et al., 1951) was used to determine protein concentration and the activity expressed as units per mg protein. Basically, the activity of uracil phosphoribosyltransferase was measured by monitoring the conversion of uracil to UMP at 271 nm. The assay solution (1 ml) contained 5 mM $MgCl_2$, 1.5 mM phosphoribosyl pyrophosphate, 0.1 mM uracil, and 50 mM Tric–HCl, pH 7.8 buffer. The reaction was initiated by the addition of 20 μL of liver homogenate and activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of UMP/min (Liu et al. 2000). The activity of β-NAD synthetase was measured by monitoring the rate of NAD formation. The reaction solution contained 1 mM deamido-NAD^+, 2 mM ATP, 0.1 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 56 mM KCl, 50 mM Tris–HCl buffer, pH 8.0 (at 37 °C). The reaction was initiated by addition of 100 μL of liver homogenate and terminated after 60 s in boiling water. NAD formed was determined by employing alcohol dehydrogenase (Yi and Dietrich, 1972). Data obtained was analyzed using Analysis of Variance (ANOVA) followed by the Duncan Multiple Range Test ($p < 0.05$).

Author's contributions

KP, EA, and RK put up the general concept and design of the study. KP and SF carried out the data analysis. SOR and GO performed the mouse experiments. YW and ML performed the *in vitro* experiments. KP, EA, and RK drafted the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.09.019.

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