Review

Anti-*Wolbachia* drugs for filariasis

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The mutualistic association between *Wolbachia* endosymbionts and their filarial nematode hosts has been exploited as a validated drug target delivering macrofilaricidal outcomes. Limitations of existing antibiotics to scale-up have driven the search for new drugs, which are effective in shorter regimens of 7 days or less. Here, we review the last 14 years of anti-*Wolbachia* drug discovery by the anti-*Wolbachia* (A·WOL) consortium, which has screened more than two million compounds, delivering thousands of hit compounds. Refined screening models integrated with robust pharmacokinetic/pharmacodynamic (PK/PD) driven optimisation and selection strategies have delivered the first two drug candidates specifically designed to target *Wolbachia*. AWZ1066S and ABBV-4083 are currently progressing through clinical trials with the aim of delivering safe and effective macrofilaricides to support the elimination of onchocerciasis and lymphatic filariasis.

Human filariasis: two neglected tropical diseases with chemotherapeutic challenges

Onchocerciasis (see Glossary) and **lymphatic filariasis** (**LF**) are disabling diseases caused by parasitic filarial nematodes transmitted by insect vectors. With millions at risk, both diseases are classed as **neglected tropical diseases** (**NTDs**) and feature in the World Health Organisation's (WHO's) NTD roadmap for 2021–2030 [1]. Standard treatment for LF involves mass drug administration (MDA) strategies using the anthelmintics albendazole, ivermectin, and diethylcarbamazine citrate (DEC), which, by targeting **microfilariae**, aim to interrupt transmission. Control programmes have made significant achievements over the past 20 years [2]. Double-drug treatment regimens (ivermectin plus albendazole, or DEC plus albendazole) have been the mainstay for control programmes, but recent trials have demonstrated the superiority of a combination of all three drugs in clearing microfilariae and sterilising adult worms [3–6]. Given the potential to accelerate elimination, this triple combination of ivermectin, DEC, and albendazole (IDA) is now being implemented in several countries [7]. However, barriers exist to using this approach as a treatment for LF in areas with an overlapping incidence of onchocerciasis or another filarial disease, **loiasis**, and IDA is not currently recommended in such areas [8]. The MDA strategy for onchocerciasis relies on community-directed treatment with ivermectin (CDTI). DEC can cause severe adverse events (SAEs), including irreversible blindness, and is contraindicated for onchocerciasis [9–11], while albendazole has no added benefit [12,13]. Ivermectin can also cause serious adverse events in individuals with high *Loa loa* microfilarial (mf) loads [14], which can be fatal, highlighting the pressing need for alternative strategies to treat onchocerciasis [15]. Elimination of both onchocerciasis and LF are hindered by total reliance on **microfilaricidal** treatment regimens and the absence of a **macrofilaricidal** (adult-killing) treatment, which has been recognised as a critical requirement for onchocerciasis elimination by the WHO NTD roadmap [1]. Furthermore, the reliance on a single class of drug for MDA for onchocerciasis risks the development of resistance, with evidence of suboptimal responses reported in Ghana and Cameroon consistent with this threat [16,17]. This, together

Highlights

Wolbachia bacterial endosymbionts are a validated drug target for onchocerciasis and lymphatic filariasis delivering macrofilaricidal outcomes.

The need for a macrofilaricide is recognised as a critical requirement for onchocerciasis elimination by the World Health Organization (WHO) neglected tropical disease (NTD) roadmap 2021–2030.

The anti-*Wolbachia* (A·WOL) consortium has screened >two million compounds delivering 20 000+ hits and dozens of new chemical series.

Two A·WOL clinical candidates – AWZ1066S and ABBV-4083 – are currently progressing through Phase I and Phase II clinical trials.

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with the widespread resistance to macrocyclic lactones in veterinary medicine, including the filarial disease, heartworm [18,19], re-enforces the need for alternative drugs to support the elimination of onchocerciasis and LF.

The causative agents of these diseases (*Onchocerca volvulus* for onchocerciasis and *Wuchereria bancrofti* and *Brugia malayi* for LF) each have a mutualistic symbiotic relationship with the obligate intracellular bacterium, *Wolbachia pipientis* [20,21]. Disruption of this symbiosis impacts on the integrity of adult worms and leads to the permanent sterility and premature death of the normally long-lived adult worms (5–14 years) and has, therefore, become a focus of filariasis drug-discovery efforts. Here, we review the past decade and a half of anti-*Wolbachia* drug discovery by the A·WOL consortium[i], the resulting outputs, and future perspectives.

Wolbachia: a validated macrofilaricidal target

Antibiotics as treatments for human filariasis

Several clinical trials, using doxycycline, have demonstrated macrofilaricidal efficacy in human patients for both onchocerciasis and LF [22–25]. The therapeutic benefits of anti-wolbachial treatments are summarized in Box 1. An important advantage of the anti-*Wolbachia* approach is safety in *L. loa*-endemic areas. *L. loa* lacks *Wolbachia* endosymbionts [26] and, therefore, is not affected by anti-*Wolbachia* treatments [25].

While community implementation of doxycycline treatment has been shown to be well tolerated, feasible, and effective in depleting *Wolbachia* [27,28], can be implemented in restricted communities where existing strategies are failing [29,30][ii], and has been used as an end-game strategy in the Brazilian and Venezuelan Onchocerciasis Elimination Program for the Americas (OEPA) programmes [31], it is generally accepted that the 4–6 weeks of treatment, and the exclusion of pregnant women and children under 8 years of age, are barriers to the scale-up of doxycycline as a community-based anti-filarial therapy with associated compliance with antimicrobial resistance stewardship. New anti-*Wolbachia* drugs, that retain the efficacy characteristics of doxycycline, but are more easily and broadly implemented, are needed.

Anti-*Wolbachia* drug discovery: a focus for investment

In 2007, the anti-*Wolbachia* (A·WOL) consortium was formed, funded by grants from the Bill & Melinda Gates Foundation awarded to the Liverpool School of Tropical Medicine[i]. The primary aim of this global consortium, made up of academic and industrial partners, was to find new

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Box 1. Targeting of *Wolbachia* with doxycycline delivers safe curative outcomes in onchocerciasis and LF

90% depletion of *Wolbachia* leads to:

- arrested development of larval stages,
- permanent blockade of embryogenesis,
- gradual loss of blood or skin microfilariae,
- macrofilaricidal outcomes.

Benefit of the anti-*Wolbachia* mode of action:

- Slow kill mechanism avoids side-effects of rapid adult worm death in tissues;
- Not microfilaricidal
  - de-risks ocular side effects in onchocerciasis,
  - safe to use in *Loa loa* coinfection.
- Blocks transmission by:
  - permanently sterilising adult worms,
  - impairing ability of mf to develop in vectors.
- Improvement in clinical disease: onchodermatitis, hydrocoele, lymphoedema.

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anti-*Wolbachia* drugs that overcome the barriers to doxycycline scale-up. A secondary aim was to optimise the regimens of registered drugs with anti-*Wolbachia* activity for use in more restricted settings. The first task was to create a Target Product Profile (TPP), which defines the desired characteristics of the new product in terms of its intended use in the target population and, ultimately, steers the drug discovery and development process. For onchocerciasis and LF, the A·WOL TPP included stipulations for any resulting drug to be available and efficacious in an oral formulation and to require no more than 7 days treatment. Applicability for pregnant women and children below 8 years of age were also included as 'ideal' TPP criteria. The complete current A·WOL screening pipeline is presented in Figure 1.

Primary screening assays: evolution of screening assay capacity
Assay development
The A·WOL consortium brought together industrial partners, with access to chemical libraries and technologies, and academics with biological expertise, to develop a robust drug-screening assay. In the absence of a cell-culture system for nematode *Wolbachia*, established cell cultures derived from *Wolbachia*-infected mosquitoes were selected [32]. Initial experiments focused on determining the optimal conditions (including cell number, media replenishment requirements, and assay duration) to establish a 96-well format assay with appropriate dynamic range between vehicle-treated and the 'gold-standard' doxycycline-treated cells. Using an established stably-infected C6/36(*w*AlbB) cell culture [33], various readouts were tested with a final selection of a

Microfilariae can also migrate to the eye. Pathology, generally caused by inflammation in response to dead or dying microfilariae, is skin disease (onchodermatitis) and visual impairment leading to blindness ('river blindness'). *O. volvulus* nematodes contain *Wolbachia* bacteria.

Pharmacokinetic/ pharmacodynamic (PK/PD): PK/PD modelling/analysis. Integration of pharmacokinetic information (how the drug is absorbed, distributed, metabolised, and excreted) with pharmacodynamic information (the effects of the drug on the infection and the body) to understand dose and effect relationships and therefore inform dosing strategies.

Structure–activity relationship (SAR): the relationship between the chemical structure of a molecule (e.g., drug) and the biological activity. Knowledge of SAR can inform the medicinal chemistry strategy to optimise parameters such as potency.
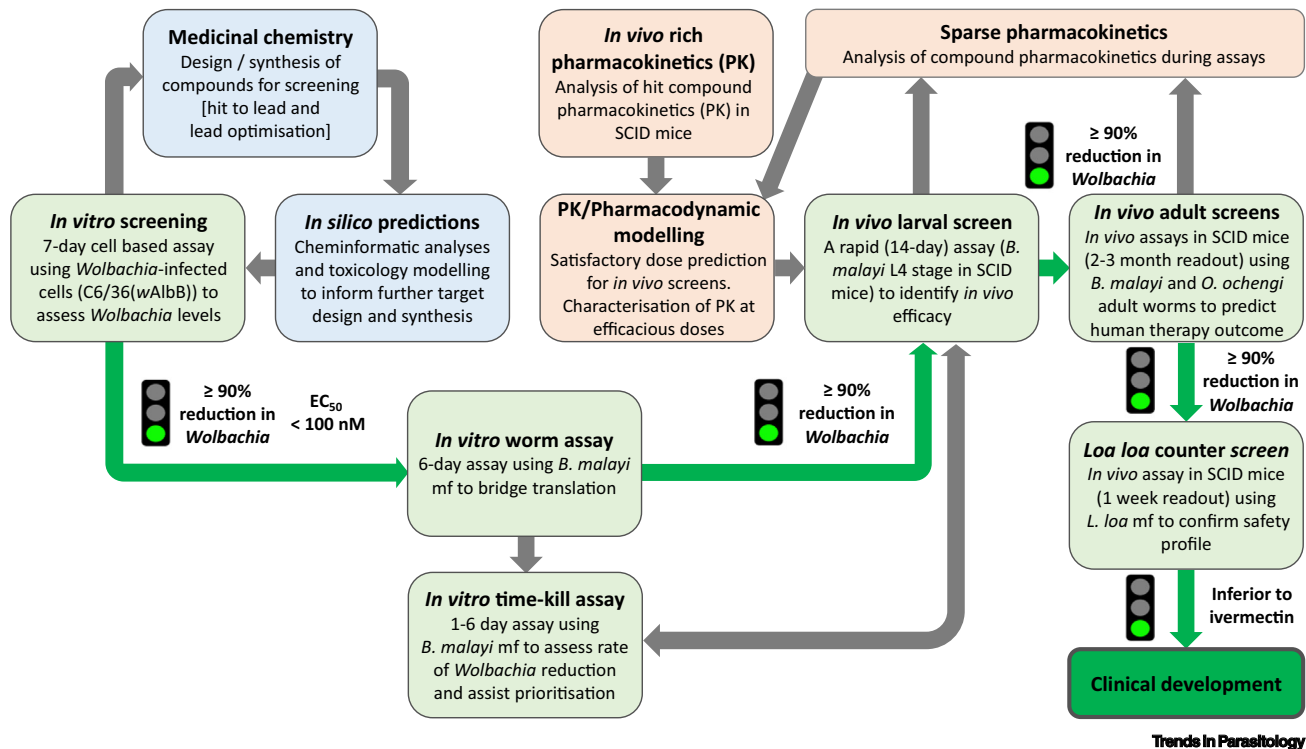
Figure 1. The A·WOL screening pipeline. The current screening assays (shown in light green) have evolved over time and involve *in vitro* assays and *in vivo* preclinical models. Movement of compounds through this pipeline is governed by strict go/no-go criteria, highlighted by green arrows, traffic lights, and associated text. The chemistry components of the pipeline (shown in blue) feed directly into an iterative process where hits are selected, optimised, and retested in order to advance the most appropriate for further testing. The incorporation of pharmacokinetic (PK) and pharmacodynamic (PD) analyses (shown in orange) at various points in the pipeline, from determining the PK of initial screening hits to modelling dose prediction, also serves to drive the selection process. Abbreviations: A·WOL, anti-*Wolbachia* consortium; *B. malayi, Brugia malayi*; mf, microfilariae; *O. ochengi, Onchocerca ochengi*, SCID, severe combined immunodeficient.

9-day assay endpoint, and a qPCR readout with toxicity assessed using a metabolism-based readout (CellTitre-Glo™) [34]. With the assay system defined, the first step in the A·WOL screening campaign was to test the complete human pharmacopoeia (2664 compounds) to identify registered drugs with anti-*Wolbachia* repurposing potential [34]. This identified tetracyclines, rifamycins, and the fluoroquinolone class of antibiotics as having anti-wolbachial activity. Further screening of focused libraries continued using this assay, including screening of a library of anti-infectives provided by Abbott (now Abbvie), that led to the discovery that the veterinary antibiotic, tylosin, had potent anti-*Wolbachia* efficacy. This prompted a medicinal chemistry programme that resulted in the development of TylAMac™ (ABBV-4083) – the first designer semi-synthetic anti-*Wolbachia* drug [35,36] (see the section 'Translational status of A·WOL outputs').

Scaling up: exploring chemical diversity

The first large diversity library to be screened was made up of 10 000 compounds from the BioFocus SoftFocus libraries [37]. To complete this screening rapidly, improvements in capacity and throughput were required through altering qPCR reagents and optimising logistics. Screening was completed in less than 12 months, and subsequent chemoinformatic analyses of the hits led to the discovery of six new chemotypes with activity against *Wolbachia* [37], one of which was the forerunner to the first fully synthetic A·WOL candidate AWZ1066S [38] (see the section 'Translational status of A·WOL outputs').

The technological advances in microscopy-based multiwell systems provided an opportunity to revisit visualisation and quantification of *Wolbachia* within cells as a drug-screening assay. The development of a new assay using the Operetta high content automated imaging system (Perkin Elmer) with *Wolbachia*-infected cells involved stabilising cell–cell fluctuations in *Wolbachia* numbers through altering the culture medium, thus improving the signal window, as well as miniaturising the assay from a 96-well to a 384-well format. The readout was much simplified, using a simple DNA stain, SYTO11, to visualise cell nuclei and *Wolbachia*, and integrated Harmony software to determine infection levels. The signal window was also retained with a reduction in assay duration from 9 to 7 days. The resulting validated assay led to a 25-fold improved throughput as well as the ability to generate EC_{50} curves [39]. These improvements facilitated progression of hits through hit-to-lead and lead optimisation workflows by, for example, allowing detailed **structure–activity relationship (SAR)** analyses to be conducted [35–38,40].

Industrial-scale A·WOL screening

The improvements in throughput of the Operetta-based assay, as well as the implementation of simple robotics, facilitated larger-scale screening of 50 000+ compound libraries (including one gifted to the consortium by the Medicines for Malaria Venture, MMV) through this in-house screen. A partnership formed between the A·WOL consortium and the pharmaceutical company AstraZeneca led to an even further improved throughput by allowing access to their leading automation, screening technologies, and expertise.

While the Operetta-based screen utilised a simple staining technique, throughput was hampered by the time taken to analyse the plates, a consequence of the magnification and number of fields of view required to achieve a robust signal-to-noise ratio. The assay developed through the shared knowledge and experience of A·WOL and AstraZeneca researchers simplified the readout from a 'per-cell' to 'whole-well' analysis, therefore accelerating data acquisition [41]. To achieve this, an immunofluorescence readout was developed, using a *Wolbachia*-specific antibody, anti-*Wolbachia* peptidoglycan-associated lipoprotein of *Brugia malayi*, *w*BmPAL [42]. The industrial automation available at AstraZeneca allowed this more complex assay to be developed and validated [41] and subsequently used to screen AstraZeneca's 1.3 million compound library in

two months [43]. Subsequent chemoinformatic analyses prioritised approximately 6000 compounds for dose–response screening, resulting in 57 prioritised chemical clusters with representatives ready to move through the pipeline [43].

Orthogonal screening assays: prioritising hits

In vitro worm-based screening

The A·WOL primary screening assay evolved over time to the point where in excess of 20 000 hits were discovered. Secondary screening assays involving in vitro worm-based assays using adult male Onchocerca gutturosa [44] or B. malayi [45] were included in earlier pipelines, but refinement of the in vivo model systems allowed these to be streamlined out [46]. However, the increased throughput of the primary assay, together with the accumulation of potency metrics from this assay, resulted in an accumulation of high-potency hits. To prevent a bottleneck in the screening cascade, it became necessary to reintroduce a worm-based triage assay to prioritise compounds for entry into the available in vivo model systems (Figure 1). A 6-day B. malayi mf assay [38,43] was developed as a way to triage hits, with higher throughput than adult worm assays, by confirming that compounds (i) could penetrate the nematode cuticle to target nematode Wolbachia and (ii) did not have direct toxicity for the nematode. Potency was also measured for active compounds and could be factored into the screening prioritisation.

Assessing dynamics of anti-Wolbachia activity

A reduction in the duration of treatment is a core component of the TPP for a new anti-Wolbachia treatment. Comparing the time-kill dynamics of compounds, therefore, has potential to offer insight into which compounds are more likely to achieve this goal. By implementing a timed wash-out strategy (i.e., through washing off the compound after 24 h or 48 h of exposure) onto the standard mf assay, the dynamics of anti-Wolbachia activity can be assessed. Importantly, compounds are compared using equipotent concentrations ($10 \times EC_{50}$) in this assay to ensure that any differences in time-kill profile are not a result of differences in potency. When compared with antibiotics with activity against Wolbachia that are approved for human use (tetracyclines, rifamycins, fluoroquinolones), AWZ1066S and five chemotypes discovered via the AstraZeneca collaboration were demonstrated to have a more rapid kill profile in this assay [38,43]. These six compounds reduce Wolbachia numbers significantly after only 24 h of treatment and can achieve equivalent reductions after 48 h to those achieved by doxycycline after 6 days. Not only does this offer hope for a reduced treatment regimen in the clinic but would also indicate that, with these compounds, anti-Wolbachia drug discovery has shifted from operating purely in the bacteriostatic space and has discovered potentially bactericidal agents. Uncovering the mechanism of action of AWZ1066S, using chemical proteomic approaches, is ongoing.

Preclinical translational models

Pharmacological evaluations of promising anti-Wolbachia candidates as antifilarial agents require assessment in the context of a whole-animal physiological system. Laboratory drug testing has traditionally utilised the rodent Meriones unguiculatus (Mongolian jird), which is naturally susceptible to a human subperiodic isolate of B. malayi. Parasites establish long-term patent infections in either the lymphatics or the peritoneum, with persistent mf production circulating in the blood or contained within the peritoneal cavity, respectively [47–49]. Thus, these models closely emulate the life cycle traits and Wolbachia growth dynamics of human LF pathogens and are useful tools to predict in vivo anti-Wolbachia drug responses and concomitant parasitological efficacies against human LF infections: B. malayi, Brugia timori, and W. bancrofti.

An alternative laboratory model adapted for drug screening utilises the Wolbachia-containing rodent filaria Litomosoides sigmodontis. L. sigmodontis is a natural parasite of cotton rats [50] and

can be maintained in jirds with patent infections establishing in the pleural cavity surrounding the lungs and mf migrating into the circulation to develop parasitaemias. *L. sigmodontis* has also been selected via passage to survive for periods sufficient to complete its life cycle within certain inbred strains of mice [51]. Advantages of this surrogate model are the increased convenience, throughput, and reduced costs of using a smaller mouse host and faster growing rodent filaria, including reducing experimental anti-*Wolbachia* drug compound synthesis requirements (around fourfold, considering weight difference between mice and jirds). More extensive murine pharmacokinetic data in public and industry domains is another advantage of undertaking filariasis testing within mice.

However, phylogenetic dissimilarities between *L. sigmodontis, Brugia* spp.*,* and the medically important filarial pathogen*, O. volvulus,* risks a lack of translation when selecting drug candidates, particularly for onchocerciasis indications. In the context of anti-*Wolbachia* pharmacology, potential variability between *Litomosoides, Brugia*, and *Onchocerca* include differences in the bioaccumulation of small molecules within *Wolbachia*-containing tissues (due to inherent differences in perfusion, active uptake, rate of metabolism, or efflux of drug). Further intrinsic variability in type of *Wolbachia* (clade C in *Onchocerca* vs clade D in LF species and *Litomosoides*) [52], and nature of the symbiosis across different filariae [53], may influence anti-*Wolbachia* efficacy via variables such as drug target expression level, the relative sensitivity of the filaria to decline in *Wolbachia* populations, the growth rate of *Wolbachia,* and ultimate target *Wolbachia* biomass.

Due to limitations in current preclinical *in vivo* filarial drug screens, the A·WOL consortium developed novel mouse infection models of human filarial pathogens for anti-*Wolbachia* research and development. By identifying the mechanisms of innate and adaptive immune control of *B. malayi* in mice, it has been possible to establish chronic patent infections of this medically important parasite in a range of transgenic mice deficient in facets of eosinophilic type-2 immunity [54,55]. Due to global commercial availability and adaptability for humanisation, CB.17 severe combined immunodeficient (SCID) mice were evaluated as a suitable long-term susceptible model of *B. malayi* infection and subsequently validated as a direct-acting or anti-*Wolbachia in vivo* drug screen using flubendazole or tetracycline antibiotics, respectively [56].

SCID mice were assessed for comparative susceptibility to *Onchocerca* adult infections*.* For this, we utilised the closely related cattle parasite *Onchocerca ochengi,* which, by identification of naturally infected cattle from farms in the North of Cameroon, provides a convenient and abundant source of adult stages of the parasite. Three approaches were evaluated: development of adult infections from experimental inoculations of isolated infectious stage larvae, xenografts of isolated cattle nodules containing male and female worms, or isolation and implantation of male *O. ochengi*. The latter approach proved reproducibly successful with approximately one-third of the implanted male *O. ochengi* surviving a minimum of 6 weeks in the peritoneum of recipient SCID mice [56]. This model was subsequently validated using oral tetracycline and rifamycin antibiotics or flubendazole as the first small-animal macrofilaricidal drug screen for the evaluation of drug candidates targeting *Onchocerca* [56–59].

Prior research has demonstrated that mf purified from cattle naturally infected with another *Onchocerca* cattle parasite, *Onchocerca lienalis,* can establish chronic microfilaridermias in SCID mice [60]. Following purifications of *Brugia* mf from jirds, we also could establish long-term circulating microfilaraemias in SCID mice. These microfilaraemic/microfilaridermic SCID mouse models are sensitive to the microfilaricide ivermectin, provoking a rapid, >90% decline in mf in the blood or skin 2 days after single-dose treatment [56,59,60]. We therefore generated a microfilaraemic SCID mouse model of *L. loa* following mf purification from experimentally

infected baboons or infected hypermicrofilaraemic loiasis individuals. *Loa* microfilaraemic mice were equally sensitive to the effects of single oral dose ivermectin with >90/98% depletions observed after 2 or 7 days, respectively [61].

Thus, a 'pan-filarial' SCID mouse drug screening model has been established, susceptible to major human filarial genera of medical importance (*Brugia, Onchocerca, Loa*) with validated responses to reference antibiotics or filaricides (Box 2). This offers the advantage of testing drug candidates for anti-*Wolbachia* or direct macrofilaricidal efficacies and their selectivity to avoid rapid microfilaricidal toxicity whilst controlling for pharmacokinetic variabilities within a single SCID mouse strain. Via scrutinising efficacy sequentially against *B. malayi* adults, *O. ochengi* adult males, and *L. loa* mf, the pan-filarial SCID mouse model has been implemented to evaluate five antifilarial and nine anti-*Wolbachia* macrofilaricide candidates [57–59,61–63] (Figure 1). The SCID models have provided robust **PK–PD** data supporting clinical selection of the anti-wolbachials ABBV-4083 and AWZ1066S as well as supporting the developmental pathway of the direct-acting clinical candidate, oxfendazole [61].

The preclinical screening models were also used to refine regimens of registered antibiotics, including minocycline [62,63], rifampicin [57,58], and fluoroquinolones [64], in which combinations of anti-wolbachial drugs enabled shorter treatment regimens. An important, but unexpected, outcome was the identification of drug synergy when combining anti-*Wolbachia* drugs with albendazole *in vivo*. The impact of this combinatorial drug synergy was to reduce treatment regimens necessary to mediate threshold anti-*Wolbachia* activity [65] and translated to deliver reduced dose time-frames in clinical trials against onchocerciasis [66] (Figure 2).

Box 2. Pan-filarial mouse model for preclinical efficacy testing

Advantages of the CB.17 SCID pan-filarial research model:

- single small-animal host for more accurate interpretation of PK/PD between filarial genera of medical importance (Figure I),
- common laboratory species/strain with historical PK data available,
- standardized commercial supply (no breeding costs or management, pedigree strains),
- potential for further technological development: for example, humanisation, longitudinal bioimaging, drug-pump implants.
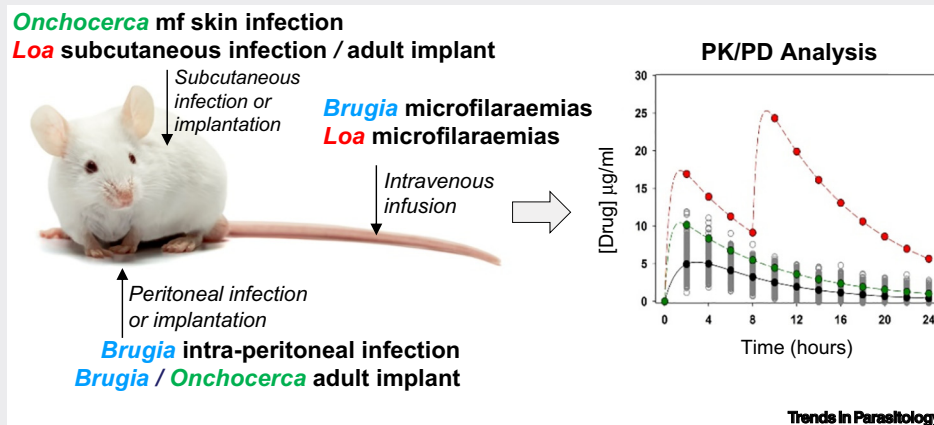

Onchocerca **mf skin infection**
Loa **subcutaneous infection / adult implant**

Subcutaneous infection or implantation

Brugia **microfilaraemias**
Loa **microfilaraemias**

Intravenous infusion

PK/PD Analysis

Peritoneal infection or implantation
Brugia **intra-peritoneal infection**
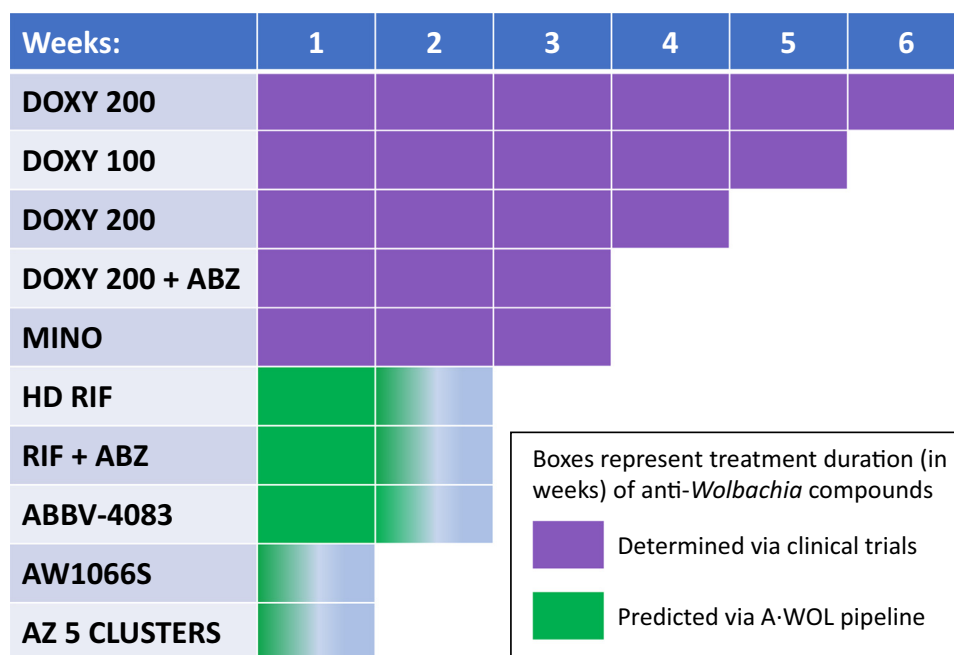*Brugia / Onchocerca* **adult implant**

Trends in Parasitology

Figure I. Schematic overview of the pan-filarial mouse model. Abbreviations: mf, microfilarial; PK/PD, pharmacokinetic/pharmacodynamic.

Weeks:	1	2	3	4	5	6
DOXY 200	■	■	■	■	■	■
DOXY 100	■	■	■	■	■	
DOXY 200	■	■	■	■		
DOXY 200 + ABZ	■	■				
MINO	■	■	■			
HD RIF	■	■				
RIF + ABZ	■	■				
ABBV-4083	■	■				
AW1066S	■					
AZ 5 CLUSTERS	■					

Boxes represent treatment duration (in weeks) of anti-*Wolbachia* compounds

■ Determined via clinical trials

■ Predicted via A·WOL pipeline

Trends in Parasitology

Figure 2. Anti-*Wolbachia* treatment regimens. The top half of the figure (purple boxes) illustrates treatment durations (in weeks) determined via clinical trials of tetracycline antibiotics at different doses (100 mg/day or 200 mg/day) with and without albendazole [22,23,25,66,72]. The bottom half of the figure (green boxes) illustrates predicted regimen reductions with adjusted doses and combinations of registered drugs or novel compounds discovered through the anti-*Wolbachia* (A·WOL) consortium [35,38,43,57,58,65]. Registered drugs included in the figure are doxycycline (DOXY), minocycline (MINO), rifampicin (RIF), high-dose rifampicin (HD RIF), and albendazole (ALB). Novel compounds shown are ABBV-4083 (TylAMac), AWZ1066S, and five chemical clusters identified from high-throughput screening in collaboration with AstraZeneca (AZ 5 CLUSTERS).

Translational status of A·WOL outputs

ABBV-4083

ABBV-4083 (Figure 3), also known as A-1574083 and TylAMac™, is the most advanced candidate in the clinical development pipeline – it has successfully completed a Phase I trial and is advancing to a Phase II trial against onchocerciasis in a partnership with Liverpool School of Tropical Medicine (LSTM), Abbvie, and DNDi[iii].

The original hits with the scaffold of ABBV-4083 were discovered in a focused macrolide library from the Abbvie antibiotic collection. Tylosin A (TylA) was identified as the most potent hit (another hit with lower potency is the analogue of TylA, tylosin B) *in vitro*. TylA is a well-established veterinary antibiotic, with a good safety record, used mainly for the treatment of Gram-positive bacterial infections in animals [67]. TylA demonstrated proof-of-concept *in vivo* efficacy in two filarial infection animal models when dosed through intraperitoneal (IP) administration, but no activity when dosed orally. Hence improving permeability and oral bioavailability were the primary goals in the medicinal chemistry optimisation process. Masking the 2′- and 4″-hydroxyl groups as esters, carbamates and benzyl ethers in TylA was investigated and resulted in two lead compounds – ABBV-4083 (benzyl ester at the 4″-position) and A-1535469 (carbamate at the 4″-position) – showing marked improvement in oral PK profiles in the mouse or jird when compared with TylA [36]. The two leads were assessed in several animal models, including *B. malayi*, *L. sigmodontis*, and *O. ochengi* to determine their anti-*Wolbachia* efficacy *in vivo* and both showed excellent
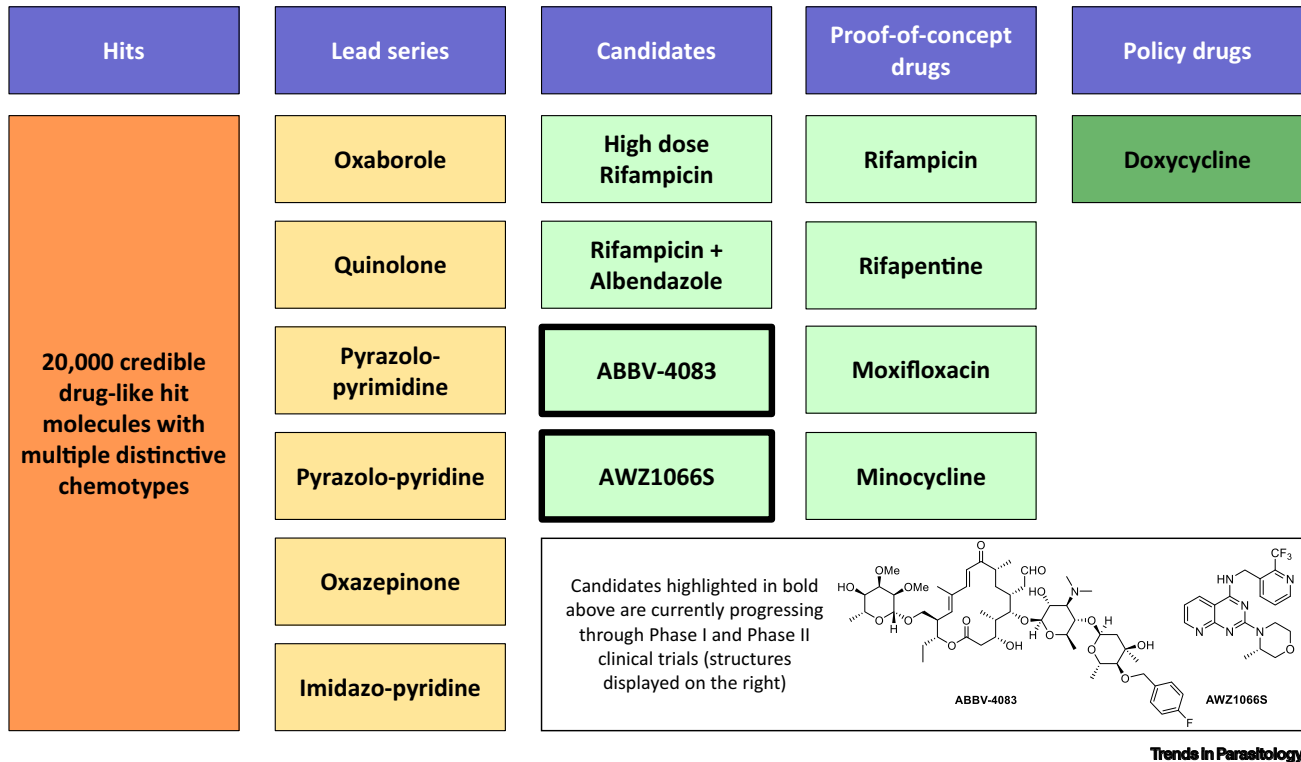
Figure 3. The anti-*Wolbachia* (A·WOL) portfolio. The portfolio of anti-*Wolbachia* compounds/drugs visualised according to their current status in the pipeline, from thousands of screening 'hits' through lead series, candidates, 'proof-of-concept' drugs to doxycycline, the only anti-*Wolbachia* drug currently in use. Candidates ABBV-4083 and AWZ1066S, highlighted in bold, are currently progressing through Phase I and Phase II clinical trials. The structures of these candidates are shown.

efficacy against *Wolbachia* (>90% reduction) in these PD studies with 14-day oral dosing regimens (Table 1). In these animal models, when given a human-equivalent dose of tetracyclines, including doxycycline and minocycline, as positive controls, tetracyclines had to be dosed for at least

Table 1. Reported efficacy of the two clinical candidates, ABBV-4083 and AWZ1066S, compared with approved anti-*Wolbachia* drug, doxycycline, in three filarial infection animal models [35,38]

	Doxycycline	ABBV-4083	AWZ1066S
Brugia malayi (adult female)	98.5% *Wolbachia* reduction at 100 mg/kg, q.d., 21 days[a]	>99% *Wolbachia* reduction at 10 mg/kg, b.i.d., 14 days[a]	97.8% *Wolbachia* reduction; 100% mf depletion (peritoneum) at 100 mg/kg, b.i.d., 7 days[b]
Litomosoides sigmodontis (adult female)	0% *Wolbachia* reduction at 40 mg/kg, b.i.d., 14 days[a]	99.7% *Wolbachia* reduction > 99.99% mf depletion at 100 mg/kg, q.d., 14 days[a]	99.7% *Wolbachia* reduction >99.99% mf depletion at 50 mg/kg, b.i.d., 7 days[a]
Onchocerca ochengi (adult male)	99.2% *Wolbachia* reduction at 25 mg/kg, q.d., 28 days[a,c]	98.8% *Wolbachia* reduction At 75 mg/kg, q.d., 14 days[a]	None reported

[a]Dosing in jirds.
[b]Dosing in SCID mice.
[c]Minocycline q.d., once a day; b.i.d., twice a day.

21 days to achieve similar levels of anti-*Wolbachia* activity (>90% reduction). From these *in vivo* studies both TylA analogues demonstrated higher efficacy than tetracyclines over a shorter treatment period of 14 days. After extensive profiling comparing the two leads in terms of efficacy and PK profiles in various animal models, ABBV-4083 was selected as the preclinical candidate for development, mainly due to its superior efficacy, whilst A-1535469 remained as a strong backup compound. The effects of ABBV-4083 were further examined in preclinical models through additional parasitological end points, such as mf production, number of adult worms recovered, and worm embryonic stages with long wash-out periods after initial dosing. For example, in a longitudinal assessment of *L. sigmodontis* microfilaraemia, both ABBV-4083 (at 100 mg/kg, once per day) and doxycycline (at 40 mg/kg, twice per day) were dosed for 14 days in jirds with established adult worm infection [68]. Although, at the initial stage, both drugs showed effects in reducing the microfilaraemia when compared with untreated animals at 10 weeks post-treatment, only the animals treated with ABBV- 4083 showed that the microfilaraemia continued to decline whilst the reduction of microfilaraemia in the doxycycline treatment group plateaued at week 11–16 post-treatment. In fact, at week 15–16 post-treatment, the ABBV-4083-treated jirds showed no microfilariae in blood despite a similar number of adult *L. sigmodontis* worms being recovered in all treated and untreated groups after 16 weeks post-treatment. Microscopic evaluation of the recovered female adult worms confirmed that significant decline in the number of intrauterine embryonic stages, for example, morulae, coiled and stretched microfilariae in the ABBV-4083 treatment group in comparison to the untreated group [35].

In terms of safety profiling, one of the most important aspects of any newly developed antifilarial drugs for consideration is their effect against the microfilariae of *L. loa* to avoid serious adverse events caused by direct microfilaricidal activity. This is a key advantage of anti-*Wolbachia* treatments due to the lack of *Wolbachia* in *L. loa*. ABBV-4083 was reported to show no effect on the motility of *L. loa* microfilariae at concentrations below 4 µM and with an $IC_{50} = 23.3$ µM *in vitro* which is far higher than the peak concentration ($C_{max} = 0.18$ µM) the experimental drug can achieve at an efficacious dose (50 mg/kg) in jirds, which predicts that ABBV-4083 treatments would avoid *L. loa* SAEs. In addition to this, through a standard preclinical toxicology and safety assessment programme, ABBV-4083 was reported to have no adverse effect at plasma concentrations in preclinical species higher than the required efficacious plasma concentrations in PD models [35]. The spectrum of activity of ABBV-4083 was investigated against a panel of other common bacterial species, which was similar to that of TylA and erythromycin, which will be taken into consideration for antimicrobial resistance stewardship.

AWZ1066S

AWZ1066S (Figure 3) is another anti-*Wolbachia* macrofilaricidal drug candidate that has completed its preclinical development successfully and is currently advancing to a first-into-human Phase I clinical trial[iv]. This candidate molecule was developed from hits identified from a phenotypic cell-based screening campaign incorporating a *Wolbachia*-infected *Aedes albopictus* cell line [C6/36 (*w*AlbB)] (see the section 'Primary screening assays') [37]. A multiparameter lead optimisation was performed with over 300 analogues synthesised and assessed for both anti-*Wolbachia* activity and drug metabolism and pharmacokinetics (DMPK) properties *in vitro*. In this process, the thienopyrimidine hit was modified to a quinazoline core and subsequently to an azaquinazoline scaffold from which the candidate AWZ1066 emerged, resolving the metabolic weakness associated with the original scaffold whilst improving potency. AWZ1066 was active against *Wolbachia* in the cell-based assay with an EC_{50} of 2.6 ± 0.5 nM and in an orthogonal secondary *in vitro* assay utilising mf of *B. malayi*, AWZ1066 with an EC_{50} of 150 nM, whilst it had no effect on the viability and motility of the mf at up to the top testing concentration of 5 µM. The two enantiomers of AWZ1066, AWZ1066S and AWZ1066R, demonstrated minor

differences in anti-*Wolbachia* potency *in vitro* with the (S)-isomer, AWZ1066S, the more potent of the enantiomers in both *in vitro* assays (EC$_{50}$s: cell assay: 2.5 ± 0.4 nM vs 14.4 ± 3.7 nM; mf assay: 122 nM vs. 408 nM). Extensive comparative analysis of the two enantiomers led to the selection of AWZ1066S as the preclinical candidate based on superiority in terms of *in vitro* potency and *in vivo* efficacy [38].

A key criterion of the A·WOL TPP was a treatment regimen of no more than 7 days. In a number of nematode infection models, including *B. malayi* and *L. sigmodontis*, AWZ1066S was able to achieve the threshold reduction of *Wolbachia* (>90%) in 7-day treatment regimens (Table 1). Furthermore, the release of mf was completely prevented in 9 out of 10 mice treated with AWZ1066S in the adult *B. malayi* SCID mouse model over the 6 weeks of observation post-treatment. Similarly, in the adult *L. sigmodontis* BALB/c mouse model, after AWZ1066S treatment (50 mg/kg, twice per day for 7 days) the peripheral blood microfilaraemia began to decline from 6 weeks post-treatment and a state of amicrofilaraemia was evident from 14 weeks post-treatment and was sustained until the end of the experiment at 18 weeks post-treatment. All of these parasitology observations from PD studies clearly demonstrated the sustained effects on filarial embryogenesis and sterilisation after only 7 days of treatment with AWZ1066S. The advantageous fast-kill character against *Wolbachia* of AWZ1066S was further demonstrated by a time-kill experiment against *Wolbachia* in the microfilaria of *B. malayi*. Comparing with other known anti-*Wolbachia* antibiotics, that is, doxycycline, moxifloxacin, and rifampicin, in this *in vitro* study, AWZ1066S was able to achieve the maximum *Wolbachia* depletion level after only 1 day of exposure whilst the other drugs took 6 days' exposure to reach the same level of depletion. Another important advantage of AWZ1066S is its high specificity against *Wolbachia* so that it would have minimum impact on the gut microbiota and the selection of resistance in clinical applications. AWZ1066S is the first (and, so far, only) macrofilaricidal drug candidate that is developed specifically against *Wolbachia* from the very beginning, and this high specificity was confirmed by the lack of activity against a panel of clinically relevant bacteria [38].

Other anti-*Wolbachia* leads

In addition to these two clinical candidates, there are two other lead series of anti-*Wolbachia* chemotypes reported. One is a series of quinazolines, exemplified by one of the leads in the series, CBR490, that shows some chemical similarities with AWZ1066S [69]. CBR490 also showed some off-target toxicity in the CEREP panel safety screen and potential human Ether-à-go-go-Related Gene (hERG) liability [69]. Another series was derived from an antibiotic scaffold of pleuromutilin by the introduction of a boron-heterocycle moiety at the C(14)-position that is exemplified by one of the leads in the series, AN11251 [40]. The lead compounds in both series (CBR analogues and pleuromutilins) demonstrated potent activity in a number of *in vitro* and *in vivo* models against *Wolbachia* [70,71]. However, their progression in the development pipeline is unclear at the moment.

Mechanism of action

The use of phenotypic screening at the core of the A·WOL drug discovery process successfully delivered candidates with specificity that was translatable through the pipeline. However, this strategy does not easily offer insights into how these new compounds exert their effects on *Wolbachia*. Given their endosymbiotic lifestyle, compounds could either target the bacteria directly or affect host pathways that are important in the host–symbiont relationship. The fact that activity of the candidates is shared between *Wolbachia* in mosquito cells and nematode hosts argues against the latter possibility, although identifying the precise targets is an important aspect of drug discovery efforts. Due to the genetic intractability of *Wolbachia*, traditional genomics-based target identification approaches are unfeasible. Instead, a chemical proteomics

approach is currently being utilised to identify the targets of AWZ1066S. Using the SAR knowledge gained via the lead optimisation process, photoaffinity labelling probes of AWZ1066S were designed and developed to perform affinity-purification of protein targets from live *Wolbachia*-infected cells. A short-list has been successfully generated [38] and future work is focused on confirming and validating the potential targets on the list, with the aim of rolling out this approach to other compounds.

Concluding remarks

Within a decade and a half, the A·WOL consortium, with its partners, has created an industry quality translationally validated screening cascade from scratch (Figure 1). The assays are capable of screening millions of compounds in days and then triaging hits for lead identification, lead optimisation, and candidate selection using disease-relevant *in vitro* and *in vivo* preclinical infection models. The outputs of this endeavour are remarkable, delivering thousands of hit compounds and dozens of new chemical series, presumably including those with novel mechanisms of action, for onward investigation and development (Figure 3). Integrating the biological outputs with robust industry standard PK/PD-driven optimisation and selection strategies has delivered the first two drugs ever developed specifically for filariasis to the point of human trials. This achievement through what is in effect a product development partnership (PDP) would be considered spectacular by any pharma industry norms.

The successes to date are really only the start of the journey. There is real optimism that AWZ1066S and/or ABBV-4083 will eventually achieve regulatory approval after further clinical evaluation, with multiple back-up options should they fail. The vast collection of hit series offers exciting opportunities not only for drug discovery but also as tool molecules to probe parasite/endosymbiont basic biology (see Outstanding questions).

After more than 10 years investment from many contributors to the A·WOL initiative we are in a strong position, subject to continued financial support for the sector, to actually deliver drugs that fully meet the TPP that was developed to deliver elimination of these important NTDs.

Outstanding questions

What are the molecular targets and precise mode of action of fast-acting compounds?

How does albendazole mediate synergy with anti-*Wolbachia* drugs?

Can fast-acting compounds be exploited as new treatments for veterinary filariasis (heartworm, zoonotic brugian filariasis)?

Do the screening outputs from A·WOL provide new opportunities for antimicrobial drug discovery?

What mechanisms of resistance are employed by *Wolbachia*?

Declaration of interests

The authors declare no competing interests.

Resources

[i]https://awol.lstmed.ac.uk/

[ii]www.sightsavers.org/blogs/2017/06/test-and-treat-tackling-river-blindness-in-cameroon/

[iii]https://dndi.org/research-development/portfolio/abbv-4083/

[iv]www.ghitfund.org/investment/portfoliodetail/detail/155/en

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