Wolbachia in Filarial Parasites: Targets for Filarial Infection and Disease Control

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Lymphatic filariasis and onchocerciasis are debilitating diseases caused by parasitic filarial nematodes. These nematodes have evolved a mutualistic symbiosis with intracellular bacteria of the genus *Wolbachia*, which are required for nematode embryogenesis and survival. The essential role of these bacteria in the biology of the nematode and their demonstrated involvement in the pathogenesis of filariasis make *Wolbachia* a promising novel chemotherapeutic target for the control of filarial infection and disease. This article reviews the recent findings, which highlight potential processes that form the basis of the symbiosis, the role of *Wolbachia* in filarial pathogenesis, and the efficacy of *Wolbachia*-targeted antibiotic chemotherapy in human trials. Future prospects for the development of an anti-*Wolbachia* treatment regimen suitable for integration into mass drug administration programs are also discussed.

Introduction

The filarial nematodes that cause lymphatic filariasis and onchocerciasis are responsible for a significant global disease burden affecting more than 200 million people throughout the tropics. Because of the high level of morbidity, both lymphatic filariasis and onchocerciasis have become the subjects of mass drug administration (MDA) programs, with the aim of eliminating filariasis as a public health problem [1]. Generous donations of drugs by the pharmaceutical industry (ivermectin from Merck and Co. Inc., Whitehouse Station, NJ, USA, and albendazole from GlaxoSmithKline, Research Triangle Park, NC, USA) have been critical to the establishment of these control programs, which aim to interrupt transmission of filariasis by annual community-directed mass treatment. Concerns over the sufficiency of current tools in achieving the goals of the programs [2–5] together with the risks of drug resistance [6,7] promote the need for further research into new treatment and control methods. A promising new approach to this problem has been the targeting of the endosymbiotic bacteria of filariae, *Wolbachia* [8].

Role of *Wolbachia* in Filarial Biology

Wolbachia are maternally inherited alpha proteobacteria and have been found in numerous arthropod species in addition to filarial nematodes. Taylor et al. [8] present a comprehensive review. Phylogenetic analyses and studies utilizing antibiotics have led to the conclusion that this intracellular symbiosis is mutualistic in contrast with the parasitic lifestyle embraced by the majority of arthropod *Wolbachia* [9]. Although the precise nature of this mutualism remains to be elucidated, the recent completion and annotation of the genome sequence of *Wolbachia* from *Brugia malayi* (*w*Bm) has offered some clues [10••,11,12].

Given that the trend in endosymbiont evolution is genetic reduction, the presence of several complete biosynthetic pathways in the *w*Bm genome suggests their importance in *Wolbachia* biology and also offers some insight into the nematode-symbiont relationship. For example, *Wolbachia* have retained complete pathways for de novo biosynthesis of purines and pyrimidines [10••], and evidence that *Wolbachia* may contribute to the nucleotide pool of the nematode host has recently been suggested using the rodent model filaria *Litomosoides sigmodontis* [13]. Differential gene display showed that depletion of *Wolbachia* using tetracycline treatment resulted in an upregulation of a putative phosphate permease gene within the nematode. As phosphate is an essential requirement for nucleotide synthesis, it was hypothesized that the nematode phosphate permease transports phosphate to the *Wolbachia* for this synthesis and that the loss of *Wolbachia* leads to an upregulation of this gene through a feedback mechanism [13].

Further evidence that *Wolbachia* contribute to the metabolic activity of the filarial nematode includes the contribution of *Wolbachia* to MTT formazan reduction, which is often used as a marker of worm viability [14]. Other likely candidates in terms of resources provided by *Wolbachia* include riboflavin, flavin adenine dinucleotide (FAD), and heme. Both riboflavin and heme biosynthetic pathways appear to be absent from the genome of *B. malayi* [15]; therefore, these products must be obtained either from exogenous sources or via their endosymbionts. Heme may be essential for molting and embryogenesis due to its involvement in the modification of ecdysteroid hormones, which are required for these processes [10••,11,12]. Interestingly, these processes are particularly susceptible to antibiotic treatment. In addition, molting and embryogenesis have also been shown to be impaired following exposure to gamma radiation, which again, was associated with the depletion of *Wolbachia* [16,17].

Role of *Wolbachia* in the Pathogenesis of Filariasis

Filarial nematodes cause a spectrum of disease manifestations associated with both acute and chronic inflammation. However, these infections are long-lived, and furthermore, pathology does not exist in all infected individuals, suggesting that these parasites are able to modulate the host's immune response to their advantage [18]. *Wolbachia* have been implicated in both the development of inflammatory-mediated pathogenesis and immune hyporesponsiveness [8], with particular emphasis on the roles played by Toll-like receptors (TLRs) in these mechanisms.

Firstly, using a murine model of onchocerciasis, *Wolbachia* were shown to be responsible for inflammatory corneal pathology. *Wolbachia* were implicated in both the recruitment and activation of neutrophils in the corneal stroma in response to the injection of *B. malayi* microfilariae into the cornea [19•]. In this study, *Wolbachia* surface protein was shown to be present within neutrophil phagolysosomes in vivo. Furthermore, both *B. malayi* extracts and whole, purified *Wolbachia* activated murine neutrophils in vitro. In contrast, this activation was greatly reduced when extracts from doxycycline-treated worms were used [19•]. Injection of purified *Wolbachia* (ie, without filarial antigens) into the cornea resulted in the recruitment of neutrophils into the stroma, thereby suggesting *Wolbachia* have a direct role in activating innate inflammation in vivo [20]. This recruitment and subsequent keratitis, however, was completely ablated in myeloid differentiation factor 88 (MyD88, an adapter protein used in TLR signaling pathways) knockout mice, thereby underlining the role of TLRs in the *Wolbachia*-mediated inflammatory response. In sub-Saharan Africa, ocular onchocerciasis presents as either a mild form (primarily in rain forested regions) in which blindness is very rare, or a severe form (presented in savannah regions) with a high prevalence of blindness. Interestingly, the savannah strains of *Onchocerca volvulus* were found to have a significantly higher *Wolbachia* DNA to nematode DNA ratio than the milder forest strains [21], further supporting a role for these bacteria in onchocercal pathogenesis.

In addition to the inflammatory activity of *Wolbachia* ligands, a role for these ligands in the immune hyporesponsiveness associated with filariasis was recently reported [22•]. In this study, murine peritoneal-elicited macrophages were pre-exposed to *B. malayi* soluble extract and subsequently restimulated with extract or TLR ligands. This pre-exposure resulted in macrophage tolerance to both self and non-self ligands. Furthermore, the induction of homo- and heterotolerance was determined to be dependent on *Wolbachia*. Investigators made this determination by comparing *Wolbachia*-containing extracts with those prepared using tetracycline-treated *B. malayi*, the aposymbiotic species *Acanthocheilonema viteae* and *Loa loa*, and by comparing the activity of extracts prepared from *Wolbachia*-infected and *Wolbachia*-free mosquito cell lines. The initiation was MyD88- and TLR2-dependent, even though the heterotolerance extended to multiple TLR ligands including MyD88-independent ligands, and CD40L, a TLR-independent stimulus [22•]. Therefore, *Wolbachia*-TLR2/MyD88 interactions are capable of modulating macrophage responsiveness, and the exposure of filariasis patients to *Wolbachia* and/or their components during a chronic infection may contribute to the establishment or maintenance of immune hyporesponsiveness.

A recent trial using doxycycline in patients with lymphatic filariasis revealed that depletion of *Wolbachia* leads to reductions in the dilation of lymphatic vessels and plasma levels of lymphangiogenic factors (vascular endothelial growth factor [VEGF]-C/soluble VEGF receptor-3) associated with improvements in the severity of lymphedema [23••], thereby providing further evidence that *Wolbachia* may contribute to lymphatic filariasis chronic disease pathogenesis. This potential restorative effect of doxycycline on patients with filarial pathology further supports the use of *Wolbachia* as a drug target for the control of filariasis.

Wolbachia-targeted Chemotherapy

Wolbachia are a compelling drug target for human filariasis following a series of experiments conducted in both animal and in vitro models [8].

In all these experimental systems, *Wolbachia* have been shown to be susceptible to tetracycline and rifampicin antibiotics [14,24,25]. Human trials have thus far focused on the use of doxycycline. The six clinical trials published since 2003 have focused on assessing macrofilaricidal effects, defining the minimum treatment duration required, and investigating the effects of *Wolbachia*-targeted drug treatment on filarial pathology.

Macrofilaricide

A randomized, placebo-controlled trial using an 8-week treatment regimen of 200 mg doxycycline per day (without the use of standard antifilarial treatment) for *Wuchereria bancrofti* infection resulted in an almost complete elimination of microfilaremia [26••], as was previously described for a 6-week treatment regimen [27]. However, for the first time in trials investigating the use of antibiotic treatment for human filariasis, macrofilaricidal effects were detected. These effects were observed through the lack of scrotal worm nests detectable by ultrasonography and a decrease in circulating filarial antigen levels in doxycycline treated individuals compared to the placebo group. The mean number of worm nests between groups differed by 80% at 14 months following treatment [26••], indicating that approximately 80% of adult worms had died as a result of the doxycycline treatment.

Notably, macrofilaricidal activity has also since been demonstrated for a 6-week treatment regimen [23••]. In this more recent study, the macrofilaricidal effect was detected at 12 months and was more pronounced at 24 months following treatment, adding to previous suggestions that the macrofilaricidal effect is gradual and may take longer than 12 months [26••].

Minimum regime

A recent trial using doxycycline in onchocerciasis patients was intended to define the minimum treatment duration with respect to achieving long-term sterilization of female adult worms and therefore long-term reduction in microfiladermia [28]. A previous trial using 100 mg doxycycline per day for 6 weeks effectively blocked embryogenesis in female *O. volvulus* [29]. This sterilization was sustained for at least 18 months following treatment. The subsequent trial tested whether shorter treatment regimens with a higher dose of 200 mg per day were equally as effective. In this study, a 2-week treatment regimen was insufficient to reduce microfiladermia in comparison with untreated controls [28]. However, treatment for 4 or 6 weeks did have an effect at the same time point, suggesting that both of these treatment regimens are effective in blocking embryogenesis. Although both treatments were effective at 8 months, one patient in the 4-week group was shown to have very low levels of microfilariae at 12 months following treatment, either indicating that the 6-week regimen is more effective at blocking embryogenesis or reflecting differences in sample size. Further investigations with larger samples are currently underway to resolve this issue.

In terms of bancroftian filariasis, a recent placebo-controlled trial conducted using a 3-week doxycycline treatment regimen sought to assess the effects of a shortened treatment course on *Wolbachia* loads, microfilaremia levels, adult worm viability, and adverse effects associated with standard treatment (ivermectin plus albendazole) [30•]. Trial participants were given doxycycline, 200 mg per day, or placebo for 21 days. Four months after treatment began, they received the standard albendazole and ivermectin doses used in MDA. This combination of doxycycline and standard treatment led to a long-term microfilaremia suppression superior to the standard treatment alone, although in contrast with the aforementioned trial using an 8-week course [26••], no macrofilaricidal effect was observed. Therefore, a 3-week course of doxycycline is not sufficient to kill adult worms, and further research is required to determine the minimum course of treatment for macrofilaricidal activity. However, another interesting outcome of this trial was the observation that doxycycline treatment may reduce the incidence of adverse events associated with standard antifilarial treatments [30•]. Although the difference was not statistically significant due to sample size, moderate adverse reactions were only observed in the placebo group, and these reactions were associated with microfilaremia in addition to levels of *Wolbachia* and proinflammatory cytokines in plasma.

Antipathology

The role of *Wolbachia* in filarial pathogenesis, in addition to the observation that doxycycline treatment reduces the levels of proinflammatory cytokines in patients' plasma [30•], led to the hypothesis that *Wolbachia*-targeted chemotherapy may lead to a reduction in filarial pathology [23••]. A 6-week placebo-controlled trial conducted on patients with *W. bancrofti* infection assessed the macrofilaricidal activity of doxycycline and also analyzed its effects on lymphatic dilation, plasma levels of the lymphangiogenic factors VEGF-C and sVEGFR-3, and the severity of pathology in lymphedema patients as measured by grade. Doxycycline was shown to reduce the pathology associated with lymphatic filariasis as the dilation of supratesticular lymphatic vessels was reduced in patients following doxycycline treatment. There was no improvement in placebo-treated patients. In addition to lymphatic dilation, an improvement was also observed in the clinical manifestations of lymphedema in patients treated with doxycycline, whereas those individuals treated with placebo demonstrated a trend towards deterioration. Both these improvements in pathology were associated with reductions in plasma levels of VEGF-C/sVEGFR-3, indicating that the VEGF-C/sVEGFR-3 system may constitute a key mediator of lymphatic pathology, and the reduction of these components in response to doxycycline is suggestive of a role for *Wolbachia* in driving lymphangiogenesis [23••].

Future Prospects

The ability of anti-*Wolbachia* chemotherapy to provide a sustained and possibly permanent block of embryogenesis together with the killing of adult worms and improvement of pathology offers a significant improvement over standard antifilarial treatments in both lymphatic filariasis and onchocerciasis. Indeed, mathematical modeling has tentatively suggested that implementation of doxycycline in a MDA setting for lymphatic filariasis would be at

least as effective as the diethylcarbamazine plus albendazole treatment regimen and most likely superior to the ivermectin plus albendazole regimen with regards to the prospects of elimination [31]. However, as described earlier, the treatment duration is restrictive with regards to MDA due to logistical difficulties. Moreover, known contraindications of doxycycline exist, which prevent its use in children under 9 years of age and in pregnant/lactating women. Therefore, current and future research must focus on identifying shorter treatment duration and alternative antibiotics effective at eliminating *Wolbachia* suitable for mass treatment strategies.

One possible improvement to the current doxycycline treatment regimen would be the addition of a second antibiotic with anti-*Wolbachia* activity. Such combination therapy has been effective in reducing the length of treatment in murine filariasis using doxycycline plus rifampicin [32].

Another factor that could influence the use of doxycycline is an improvement in the mode of delivery in order to increase the absorption and retention of the drug. One study has investigated the use of a liposomized form of tetracycline to treat *B. malayi* infection in the rodent *Mastomys coucha* [33]. In this investigation, tetracycline entrapped within liposomes was given subcutaneously to infected rodents, and both microfilarial counts and macrofilaricidal activity were compared with those animals treated with free tetracycline via subcutaneous injection and those treated with free tetracycline orally. The liposomized tetracycline treatment was significantly more effective than the free form of the drug. A 12-alternate-day treatment regimen resulted in a microfilaricidal effect, which was evident as early as 15 days post-treatment and complete at day 90. Subcutaneous injections of free tetracycline did not suppress the microfilarial load. Furthermore, tetracycline given orally required 45 days of treatment to show evidence of microfilaricidal effects [33]. Evidently, the absorption and retention of doxycycline can be improved, although further research is required to validate the use of liposomized antibiotics in *Wolbachia*-targeted filarial chemotherapy in humans.

Another study using an animal model of filariasis has offered some information on the dynamics of the macrofilaricidal effects achieved with tetracycline-based drugs. Using cattle naturally infected with *Onchocerca ochengi*, the study was designed to compare different treatment regimens on the viability of adult worms and their *Wolbachia* [34]. When comparing short intensive treatments with prolonged intermittent treatments or a combination of the two, it was demonstrated that although the prolonged intermittent and combination treatment affected a sustained depletion of *Wolbachia* and a macrofilaricidal effect, the short intensive treatment only produced a transient reduction in *Wolbachia* without any associated death of adult worms. The authors suggested macrofilaricidal activity against *O. volvulus* may be achieved by treating patients intermittently but for a longer duration (eg, 3- to 5-day treatments monthly for 6 months). The merits and logistics of such treatment regimens in human filariasis remain to be evaluated.

When considering future *Wolbachia*-targeted chemotherapy, the annotation of the sequenced *Wolbachia* genome *w*Bm is an obvious source for exploration [10••,11,12]. Potential drug targets that would interfere in the mutualistic association between the nematode and endosymbiont are high-priority targets for future drug discovery and development [12].

Conclusions

Filarial disease is still a leading cause of global disability despite the implementation of global MDA programs. As essential endosymbionts of the parasites responsible for filarial disease, *Wolbachia* are a promising chemotherapeutic target, and antibiotics targeting these bacteria have already shown an improved efficacy over standard antifilarial treatments in terms of the induction of long-term sterility in addition to macrofilaricidal effects. Future research should be directed toward developing an anti-*Wolbachia* treatment regimen that can be utilized within a field setting and will complement current filariasis control programs. The focus should not be placed solely on shortening current antibiotic courses but should also investigate the use of drug combinations and exploit the wealth of information in the recently annotated *B. malayi Wolbachia* genome to identify novel drug targets.

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