The Heme Biosynthetic Pathway of the Obligate Wolbachia Endosymbiont of Brugia malayi as a Potential Anti-filarial Drug Target

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Abstract

Background: Filarial parasites (e.g., Brugia malayi, Onchocerca volvulus, and Wuchereria bancrofti) are causative agents of lymphatic filariasis and onchocerciasis, which are among the most disabling of neglected tropical diseases. There is an urgent need to develop macro-filaricidal drugs, as current anti-filarial chemotherapy (e.g., diethylcarbamazine [DEC], ivermectin and albendazole) can interrupt transmission predominantly by killing microfilariae (mf) larvae, but is less effective on adult worms, which can live for decades in the human host. All medically relevant human filarial parasites appear to contain an obligate endosymbiotic bacterium, Wolbachia. This alpha-proteobacterial mutualist has been recognized as a potential target for filarial nematode life cycle intervention, as antibiotic treatments of filarial worms harboring Wolbachia result in the loss of worm fertility and viability upon antibiotic treatments both in vitro and in vivo. Human trials have confirmed this approach, although the length of treatments, high doses required and medical counter-indications for young children and pregnant women warrant the identification of additional anti-Wolbachia drugs.

Methods and Findings: Genome sequence analysis indicated that enzymes involved in heme biosynthesis might constitute a potential anti-Wolbachia target set. We tested different heme biosynthetic pathway inhibitors in ex vivo B. malayi viability assays and report a specific effect of N-methyl mesoporphyrin (NMMP), which targets ferrochelatase (FC, the last step). Our phylogenetic analysis indicates evolutionarily significant divergence between Wolbachia heme genes and their human homologues. We therefore undertook the cloning, overexpression and analysis of several enzymes of this pathway alongside their human homologues, and prepared proteins for drug targeting. In vitro enzyme assays revealed a ~600-fold difference in drug sensitivities to succinyl acetone (SA) between Wolbachia and human 5-aminolevulinic acid dehydratase (ALAD, the second step). Similarly, Escherichia coli hemH (FC) deficient strains transformed with human and Wolbachia FC homologues showed significantly different sensitivities to NMMP. This approach enables functional complementation in E. coli heme deficient mutants as an alternative E. coli-based method for drug screening.

Conclusions: Our studies indicate that the heme biosynthetic genes in the Wolbachia of B. malayi (wBm) might be essential for the filarial host survival. In addition, the results suggest they are likely candidate drug targets based upon significant differences in phylogenetic distance, biochemical properties and sensitivities to heme biosynthesis inhibitors, as compared to their human homologues.

Introduction

Human filarial nematodes affect more than 150 million people worldwide with 1 billion people at risk in over 80 countries, and lead to some of the most debilitating tropical diseases, including elephantiasis and African river blindness [1,2]. The current anti-filarial treatments e.g. DEC, ivermectin, albendazole (all suitable for lymphatic filariasis; ivermectin for onchocerciasis) interrupt the cycle of transmission of the causative filarial parasites Brugia malayi, Onchocerca volvulus and Wuchereria bancrofti, by predominantly killing microfilaria. However, a lower activity against adult worms, which can survive in human hosts for up to decades, is known. DEC and albendazole produce macrofilaricidal activity only after repeated rounds of mass drug administration (MDA) [3]. Since the current treatments have to be administered annually on a community-wide basis for many years to break the infection cycle, and drug resistance may be emerging [4,5], there is still an urgent need to develop novel drugs (particularly macrofilaricidal). Numerous lines of evidence, in both laboratory and human trials, show that depletion of Wolbachia in filarial parasites by antibiotics (e.g.
doxycycline, tetracycline) can kill adult worms in addition to affecting embryogenesis, mf output and worm development [6,7,8,9,10,11,12,13]. These studies indicate that these vertically transmitted Wolbachia endosymbionts are indispensable for their filarial hosts and represent a promising therapeutic strategy for filariasis control.

Comparative analysis of available genomic sequences for Wolbachia (wBm, GenBank accession no. AE017321) and its B. malayi nematode host (GenBank accession no. EF588824 to EF588901) provides insight into metabolic pathways that might contribute to the mutualistic symbiotic relationship [14]. This approach can be used to aid identification of potential anti-filarial drug targets. One biochemical pathway identified as potentially important in the symbiotic relationship between wBm and its nematode host is heme biosynthesis. Heme, an iron-containing tetrapyrrole, is an essential cofactor for many proteins such as cytochromes, hemoglobins, peroxidases, and catalases, which are involved in a wide range of critical biological processes, including oxidative metabolism and electron transport. All but one of the C₄-type heme biosynthetic genes are readily identified from the wBm genome (Fig. 1). The only missing step, protoporphyrinogen-IX oxidase (PPO/hemG), has not been identified in many heme-producing bacteria [15]. However, all but one heme biosynthetic gene (FC/hemH, ferrochelatase, the last step in heme biosynthesis) is absent in the B. malayi genome [16], implying filarial nematodes are incapable of de novo heme biosynthesis, a condition that seems to be characteristic of all or most nematodes, including Caenorhabditis elegans [17]. Filarial worms presumably salvage heme/intermediates from their surroundings and/or acquire them from their Wolbachia endosymbionts. Heme deprivation may at least partially account for the effects caused by elimination of wBm following antibiotic treatment of filarial worms. For example, it is already known that antibiotic treatment disrupts the L4 to L5 molt in B. pahangi [18]. Furthermore, heme-containing enzymes such as peroxidases have critical functions in the molting of C. elegans and orthologs exist in B. malayi [19,20,21]. In this report, we indicate that Wolbachia heme biosynthesis likely contributes to filarial worm survival and thus could be a potential anti-filarial drug target set.

Materials and Methods
Cloning, expression and purification of human and Wolbachia heme biosynthetic enzymes
Human heme gene cDNA clones were purchased from the Invitrogen human cDNA clone collection, except for the 5'-aminolevulinic acid synthetase cDNA clone which was purchased...
from Open Biosystems. *B. malayi* worms were purchased from TRS Labs, Athens, GA. *B. malayi* DNA (including *Wolbachia* DNA) was extracted using DNeasy extraction (Qiagen) according to the manufacturer’s protocol. Based on available human, *Wolbachia* and *E. coli* sequences in the NCBI database, primers were designed with restriction endonuclease sites (Table S1) and used for full-length open reading frame (ORF) amplification by PCR with Phusion polymerase (New England Biolabs, NEB). After purification by QIAquick PCR purification (Qiagen) and digestion with corresponding restriction endonucleases (NEB), resulting PCR products were cloned into the pET21a+ vector (Novagen) for protein expression with a C-terminal 6XHis-tag. Correct clones were first identified by lysed-colony PCR and then verified by DNA sequencing. For improving protein expression and solubility, human 5′-aminolevulic acid dehydratase (ALAD), *Wolbachia* porphobilinogen deaminase (PBGD) and *Wolbachia* ferrochelatase (FC) genes were codon-optimized by gene re-synthesis using DNAlworks oligonucleotide designing software [22] and USER cloning methods [23].

All cloned heme genes were expressed in 17 Express competent *E. coli* (NEB), either with or without the RIL plasmid (Stratagene) which encodes *E. coli* rare tRNAs for arginine, isoleucine and leucine. Protein expression was induced with starting OD600 0.3–0.4, 10–100 μM isopropyl β-D-thiogalactopyranoside (IPTG, Sigma), 18–48 hours at 14–16°C. The 6XHis-tagged proteins were purified under native conditions, using a nickel resin (Qiagen) according to a modified manufacturer’s protocol. Buffers (100 mM Tris-HCl pH 8.0, 300 mM NaCl) containing different concentrations of imidazole (10–20 mM, 40–50 mM and 250 mM) were used as the lysis, wash and elution buffers, respectively. Purity of the proteins was verified on 4–20% SDS-PAGE gels (Invitrogen) and protein concentrations were measured on a Nanodrop ND-1000 (Thermo Scientific). Proteins were stored at −80°C in 10% glycerol for long-term storage or stored at 4°C for no more than 1 month for further analysis.

Phylogenetic analysis

Homologous protein sequences were retrieved from the NCBI database via protein-protein BLAST similarity searches and were aligned using CLUSTAL x1.83 [24]. The sequence alignments were further refined manually after the removal of large gaps and evolutionarily diverse regions. Based on protein sequence alignments, gene phylogenies for the *B. malayi Wolbachia* heme synthesis genes were derived from both Bayesian inference (BI) [25] and Maximum likelihood (ML) [26] methods. ML trees were constructed by the PROML programs of PHYLP package version 3.65 [27] with global rearrangements and randomized input order options in conjunction with estimated parameter gamma and the proportion of invariable sites obtained from TREE-PUZZLE 5.1 [28] calculation, in which Quartet puzzling maximum likelihood (QP) analysis was carried out employing the JTT-1 amino acid substitution probability model with a mixed eight category gamma+invariable-sites model of rate heterogeneity and 10,000 puzzling steps. ML analyses were performed by subsequent applications of SEQBOOT (100 replicates), PROML and CONSENSE. BI analyses were conducted with randomly produced starting trees, JTT amino acid substitution frequencies, four category gamma+invariable-sites model, 200,000 generations of searches. Posterior possibilities for the best trees were calculated using a 50% majority rule.

*B. malayi* worm ex vivo motility assays

Fresh live adult male and female *B. malayi* worms were incubated with different concentrations of succinyl acetone (SA, Sigma) or methyl mesoporphyrin (NMMP, Frontier Scientific) (3 replicates/experiment, 1 adult female or 3 adult males/replicate, experiment repeated three times), which target ALAD and FC, respectively. Worms were cultured in RPMI-1640 with 2 mM glutamine, 25 mM HEPES (Gibco) with 10% Fetal Calf Serum (Gibco) and 100 U/ml streptomycin, 100 μg/ml penicillin, 0.25 μg/ml amphotericin B (Sigma). Medium was changed every 2 days. SA was freshly made in water at a concentration of 500 mM before use. Both NMMP and hemin (Frontier Scientific) were freshly prepared as 5 mM stock concentrations in 50% ethanol containing 0.02 N NaOH. In NMMP tests, control worms were cultured in medium containing 1% ethanol and 0.0004 N NaOH (“solvent only”) with and without 100 μM hemin. Motility was measured daily [similar to the method used by Rao et al [11]] as, 0, no motility; 1, slight movement clearly observed under microscope; 2, minor movement readily observed by eye; 3, non-continual moderate movement; 4, continual moderate movement; 5, continual active movement.

*C. elegans* growth assays

*C. elegans* Bristol N2 was cultured and maintained according to standard protocols [29]. Drug testing was performed as follows. Eggs were extracted from gravid hermaphrodites using alkaline hypochlorite treatment followed by extensive washes in M9 buffer (22 mM KH2PO4, 42 mM Na2HPO4, 86 mM NaCl, 1 mM MgSO4) [29]. Eggs were allowed to hatch overnight in S-basal buffer (0.1 M NaCl, 0.05 M KH2PO4 pH 6, 5 mg/ml cholesterol). The concentration of first-stage larvae (L1) was adjusted to obtain an average of 5 to 6 live animals per well. Pre-grown concentrated *E. coli* OP50 was added as a food source. The compounds to be tested were added at the appropriate concentration. Worms were cultured in 96-well plates (NUNC, Rochester, NY) in a 100 μl volume for three days at 20°C. Twenty-four wells were cultured for each compound at a given concentration. Only one generation was followed. The number of parental animals reaching adulthood was scored.

Enzyme assays

Enzyme activities were assayed using purified recombinant C-terminal 6XHis-tagged *Wolbachia* and human ALAD proteins (wALAD & hALAD) at 37°C for 15–30 min. The enzyme reactions were carried out in 100 mM Bis-Tris Propane (BTP) buffer (Sigma, pH range 6.5–9.5) containing 1 μg protein, 5 mM substrate 5′-aminolevulinic acid (ALA) (Sigma) and 10 mM β-mercaptetoethanol (Sigma) unless otherwise stated, in a total volume of 100 μl. All assays were initiated by the addition of ALA after enzyme pre-incubation for 20–30 min with various metal ions (e.g. Zn2+, Mg2+) and/or other reagents (e.g. the metal ion chelator EDTA, specific enzyme inhibitor SA). After determining optimal reaction pH, enzyme assays were further conducted in the presence of different concentrations of substrate ALA (for determination of *Km* and *Vmax*) or inhibitor SA (for calculation of *EC50*). The reaction was stopped by mixing with an equal volume of stop buffer (0.1 M HCl in 12% Trichloroacetic acid) followed by the addition of 800 μl modified Ehrlich reagent for 10 min [30]. The product porphobilinogen (PBG) was subsequently estimated by measuring the absorbance at OD555. The molar extinction coefficient for PBG (60,200 M−1 cm−1) was used in calculation of PBG concentration (μmol PBG/mg of protein/h).

Complementation assays in *E. coli*

*E. coli* hemB (ALAD) mutant strain RP523 [31] and HemD (Uroporphyrinogen III synthase, UROS) deletion mutation strain SASZ31 were obtained from the *E. coli* Genetic Stock Center
E. coli hemG (PPO) deletion strain SASX36 [32] and hemH (FC) deletion strain VS200 [33] were generously provided by Dr. Harry A. Dailey, University of Georgia. The pET21a+ vectors carrying the corresponding human, Wolbachia and E. coli heme gene inserts were transformed into the above-mentioned E. coli mutant strains, both with and without RIL plasmid co-transformation alongside a vector only control, and with appropriate antibiotic selection. Transformants were selected on 20 μM hemin-containing LB plates with appropriate antibiotics and incubated at 37°C overnight. The selected transgenic clones were further tested on LB plates with no hemin addition.

E. coli-based growth assays

E. coli hemH mutants containing human, Wolbachia or E. coli FC genes, were used in growth assays. The fresh transgenic E. coli mutants (grown to 0.6–0.8 OD600) were diluted to 0.01 OD600 before initiating growth assays in the presence or absence of different concentrations of the specific FC inhibitor, NMMP in LB medium. The E. coli cells were grown in a 30°C shaker (180 rpm) for 3 h before estimating the cell growth level by measuring the final OD600 values. The final cell density for the untreated controls varied from 0.4 to 1.0 OD600. Relative cell density was taken as a measure of toxicity. The average cell growth ratio (final OD600/0.01) for untreated control is set at 1.0.

Results

Phylogenetic analyses reveal significant evolutionary divergence between Wolbachia and human heme biosynthesis gene homologues

Based upon genomic DNA sequence analyses, both humans and Wolbachia share the C4-type heme biosynthetic pathway, usually consisting of eight components (Fig. 1) and phylogenies inferred by both ML and BI analyses indicate a deep evolutionary distance existing between homologues in this pathway. Sequences for all heme biosynthetic enzymes (except for the missing PPO gene) were obtained by database queries from diverse organisms (17–30 species, with exclusion of archaeal sequences due to their extreme divergence). They were aligned using ClustalX (with manual refinement). Conserved regions (157–312 sites) were used for phylogenetic reconstruction. The unrooted gene trees (except for ALAS), presented in Fig. 2 and Fig. S1, show that the gene homologues from both nematode and insect Wolbachia consistently group together and mostly within the Rickettsia subgroup of the alpha-proteobacterial cluster, sharing high amino acid sequence similarity.

Figure 2. Gene phylogeny of ALAD and FC. A) ALAD, B) FC. The scaled Maximum likelihood (ML) consensus trees were inferred by ProML program of PHYLP 3.65 package [27]. Two methods – Bayesian inference (BI) and ML analyses were used in gene phylogeny reconstruction and yielded similar tree topologies (details see Materials and Methods). The supporting values shown at nodes were obtained from ML and BI analyses, respectively and the values below 50% indicated by hyphens. The branch length scale shown below ML tree represents estimated substitutions per site. Available GenBank accession numbers follow the corresponding sequences. The asterisk indicates sequences retrieved from the organism’s genome data; details are listed in the Text S1.

doi:10.1371/journal.pntd.0000475.g002

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www.plosntds.org 4 July 2009 | Volume 3 | Issue 7 | e475
identities/similarities (70–87% / 80–97%). By comparison, *B. malayi Wolbachia* heme synthesis genes only share 22–34% identities (29–33% similarities) with their human homologues. Among the seven identified components of *Wolbachia* heme pathway (Fig. 1), two were of particular interest (gene trees are presented in Fig. 2) owing to their significant divergence and biochemical properties. The ALAD gene tree shows that *Wolbachia* and human homologues belong to *Zn<sup>2+</sup>*-independent and *Zn<sup>2+</sup>*-dependent groups, respectively (Fig. 2A), which is confirmed by the absence of the critical cysteine residues in the *Zn<sup>2+</sup>*-binding sites in *Wolbachia* ALADs, while present in human ALAD (Text S1). Similarly, it is known that human FC contains both an N-terminal extension (encoding a mitochondrion-targeting signal) and a C-terminal extension (involved in formation of homodimers), and harbors an [Fe-S] cluster binding site (formed by 4 cysteine residues) [34,35]. However, sequence analysis of *Wolbachia* FCs revealed that they do not have any of these features (Text S1). This is in line with the FC phylogenetic analysis that shows significant evolutionary divergence between human and *Wolbachia* FCs (Fig. 2B).

**Wolbachia** heme biosynthesis might be crucial for *B. malayi* worm survival

Two analog inhibitors, SA (1–3 mM) and NMMP (10–100 μM), were used in *B. malayi* worm *ex vivo* motility assays, which specifically target ALAD and FC, respectively. The results are shown in Fig. 3A-D. Motility was measured (similar to the method used by Rao et al [11]). Compared to the untreated controls, both SA and NMMP lead to significantly reduced motilities of adult worms during the nine-day treatments, independent of the addition of heme to the medium (Fig. 3A-D). The tissue/cell structure of immotile worms (scaled as 0) seemed degenerative after treatment and no recovery was observed even after transferring these worms to fresh medium without inhibitor. Similar results were also observed in *B. malayi* mf larvae assays (data not shown). The effect of the inhibitors on female adult worms (Fig. 3B, D) appears more severe than that on male adult worms (Fig. 3A, C). The free-living nematode *C. elegans* was used as a worm control as it lacks the heme biosynthetic pathway and does not harbor an obligate endosymbiont, so has to salvage heme from the medium for viability [17]. In the presence of heme, NMMP (10–100 μM) did not affect the growth of *C. elegans* larvae into adults (Fig. 3E) or worm fertility (data not shown) as expected. However, SA (1–3 mM) appeared to have a nonspecific inhibitory effect on *C. elegans* larval development (Fig. 3E) and resulted in 100% sterility even at the lowest concentration tested (data not shown).

Biochemical characterization of purified recombinant *Wolbachia* and human ALAD enzymes
cDNA clones in pET 21a+ of *Wolbachia* and human ALADs were transformed into *E. coli* containing the RIL plasmid and expressed as C-terminal 6XHis-tagged proteins. However, expression for hALAD was very poor, thus a codon-optimized version was made for improvement of expression. Examples of purified full-length C-terminal 6XHis-tagged wALAD (37.7 kDa) and hALAD (37.4 kDa) are presented in Fig. 4.

The pH profiles (Fig. 5) for hALAD and wALAD enzyme activities indicate an overlapping optimal pH range (pH 6.5–7.5 is pH 7.0–8.5), hALAD is *Zn<sup>2+</sup>*-dependent. At optimal pH 7.0, its activity is inhibited by the metal ion chelator EDTA and recovered by addition of *Zn<sup>2+</sup>* (Fig. 6A). wALAD activity (at its optimal pH 8.0) is also sensitive to EDTA inhibition, however, its activity is only restored by Mg<sup>2+</sup> addition (Fig 6B). This suggests that wALAD is *Zn<sup>2+</sup>*-independent, which agrees with the absence of a putative *Zn<sup>2+</sup>* binding site in wALAD, while it is present in hALAD. The maximum activity (*Vmax*) of hALAD (pH 7.8) is measured as 57.8±2.2 μmol porphobilinogen (PBG)/mg of protein/h and the *Km* value for substrate ALA is 0.33±0.06 mM (Fig. S2A), while for wALAD (pH 8.0), the *Vmax* and *Km* values are 22.5±1.1 μmol PBG/mg of protein/h and 0.32±0.07 mM, respectively (Fig. S2B). hALAD activity is about 2.5 times higher than that of wALAD with similar ALA substrate binding affinity.

SA is a specific ALAD inhibitor with different potency depending on the particular ALAD species involved. The sensitivities of *wALAD* and *hALAD* to SA are presented in Fig. 7. Both enzymes could be inhibited by SA, but with strikingly different inhibition profiles - *EC<sub>50</sub>* for wALAD and hALAD were ~109 μM and ~0.18 μM, respectively, a 600 fold difference. This likely reflects a significant structural variation between these two enzymes.

**Functional complementation of *E. coli* heme deficient mutants and *E. coli*-based drug sensitivity assays**

To test whether the cloned *Wolbachia* hemo biosynthetic genes are functional, we performed complementation assays using *E. coli* heme deficient mutant strains (*hemB*, *hemD*, *hemG* and *hemH*). These assays complement the *in vitro* enzyme assays described above. The corresponding *Wolbachia* and human heme genes, cloned in the pET21a+ vector, were tested for activity, alongside a pET21a+ vector negative control.

An *E. coli* *hemB* mutant, transformed with the pET21a+ plasmid fails to grow on LB plates, unless heme is added to the media. Transformations of the *E. coli* *hemB* mutant strain with *wALAD* or *hALAD* constructs result in strong colony growth on LB plates without heme addition, similar to wild type *E. coli* growth, indicating functional expression of these two heme genes in *E. coli*. Similar complementation was observed for *E. coli* *hemD* and *hemH* mutants with *Wolbachia/human* UROS and *Wolbachia/human* FC genes, respectively. As mentioned previously, *Wolbachia* PPO is still un-recognized. It has been reported that overexpression of *E. coli* CPO might function as PPO [36] and therefore we tested an *E. coli* *hemG* mutant with the *Bm* CPO construct. No complementation was observed, even under IPTG induction. As a positive control, an *E. coli* *hemG* mutant was functionally complemented by transformation with the human PPO construct. Our enzyme assays and *E. coli* complementation tests verified that *Wolbachia* ALAD, UROS and FC genes are functional. Since ALAD is the second step and FC is the last step of the heme biosynthesis pathways, our results indicate that *Wolbachia* has the ability to synthesize endogenous heme.

FC is a potential drug target based on its evolutionary divergence and differing protein features compared to its human homologue. Human FC (hFC) is an [Fe-S] protein, while *Wolbachia* FC (wFC) lacks [Fe-S] clusters. A potent FC inhibitor - NMMP was used in growth inhibition assays using *E. coli* *hemH* mutants transformed with wFC, hFC or *E. coli* FC (EcFC). The growth of all transformed strains was inhibited by NMMP with significantly different drug sensitivities (sensitivity level: hFC>EcFC>wFC) as compared to their non-treated controls (Fig. 8). The inhibition was readily overcome by the inclusion of heme in the growth medium (Fig. 8). This assay further supports the possibility of using a rapid *E. coli*-based complementation assay to screen for specific inhibitors that will differentially target the *Wolbachia* heme synthesis enzymes.
Figure 3. Effects of succinyl acetone (SA) and N-methyl-mesoporphyrin (NMMP) on the motility of *B. malayi* adult worms *ex vivo* and *C. elegans* larval growth. A) Effect of SA on *B. malayi* adult males, B) Effect of SA on *B. malayi* adult females, C) Effect of NMMP on *B. malayi* adult males, D) Effect of NMMP on *B. malayi* adult females, E) Effects of SA and NMMP on growth of *C. elegans*. The scale of viability (A–D) is arbitrarily measured by the relative motility of the adult worms: 0, no motility; 1, slight movement clearly observed under microscope; 2, minor movement readily observed by eye; 3, non-continual moderate movement; 4, continual moderate movement; 5, continual active movement. Control *B. malayi* worms (C, D) were cultured in medium containing 1% ethanol and 0.0004 N NaOH ("solvent only") with and without 100 µM hemin. Data is compiled from 3 independent experiments (see Materials and Methods). Control *C. elegans* worms (E) were grown in 1% ethanol with 0.0004 N NaOH, which corresponds to the concentration of ethanol and NaOH present in the 100 µM NMMP test.
doi:10.1371/journal.pntd.0000475.g003
Discussion

Anti-Wolbachia chemotherapeutic treatment is an emerging approach for filariasis control [37]. Comparative genomic and bioinformatic analyses are shedding light onto the mutualistic symbiotic relationship between Wolbachia and its filarial host, revealing essential biochemical pathways in the bacterial endosymbiont that might provide critical metabolites for its worm host survival [14]. Based on our data from ex vivo worm assays, phylogenetic analyses, gene expression and purification profiles, in vitro enzyme assays, and E. coli complementation results, we have evaluated the possibility of the Wolbachia heme pathway as an anti-filarial drug target set. No heme biosynthetic genes except ferrochelatase (FC, the last step) have been identified from the worm host B. malayi genome sequence [16]. It appears that the nematode is not capable of synthesizing heme de novo, and thus may have to acquire heme from its Wolbachia endosymbiont or salvage environmental heme or both. The motilities of both B. malayi male and female worms are significantly reduced when exposed to the heme biosynthesis inhibitor SA (targeting ALAD), even in the presence of hemin in the medium (Fig. 3A–B). However, we believe that this effect was non-specific because a similar phenotype was observed when C. elegans, a heme auxotroph, which does not have the biosynthetic pathway at all, was exposed to SA (Fig. 3E), suggesting that SA may have some unspecific effect on B. malayi. In contrast, NMMP appears to be potent and specific in its inhibitory effect on the heme pathway, since it has an in vivo effect on B. malayi (Fig. 3C–D), but not on C. elegans (Fig. 3E). This inhibition can not be rescued by hemin (Fig. 3C–D), implying that B. malayi possibly lacks the capability of salvaging environmental heme, as has been demonstrated for C. elegans [38]. However, it should be noted that the B. malayi genome encodes for FC [16] and the effect of NMMP on B. malayi ferrochelatase (BmFC) could contribute to the inhibition of the worm viability (Wu et al, in preparation). We have no experimental evidence for a direct effect of NMMP on Wolbachia. Thus, the survival of B. malayi might be dependent on bacterial derived heme from the Wolbachia heme biosynthetic pathway and/or a functional BmFC which may utilize porphyrin intermediates from the endosymbiont or the environment. Given the observed differences between human and B. malayi FC proteins, specific inhibition of nematode FC in infected humans could be a potential drug target (Wu et al, in preparation).

Because mammals also synthesize heme via the C4-type pathway like Wolbachia, caution is needed when considering Wolbachia heme biosynthetic enzymes as anti-filarial drug targets. However, phylogenetic analyses (Fig. 2 & Fig. S1) revealed that significant evolutionary distances exist among the human and Wolbachia heme genes (except for ALAS), as shown by their low sequence similarities/identities (22–34%/29–53%). We corroborate these in silico studies with biochemical/pharmacological assays by cloning the Wolbachia and human genes and expressing the proteins. Difficulty in protein expression or purification of soluble proteins for hALAD, wPBGD & wFC was addressed by codon optimization. This helped improve expression levels, but still failed to yield soluble proteins, with the exception of synthetic hALAD. Based on the significant difference (~600 fold) in drug sensitivities between wALAD and hALAD enzymes (Fig. 7), large amounts of recombinant wALAD and hALAD proteins are currently being prepared for high throughput screening as part of anti-Wolbachia (A-WOL) drug discovery and development program (http://www.a-wol.com). Crystal structures are available for ALAD from human, mouse, yeast, Pseudomonas aeruginosa and Chlorobium vibrioforme (including a structure for yeast ALAD in complex with SA) from Protein Data Bank (PDB, http://www.rcsb.org). With this information, structural studies of Wolbachia and

![Figure 4. Purification profiles for C-terminally 6XHis-tagged wALAD and hALAD recombinant proteins.](doi:10.1371/journal.pntd.0000475.g004)

**Figure 4. Purification profiles for C-terminally 6XHis-tagged wALAD and hALAD recombinant proteins.**

**Figure 5. pH profiles for wALAD and hALAD enzyme activities.** The enzyme activity is expressed as µmol PBG/mg of protein/h. [doi:10.1371/journal.pntd.0000475.g005]
human ALAD by homology modeling may help explain the observed differences in SA sensitivity and help optimize identification of lead compounds obtained from the on-going drug screening effort.

Functional Wolbachia heme synthesis activity for several genes (ALAD, UROS and FC) along with their human homologues was demonstrated by complementation tests using the corresponding E. coli heme deficient mutants (\textit{hemB}, \textit{hemD} and \textit{hemH}). As mentioned above, PPO, the penultimate step in heme pathway, is missing in \textit{Wolbachia} and is unidentifiable from many other bacterial genomes, e.g. \textit{Rickettsia} [15]. The \textit{E. coli hemG} mutant was readily complemented by the human PPO gene; however, unlike \textit{E. coli} CPO, \textit{Wolbachia} CPO was incapable of rescuing PPO deficiency. Since PPO function is required for heme biosynthesis, it is possible that an unidentified oxidase may function as PPO in \textit{Wolbachia}.

We have attempted to express and purify recombinant wFC (codon-optimized) and hFC proteins, however the yield of pure soluble protein was limited due to the formation of inclusion bodies. The availability of an \textit{E. coli hemH} deficient mutant and functional complementation by wFC and hFC genes permit an alternative \textit{E. coli}-based inhibition assay and drug-screening strategy. FC, the final step in heme biosynthesis, is responsible for insertion of iron into protoporphyrin IX (PPIX) to form heme. NMMP is a strong PPIX analog, and competitively binds to the FC active site with \textit{Ki} values in the nM range [39]. It was reported previously that FC enzymes could have dramatically different sensitivities to NMMP inhibition (>1000 fold, e.g. Human FC vs chicken FC) due to the differences existing in their active sites [40]. The \textit{E. coli hemH} mutant, complemented with wFC, hFC and EcFC, is not sensitive to NMMP when grown in the presence of hemin. Significantly different sensitivities to NMMP were detected between human and \textit{Wolbachia} FCs in absence of hemin, with human FC being much more sensitive. This may be accounted for by the structural difference in their active sites. Cell-based drug screening may help identify compounds more specifically targeting wFC instead of hFC. FC crystal structures from several species (human, yeast and Bacillus) are also available in PDB with a crystal
structure of *Bacillus subtilis* FC complexed with NMMP [41]. *In silico* comparative studies based on molecular modeling can be conducted for *Wolbachia* and human FCs. *E. coli* mutants complemented by other *Wolbachia*/human heme genes (ALAD, UROS) will be used as potential screens as well for identification of compounds specifically inhibiting selected *Wolbachia* heme biosynthesis enzymes.

Our data suggest that the *Wolbachia* heme biosynthetic pathway is a potential anti-filarial drug target due to its requirement for survival of both *Wolbachia* and its filarial host. The presumptive transporters, responsible for heme trafficking, could be drug targets as well. However, it still remains unknown how the heme/heme intermediates might transfer from *Wolbachia* to its filarial host. No enzymes involved in traditional heme catabolism (e.g. heme oxygenase) have been identified from the *B. malayi* genome sequence. It is still an open question how transport, degradation, and regulation of heme occur in filarial parasites.

**Supporting Information**

**Figure S1** Gene phylogeny of ALAS, PBGD, UROS, UROD and CPO. A) ALAS, B) PBGD, C) UROS, D) UROD, E) CPO. The scaled Maximum likelihood (ML) consensus trees were inferred by ProML program of PHYLYP 3.65 package [27]. Two methods - Bayesian inference (BI) and ML analyses were used in gene phylogeny reconstruction and yielded similar tree topologies (details see Materials and Methods). The supporting values shown at nodes were obtained from ML and BI analyses, respectively and the values below 50% were indicated by hyphens. The branch length scale shown below the ML tree represents estimated substitutions per site. Available GenBank accession numbers follow the corresponding sequences. * Sequences were retrieved from the organism’s genome data directly; details are listed in the supplementary sequence alignment file.

**Figure S2** The Vmax and Km of the purified recombinant wALAD and hALAD enzymes. A) wALAD and B) hALAD were assayed at pH 8.0 and 7.0, respectively. The enzyme activity is expressed as μmol PBG/mg of protein/h.

**Table S1** *Wolbachia*, human and *E. coli* heme gene specific primers were used for acquiring the full-length coding sequence by polymerase chain reaction (PCR) amplification, and were subsequently cloned into pET21a+ vector. Primers were designed according to information acquired from available *B. malayi*, *Wolbachia* (*wBm*), human and *E. coli* genome databases. Restriction enzyme sites in primers are underlined. Abbreviations used: w: *Wolbachia*, h: human, Ec: *E. coli*, f: forward primer, r: reverse primer. The full names of the abbreviations for the heme biosynthetic enzymes are listed in the caption of Figure 1.

**Text S1** Multiple sequence alignment for heme biosynthetic genes ALAS, ALAD, PBGD, UROS, UROD, CPO and FC.

![Figure 7. Biochemical characterization of recombinant wALAD and hALAD enzymes. A) Inhibition of wALAD enzyme activity by SA, B) Inhibition of hALAD enzyme activity by SA. The enzyme activity is expressed as μmol PBG/mg of protein/h. doi:10.1371/journal.pntd.0000475.g007](https://www.plosntds.org/doi/10.1371/journal.pntd.0000475.g007)

![Figure 8. Growth inhibition assays by N-methyl mesoporphyrin (NMMP) of *E. coli* HemH mutants complemented with human, *Wolbachia* or *E. coli* FC genes. The average cell growth rate (final OD_{600}/0.01) for untreated control is set as 1.0. doi:10.1371/journal.pntd.0000475.g008](https://www.plosntds.org/doi/10.1371/journal.pntd.0000475.g008)
Acknowledgements

We are grateful to Dr. Harry A. Dailey (University of Georgia) for sending us *E. coli* hemG and hemH deletion mutants. We also acknowledge the kind assistance of Dr. Marion Sibley, Beth A. Cantin, and Jennifer Ware. We also appreciate Dr. Donald Comb for his encouragement and Dr. Clotilde Carlow, Dr. Elisabeth Raleigh, and Dr. Mark Taylor for valuable suggestions.

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