

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Repurposing of approved drugs from the human pharmacopoeia to target *Wolbachia* endosymbionts of onchocerciasis and lymphatic filariasis



Kelly L. Johnston^{a,1}, Louise Ford^{a,1}, Indira Umareddy^{b,1}, Simon Townson^c, Sabine Specht^d, Kenneth Pfarr^d, Achim Hoerauf^d, Ralf Altmeyer^{b,e}, Mark J. Taylor^{a,*}

^a Filariasis Research Group, Department of Parasitology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK

^b CombinatoRx-Singapore Ptd Ltd, 11 Biopolis Way, 138667 Singapore, Singapore

^c Tropical Parasitic Diseases Unit, Northwick Park Institute for Medical Research, Watford Road, Harrow, Middlesex HA1 3UJ, UK

^d Institute of Medical Microbiology, Immunology & Parasitology, University Hospital of Bonn, Sigmund Freud Strasse 25, 53105 Bonn, Germany

^e Institut Pasteur Shanghai, Chinese Academy of Sciences, 320 Yueyang Road, 200031 Shanghai, People's Republic of China

ARTICLE INFO

Article history:

Available online 16 September 2014

Keywords:

Wolbachia

Filariasis

Pharmacopoeia

Drug discovery

Library screening

Anti-*Wolbachia* Consortium (A-WOL)

ABSTRACT

Lymphatic filariasis and onchocerciasis are debilitating diseases caused by parasitic filarial nematodes infecting around 150 million people throughout the tropics with more than 1.5 billion at risk. As with other neglected tropical diseases, classical drug-discovery and development is lacking and a 50 year programme of macrofilaricidal discovery failed to deliver a drug which can be used as a public health tool. Recently, antibiotic targeting of filarial *Wolbachia*, an essential bacterial symbiont, has provided a novel drug treatment for filariasis with macrofilaricidal activity, although the current gold-standard, doxycycline, is unsuitable for use in mass drug administration (MDA). The anti-*Wolbachia* (A-WOL) Consortium aims to identify novel anti-*Wolbachia* drugs, compounds or combinations that are suitable for use in MDA. Development of a *Wolbachia* cell-based assay has enabled the screening of the approved human drug-pharmacopoeia (~2600 drugs) for a potential repurposing. This screening strategy has revealed that approved drugs from various classes show significant bacterial load reduction equal to or superior to the gold-standard doxycycline, with 69 orally available hits from different drug categories being identified. Based on our defined hit criteria, 15 compounds were then selectively screened in a *Litomosoides sigmodontis* mouse model, 4 of which were active. These came from the tetracycline, fluoroquinolone and rifamycin classes. This strategy of repurposing approved drugs is a promising development in the goal of finding a novel treatment against filariasis and could also be a strategy applicable for other neglected tropical diseases.

© 2014 The Authors. Published by Elsevier Ltd. on behalf of Australian Society for Parasitology Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

1. Introduction

Lymphatic filariasis (LF) and onchocerciasis (river blindness) are debilitating diseases caused by filarial nematodes, officially recognised as neglected tropical diseases (NTDs) (WHO, 2007). Although these nematode infections are currently being effectively managed using mass drug administration (MDA) of drugs donated by large pharmaceutical companies (Chu et al., 2010; Coffeng et al., 2013), elimination is hampered by several challenges including the incomplete efficacy of available drugs against the long-lived adult filarial worms (Liu and Weller, 1996; Richard-Lenoble et al., 2003;

Bockarie and Deb, 2010; Mackenzie et al., 2012), problems associated with adverse events in areas of co-endemicity of *Loa loa* with either *Wuchereria bancrofti* or *Onchocerca volvulus* (Gardon et al., 1997; Bockarie and Deb, 2010; Taylor et al., 2010), and the risk that filarial worms will develop resistance to the drugs currently available for MDA (reviewed in Smits, 2009; Prichard et al., 2012).

Targeting the bacterial endosymbiont, *Wolbachia*, of these filarial nematodes offers solutions to these problems as the removal of *Wolbachia*, using tetracycline-based antibiotics, results in the slow death of the adult worm (reviewed in Taylor et al., 2010) and, given that *L. loa* does not harbour these endosymbionts (McGarry et al., 2003), does not lead to adverse events following treatment (Wanji et al., 2009; Turner et al., 2010). The use of doxycycline in field trials has demonstrated that this antibiotic can be used successfully to permanently sterilise adult female worms and, if given

* Corresponding author. Tel.: +44 151 705 3112; fax: +44 151 705 3371.

E-mail address: mark.taylor@lstmed.ac.uk (M.J. Taylor).

¹ These authors contributed equally to this work.

for an appropriate length of time, lead to a macrofilaricidal effect (reviewed in Johnston and Taylor, 2007; Hoerauf, 2008; Taylor et al., 2010); an important improvement over current treatments. The current 4–6 weeks of daily treatment, is the main barrier to wide-spread scale-up of this treatment regimen into MDA programmes due to logistical constraints, although community-directed treatment with doxycycline for six weeks, achieving a therapeutic coverage of 73.8% and 98% compliance, is feasible and effective in restricted populations (Wanji et al., 2009; Tamarozzi et al., 2012). Doxycycline, however, also has limitations for mass use due to contraindications that make it unsuitable for treating children under eight and pregnant women (reviewed in Johnston and Taylor, 2007; Hoerauf, 2008).

The A-WOL Consortium was established to find a new anti-wolbachial drug or combination of drugs that is compatible with MDA with a secondary goal to optimise regimens using the currently known active antibiotics (doxycycline and rifampicin) (www.a-wol.com; Johnston et al., 2014; Taylor et al., 2014). Screening large chemical libraries to identify compounds with macrofilaricidal activity has been hindered in the past by the lack of efficient screening assays with available assays being labour intensive (Townson et al., 2000; Rao et al., 2002; Townson et al., 2006; Townson et al., 2007). To overcome this limitation the A-WOL Consortium developed a *Wolbachia* cell-based assay with a quantitative PCR (qPCR) readout which has been optimised as an *in vitro* drug screening tool. Here, we briefly describe the validation of this assay which utilises a *Wolbachia pipientis*-containing *Aedes albopictus* cell line (C6/36 (wAlbB)), in a 96-well format, and quantifies the 16S rRNA gene copy number of intracellular *Wolbachia* bacteria in the presence or absence of a drug, as well as an ATP–luminescence based cytotoxicity assay to examine off-target toxic effects of the drug on the mosquito host cells. The assay can be adapted to automated high throughput-screening and represents a rapid, sensitive and efficient assay for screening chemical libraries to identify anti-*Wolbachia* compounds. Hits from this primary *in vitro* cell-based screening assay are then selected for progression down the screening pipeline into both *in vitro* and *in vivo* nematode screening.

Repurposing or repositioning of drugs provides a less risky route to drug discovery given that candidates will already have well-known safety and pharmacokinetic profiles (Ashburn and Thor, 2004; Tobinick, 2009; Mucke, 2010; Grimberg and Mehlotra, 2011). Here, we describe screening efforts against *Wolbachia* using the A-WOL assay to screen a compound library of 2664 approved drugs, bioactive compounds and natural products (CRX; Combina-toRx Singapore). This strategy identified 121 hits that had anti-*Wolbachia* activity, of which 69 were orally available hits from different drug categories, and several drugs were progressed further down the screening pipeline into *in vitro* nematode screening assays and the primary *in vivo* screening model (*Litomosoides sigmodontis* mouse model). This approach has identified several classes of registered drugs with anti-*Wolbachia* activity, which has expanded the options for improving macrofilaricidal therapeutic regimes against onchocerciasis and lymphatic filariasis.

2. Materials and methods

2.1. *In vitro* *Wolbachia* cell-based screening assay

An *A. albopictus* cell line C6/36 (ATCC number CRL-1660) stably infected with *W. pipientis* wAlbB (C6/36 (wAlbB)) was routinely cultured in L15 Leibovitz medium containing 2 mM L-glutamine, 1% non-essential amino acids, 2% tryptose phosphate broth (Sigma-Aldrich, UK), and 5% heat-inactivated FCS (Cambrex Bio Science, Walkersville, MD) at 26 °C (Turner et al., 2006). A C6/36 (wAlbB) cell-based assay developed to screen drugs/compounds

active against *Wolbachia in vitro* was used as previously described (Johnston et al., 2010). C6/36 (wAlbB) cells, sub-cultured 24 h previously, were seeded at 10,000 cells per well in 96-well flat bottom culture plates. Test compounds were dissolved in DMSO (Sigma) and diluted to appropriate concentration (μM) in culture medium, added to test wells and cells cultured in a total volume of 200 μl at 26 °C for 9 days. Medium alone and vehicle-treated (DMSO) medium were used as negative controls. Compounds and controls were added in triplicate and medium/drug was replaced on day 4. At the end of the screening assay, samples were collected by washing adherent cells once in sterile Dulbecco's PBS (Sigma) and adding 150 μl Wizard[®] SV Lysis Buffer (Promega, UK) for genomic DNA (gDNA) extraction.

In total, 2664 compounds from the CRX library, plated onto 37 master plates (72 compounds per plate), were screened at 10 μM in comparison to the gold standard doxycycline (7 μM) (Sigma). Cytotoxicity was measured in parallel using the CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega), according to the manufacturer's instructions. The level of cytotoxicity for each compound was determined by comparing the CellTiterGlo[®] luminescence readout against the vehicle-treated control wells, with compounds that reduced the % luminescence by 30% or greater being classed as cytotoxic. Screening was executed with a "front loading" of the library wherein approximately 200 anti-infectives were used in the first three master plates to maximise the number of hits in the first phase of screening. A pre-production run was done to evaluate assay performance and dynamic range with Z' factor being used as a primary quality control tool for data generation/analysis. With acceptable Z' factors achieved (greater than 80% of the plates showed Z' factors of 0.4 and above), the assay was validated for use.

Hits were cherry-picked from master plates and examined in a titration series (6 doses from 10 to 0.3 μM) for dose response effects set up in quadruplicates (inter and intra plate duplicates) and were also tested in parallel for cytotoxicity. Hits were also validated using compounds sourced externally, where available. Prioritisation of hit compounds for further screening was based on the following criteria: (1) suitability/approval status, (2) potency in screening assay, (3) repeat validation (both library and sourced compounds), (4) paediatric use, and (5) pregnancy category (US–FDA categories, www.fda.gov).

2.2. *In vitro* *Onchocerca gutturosa* screening assay

Adult male *O. gutturosa* were dissected from the nuchal ligament connective tissues obtained from naturally infected cattle in The Gambia, as previously described (Townson et al., 2006). Worms were maintained individually in the wells of a 24-well plate in 2 ml of Minimum Essential Medium containing 10% heat-inactivated FCS, 200 U/ml penicillin, 200 $\mu\text{g}/\text{ml}$ streptomycin and 50 $\mu\text{g}/\text{ml}$ amphotericin B (Sigma), at 36.5 °C with 5% CO₂ for 24 h until the addition of drugs. Compounds, dissolved in 99% DMSO, were prepared as previously described (Townson et al., 2006) in medium and each compound was tested against ten individual worms for 5 days. Daily microscopic observations were carried out to determine worm motility using a scale of 0 (immobile) to 10 (maximum motility) and the mean % motility reduction was derived by the comparison to untreated controls. At assay termination, each group of 10 worms (per compound) were transferred to RNAlater (Ambion, Applied Biosystems, UK) for 24 h at 4 °C and stored at –20 °C for gDNA extraction from individual worms at a later date.

2.3. *In vivo* *L. sigmodontis* screening assay

Treatment groups of BALB/c female mice (6–8 week old) received intraperitoneal (IP) injections with the test compounds,

in comparison to doxycycline, at appropriate concentrations (MKD, mg/kg/day) for 14 days beginning the day after natural mite (*Ornithonyssus bacoti*) infection with *L. sigmodontis*. Compounds were formulated in appropriate delivery vehicles (eg. methacycline was formulated in 0.5% hydroxypropyl methylcellulose, HPMC) and doses calculated based on the recommended human dosage and in a volume of 10 ml/kg based on body weight. At 35 days post-infection, worms were recovered from the pleural cavity, counted, staged for development and measured for length (mm). Worms were frozen at -80°C for gDNA extraction. All animal experiments were performed according to the European Union animal welfare guidelines. All protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Cologne, Germany (AZ.: 8.87-50.10.35.08.024).

2.4. DNA isolation and quantitative real-time PCR (qPCR)

Genomic DNA was extracted from C6/36 (*wAlbB*) cell lysates using the Wizard[®] SV 96 Genomic DNA Purification System (Promega) according to the manufacturer's instructions and eluted in 100 μl water. Quantification of the ribosomal genes; *W. pipientis* 16S and *A. albopictus* 18S, was performed as described previously (Makepeace et al., 2006) with modifications. Briefly, qPCR was carried out on a DNA Engine PTC-200 thermocycler (MJ Research, GRI, UK) with Chromo4 real-time PCR detection system (Bio-Rad Laboratories Ltd, UK) under the following conditions: 95 $^{\circ}\text{C}$ for 15 min, 40 cycles of 95 $^{\circ}\text{C}$ for 15 s, 55 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 15 s; and melting curve analysis between 50 and 95 $^{\circ}\text{C}$ to confirm the specificity of the amplification products. qPCR reactions were performed in 20 μl Quantitect SYBR Green (Qiagen, UK) reactions containing 1 μl gDNA for 18S or 2 μl gDNA for 16S, 0.2 μM of each primer (Supplementary Table S1) in 1 \times SYBR Green PCR reaction mix. Quantification was calculated by reference to a linear standard curve of log₁₀ diluted (5×10^6 – 5×10^0) full-length amplicons synthesised as single-stranded oligonucleotides (Sigma-Genosys, UK).

Following *in vitro* culture, gDNA was extracted from individual adult male *O. gutturosa* using the Wizard[®] SV 96 Genomic DNA Purification plate (Promega) and QiaAmp DNA mini-kit reagents (Qiagen) and eluted in 100 μl water. Quantification of the *Wolbachia* surface protein (*wsp*) and nematode glutathione S-transferase (*Ov-gst*) gene copy numbers was performed by qPCR carried out on a DNA Engine PTC-200 thermocycler (MJ Research) with Chromo4 real-time PCR detection system (Bio-Rad) under the following conditions: 95 $^{\circ}\text{C}$ for 15 min, 40 cycles of 95 $^{\circ}\text{C}$ for 15 s, 57 $^{\circ}\text{C}$ (*gst*) or 60 $^{\circ}\text{C}$ (*wsp*) for 30 s, and 72 $^{\circ}\text{C}$ for 15 s; and melting curve analysis between 60 and 97 $^{\circ}\text{C}$. qPCR reactions were performed in 20 μl Quantitect SYBR Green (Qiagen) reactions containing 1 μl gDNA, 3 mM MgCl₂ and 0.3 μM of each primer (Supplementary Table S1) for *gst* or 2 μl gDNA, 3.5 mM MgCl₂ and 0.35 μM of each primer (Supplementary Table S1) for *wsp*, in 1 \times SYBR Green PCR reaction mix. The gene copy number was determined using a gene specific standard curve of plasmid DNA.

At 35 days post-infection, *L. sigmodontis* worms were recovered from the pleural cavity and gDNA extracted using the QiaAmp DNA mini-kit (Qiagen) according to the manufacturer's instructions and eluted in 50 μl water. Quantification of the *Wolbachia* *ftsZ* (*wLs-ftsZ*) and *L. sigmodontis* β -actin (*Ls-act*) gene copy numbers was performed by qPCR (Arumugam et al., 2008; Strübing et al., 2010) carried out on a RotorGene 3000 (Corbett Research, Sydney, Australia). The following cycling conditions were used: 95 $^{\circ}\text{C}$ for 15 min, 45 cycles of 95 $^{\circ}\text{C}$ for 15 s, 58 $^{\circ}\text{C}$ for 45 s, and 72 $^{\circ}\text{C}$ for 15 s; and melting curve analysis between 62 and 99 $^{\circ}\text{C}$. qPCR reactions were performed in 10 μl reactions volumes using the following conditions: 1 \times PCR buffer (Qiagen), 0.2 mM dNTPs, 3 mM MgCl₂, 0.1 μl SYBR Green (1:1000 dilution of stock in DMSO; Roche,

Mannheim, Germany), 0.25 U HotStar Taq polymerase (Qiagen) and 2 μl DNA. The gene copy number (copy numbers/ μl) was determined using a gene specific standard curve of plasmid DNA.

For all qPCR reactions results were expressed as *Wolbachia* gene:host gene ratios to normalise the data and obviate differences in the quality and quantity of DNA. The log drop in the ratio in comparison to the control gives a quantitative measure of the effect of the compound on *Wolbachia*.

2.5. Statistical analysis

Student *t*-test was performed for statistical analysis using Prism (GraphPad Software, Lajolla, CA).

3. Results

3.1. Development of a screening assay which identifies anti-*Wolbachia* activity *in vitro*

In this report, we describe the development, validation and use of an assay for the *in vitro* cell-based screening of anti-*Wolbachia* compounds. Using doxycycline as a gold-standard, critical features such as reproducibility, assay duration and dynamic range were evaluated. Initial experiments were conducted over 21 days in order to assess the dynamic range of the doxycycline response over time (Fig. 1). Assay quality and robustness were determined during the optimisation as well as during the screening process by calculation of the statistical parameters *Z* (and *Z'*) (Zhang et al., 1999), and having achieved acceptable *Z'* factors the assay was validated for use (Supplementary Tables S2 and S3). Acceptable *Z'* factors were achieved at day 9 but not at day 5 (Supplementary Table S2), demonstrating that the optimal duration of the assay was 9 days. A pre-production run of 8 replicate plates (master plate MS-250501) was used to calculate the intra-plate variability (*Z'* factors) of the 16S qPCR assay read-out (Supplementary Table S3). Greater than 80% of the plates showed *Z'* factors of 0.4 and above and signal window of 2 allowing us to be confident that the assay could be used for screening of the CombinatoRx (CRX) library.

3.2. Screening of the human pharmacopeia identified drugs with activity against *Wolbachia*

The CRX library of 2664 compounds, representing the approved human drug pharmacopeia, was screened using the validated assay. Initial screening was executed with a "front loading" of the library where 200 anti-infectives were used in the first three master plates to maximise the number of hits in the first phase of screening and further validate the assay prior to screening the complete library. From the 2664 compounds tested, we identified 121 compounds that inhibited *Wolbachia* by 0.5 logs (70% inhibition at 10 μM); this represents a primary hit rate of 4.54%. We then further defined hits as those compounds that along with these *in vitro* hit criteria (≥ 0.5 logs inhibition of *Wolbachia* 16S and $\leq 30\%$ cytotoxicity) are also available in an oral formulation in order to align our hit picking strategy to the Target Product Profile (TPP) criteria.

Of the 121 active compounds, 69 compounds (2.59% of the total screened), over several drug classes (Table 1), satisfied hit criteria (Table 2). Hits identified in the screening campaign were interesting and diverse (Table 1), and included anti-infective compounds (35%) such as antibiotics, anti-viral, anti-parasitic and anti-fungal compounds, as well as non anti-infective compounds (65%) constituting anti-psychotic compounds, natural products/nutraceuticals, receptor antagonists, anti-hypertensives, muscle relaxants, non-steroidal anti-inflammatory drugs and other drug classes, pointing towards potentially novel mechanisms of countering intra-cellular *Wolbachia* bacteria which could be exploited.

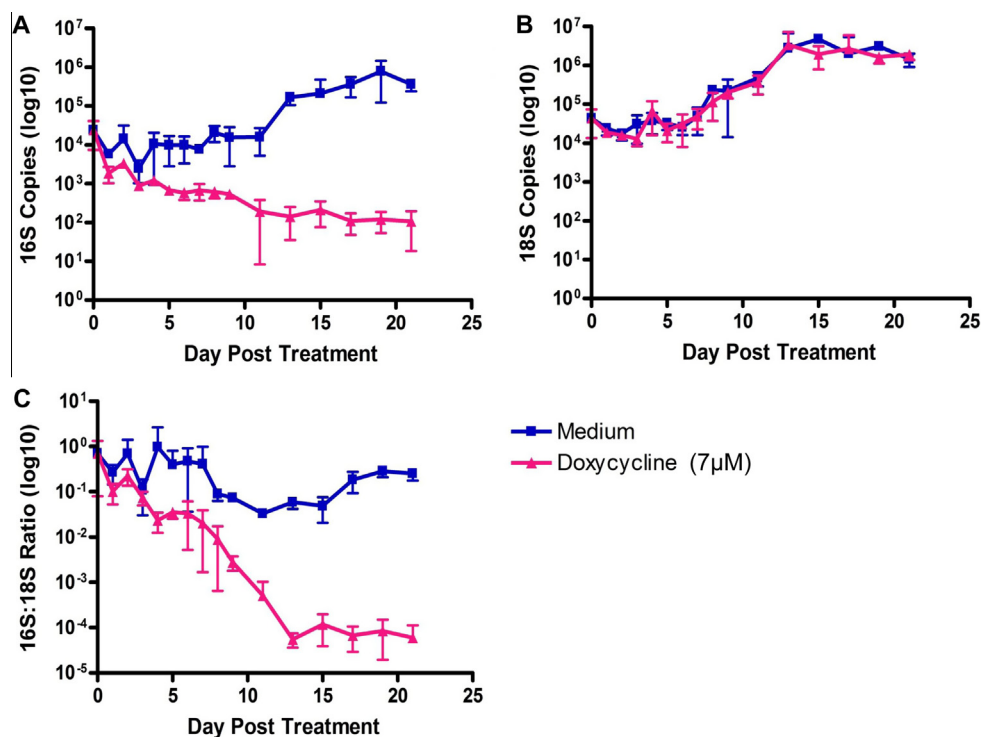


Fig. 1. Dynamics of cell and *Wolbachia* response to doxycycline over 21 days. *Wolbachia* growth was assessed by qPCR targeting the 16S rRNA gene (A). C6/36 cell growth was assessed by qPCR targeting the 18S rRNA gene of *Aedes albopictus* (B). Data was normalised using the ratio of 16S copies to 18S copies (C).

Table 1

Distribution of drug classes across 69 hit anti-*Wolbachia* compounds. 121 compounds inhibited intracellular *Wolbachia* bacteria by 0.5 logs or more. Out of these, 69 belong to diverse classes of approved drugs and are available in an oral formulation and hence constitute hits for further analysis.

Drug class	Number of hits	Percent of hits (%)
Anti-infectives	24	35
Anti-psychotics/anti-convulsants	8	12
Natural products/nutraceuticals	7	10
Receptor antagonists	6	9
Anti-hypertensives	6	9
Muscle relaxants	5	7
Others	5	7
Non-steroidal anti-inflammatories	4	6
Anti-neoplastic agents	4	6

Encouragingly, we identified hits among classes of antibiotics (the tetracyclines, rifamycins and fluoroquinolones) previously shown to reduce *Wolbachia* (Hoerauf et al., 2000; Townson et al., 2000; Hermans et al., 2001; Rao et al., 2002; Fenollar et al., 2003; Volkman et al., 2003; Townson et al., 2006), thus giving us confidence in the screening outcome. Out of the 69 hits, 24 compounds inhibited *Wolbachia* by 1 log or more which corresponds to 90% inhibition. Moreover, 10 of these 24 compounds showed comparable or better activity than that of doxycycline (≥ 1.6 logs or 95% inhibition). Compounds which were equivalent to or better than doxycycline *in vitro* were: ciprofloxacin hydrochloride, ethosuximide, indomethacin, kitasamycin, methacycline hydrochloride, minocycline, paromomycin sulfate, piracetam, rifapentine, and sulfamethizole (Table 2).

3.3. Validation and prioritisation of hits

To further characterise and validate the hit compounds, dose response assays were performed with 66 of the hit compounds

to examine the dose-dependent effects. Of the re-tested compounds, 16 compounds failed to show any activity (>50% inhibition of *Wolbachia*) in the repeat assays and were termed as drop-outs (“validated – library compound” column, Table 2). The remaining compounds showed varying degrees of activity from 98% to 50% inhibition at 10 µM. In the dose response assays, 36 of the hits showed a dose response in the dose range tested (e.g. paromomycin sulfate and loratadine), while other hits did not show a dose response. Ten compounds were active at all tested concentrations to the same extent (e.g. methacycline hydrochloride and sulfaguanidine), while four compounds showed activity only at the highest concentration (10 µM) (e.g. ciprofloxacin hydrochloride and curcumin). In addition, hit compounds were further validated by re-screening using, where available, externally sourced compounds (30/69 compounds) (“validated–sourced compound” column, Table 2).

The hit compounds were then ranked based on the following criteria: (1) suitability/approval status, (2) potency in screening assay, (3) repeat validation (both library and sourced compounds), (4) paediatric use, and (5) pregnancy category (US–FDA categories, www.fda.gov), and then were prioritised for progression through the A-WOL screening pipeline into *in vitro* nematode screening assays and the primary *in vivo* screening model (*L. sigmodontis* mouse model) (Table 2). Seventeen compounds, that were validated using both library and sourced compounds, were classed as top priority hits. A further 21 compounds, that were validated using library compound only, were classed as second priority hits and ranked using the defined criteria. The remaining 31 hits were classed as de-prioritised hits for various reasons. For example, ciprofloxacin was de-prioritised as it had previously been tested in the *in vivo* mouse model (Hoerauf et al., 2000), as well as compounds that were subsequently found to be inactive, or variably active, in repeat screening. Furthermore, compounds that had not been validated using either library compound or sourced compound as well as hits that were anti-neoplastics, clinical trial compounds

Table 2

Prioritisation of hit compounds. The 69 hits obtained in single agent screening were first validated using either library or sourced compound, where available, and then prioritised for further screening based on (1) suitability/approval status, (2) potency in 16S assay, (3) repeat validation (both library and sourced compounds) (4) paediatric use and (5) pregnancy category (US-FDA pregnancy categories). Compounds were classed as top priority, second priority or deprioritised hits and are listed in the following table in rank order based on activity in the cell-based screen. nd = not determined, ? = evidence unclear.

Compound	16S log drop	Teratogenic/embryocidal	Pregnancy category ^a	Use in pediatric indications	Validated-library compound	Validated-sourced compound	Characteristics	Comments
<i>Top priority hits</i>								
Methacycline hydrochloride	1.8	Yes	D	Not evaluated in children under 8 years	Yes	Yes	Anti-biotic	
Indomethacin	1.7	None observed	C	Not evaluated in children under 14 years	Yes	Yes	Non-steroidal anti-inflammatory	
Paromomycin sulfate	1.7	Yes	D	Approved	Yes	Yes	Anti-biotic	
Rifapentine	1.7	Yes	C	Not evaluated in children under 12 years	Yes	Yes	Anti-TB	
Minocycline	1.6	Yes	D	Not evaluated in children under 8 years	Yes	Yes	Anti-biotic	
Naftopidil	1.2	Not evaluated	?	Not evaluated	Yes	Yes	Anti-hypertensive	Treatment of enlarged prostate
Abacavir Sulfate	1	Yes?	C	Yes	Yes	Yes	Anti-viral	
Sparfloxacin	1	Yes	C	Not evaluated in children under 18 years	Yes	Yes	Anti-biotic	
Docusate Calcium	0.8	Yes	C	Not evaluated in children under 3 years	Yes	Yes	Laxative	
Loratadine	0.8	None observed	B	Not evaluated in children under 2 years	Yes	Yes	Allergy medication	
Ethoxzolamide	0.7	?	?	?	Yes	Yes	Diuretic	
Bepiridil	0.6	Yes	C	Not evaluated	Yes	Yes	Ca channel blocker	
Furazolidone	0.6	None observed	B	Contraindicated in infants < one month	Yes	Yes	Anti-protozoal	
Nefazodone hydrochloride	0.6	Yes	C	Not evaluated	Yes	yes	Anti-depressant	
Curcumin	0.5	None observed	?	Yes?	Yes	Yes	Hepatoprotective agent	Experimental drug
Diacerein	0.5	Not evaluated	?	Not evaluated	Yes	Yes	Osteoarthritis drug	
Isoniazid	0.5	Yes	C	Yes	Yes	Yes	Anti-TB	
<i>2nd Priority hits</i>								
Ethosuximide	2	Yes	C	Not evaluated in children under 3 years	Yes		Anti-epileptic	
Piracetam	1.6	Not evaluated	?	Not evaluated	Yes		Nootropic	
Sulfamethizole	1.6	Yes	Not safe	Not evaluated	Yes		Anti-biotic	
Nevirapine	1.5	None observed	B	Approved	Yes		Anti-viral	
Oxycodone hydrochloride	1.4	None observed	B	Not evaluated	Yes		Opioid agonist	
Sulfaguanidine	1.4	Yes	?	Not evaluated	Yes		Sulfa drug	
Valacyclovir hydrochloride	1.3	None observed	B	Not evaluated in children under 2 years	Yes		Anti-viral	
Ibuprofen	1.2	Yes?	C	Not evaluated	Yes		Non-steroidal anti-inflammatory	
Phenytoin	1	Yes	D	Yes	Yes		Anti-epileptic	
Mefexamide hydrochloride	0.9	Not evaluated	?	Not evaluated	Yes		Anti-depressant	
Nitrazepam	0.9	Yes	D	Yes	Yes		Hypnotic	
Benznidazole	0.8	Not evaluated	?	Not evaluated	Yes		Anti-parasitic	
Sorbic acid	0.8	None observed	B	?	Yes		Anti-infective, food preservative	
Acyclovir	0.7	None observed	B	Not evaluated in children under 2 years	Yes		Anti-viral	
Tolterodine tartrate	0.7	Yes	C	not evaluated	Yes		Muscle relaxant	
Trifluoperidol	0.7	Not evaluated	?	Not evaluated in children under 6 years	Yes		Anti-psychotic	
Benzydamine hydrochloride	0.6	No contraindications	?	?	Yes		Non-steroidal anti-inflammatory	
Bumetanide	0.6	Yes	C	Not evaluated in children under 18 years	Yes		Anti-hypertensive	

Table 2 (continued)

Compound	16S log drop	Teratogenic/embryocidal	Pregnancy category ^a	Use in pediatric indications	Validated-library compound	Validated-sourced compound	Characteristics	Comments
Riboflavin	0.6	None observed	?	?	Yes		Micronutrient	Micronutrient
Phytonadione	0.5	None observed	C	Not evaluated in pediatric populations	Yes		Micronutrient	
Pyrimethamine	0.5	Yes	C	Yes	Yes		Anti-parasitic	
<i>Deprioritised</i>								
Kitasamycin	2.1	?	?	?	Yes		Anti-biotic, macrolide	Safe for livestock
Ciprofloxacin hydrochloride	2	None observed	C	Approved	Yes	Yes	Anti-biotic	Tested previously (Hoerauf et al., 2000)
Oxfendazole	1.2	Not evaluated	?	Not evaluated	Yes		Anti-helminthic	Safe for livestock
Sodium Caseinate	1.2	None observed	A	Approved	Nd		Nutrient	
Morantel Tartrate	1.1	Not evaluated	?	Not evaluated	Yes		Anti-helminthic	Safe for livestock
Benactyzine (Hydrochloride)	1	Not evaluated	?	Not evaluated	Yes	Yes	Anti-cholinergic	No longer widely used in medicine due to side effects
Neratinib	1	Not evaluated	?	Not evaluated	Nd		Inhibitor of ErbB1 and ErbB2	Phase I compound
Eliprodil	0.9	Not evaluated	?	Not evaluated	No		NMDA receptor antagonist	Other NMDA antagonists are category B
Geftinib	0.9	Yes	D	Not evaluated	Yes	Yes	Anti-neoplastic	In clinical trials
Narasin	0.9	Not evaluated	?	Not evaluated	Yes	Yes	Anti-biotic	Safe for livestock
Dichlorophen	0.7	Not evaluated	?	Not evaluated	Yes		Anti-parasitic	Safe for livestock
L-Dopa	0.8	Yes	C	Not evaluated	No	Yes	Dopamine enhancer	
Selenium Powder	0.8	Safe	Safe	Not evaluated	No	Yes	Nutrient supplement	
Nitrofurantoin	0.7	None observed	B	Contraindicated in infants < one month	Nd		Anti-biotic	
Quinidine	0.7	Yes	C	Yes	No		Na-antagonist	
Ubenimex	0.7	Not evaluated	?	Not evaluated	No		Aminopeptidase inhibitor	In clinical trials
Baclofen	0.6	Yes	C	Not evaluated in children under 12 years	No	Yes	Muscle relaxant	
Chlorphenesin Carbamate	0.6	None observed	?	Not evaluated	No		Muscle relaxant	
Dasatinib	0.6	Yes	D	Not evaluated in children under 18 years	No		Anti-neoplastic	
Nicarbazin	0.6	Not evaluated	?	Not evaluated	Yes	Yes	Anti-biotic	Safe for livestock
Sulfantran	0.6	Not evaluated	?	Not evaluated	Yes		Anti-protozoal	Safe for livestock
Trifluoperazine hydrochloride	0.6	Yes	C	Not evaluated in children under 6 years	No	Yes	Anti-psychotic	Long-term medication
Betazole hydrochloride	0.5	Not evaluated	?	Not evaluated	No		Histamine analogue	Diagnostic agent
Carbinoxamine maleate	0.5	Not evaluated	C	Yes	No		Anti-histamine	
Diflunisal	0.5	Yes	C	Not evaluated in children under 12 years	No	Yes	Non-steroidal anti-inflammatory	
Fluoxetine hydrochloride	0.5	Yes	C	Not evaluated in children under 7 years	No	Yes	Anti-depressant	
Hydrochlorothiazide	0.5	None observed	C	Not evaluated in pediatric populations	No		Anti-hypertensive	
Isoxsuprine hydrochloride	0.5	Not evaluated	?	Not evaluated	Yes		Vasodilator	Safe for livestock
Nilutamide	0.5	Yes	C	Not evaluated	No	Yes	Androgen receptor blocker	
Scopolamine methylnitrate	0.5	Yes	C	Not evaluated in pediatric populations	No		Anti-cholinergic	
Troleandomycin	0.5	Yes?	C	Not evaluated in pediatric populations	Nd		Anti-biotic	

^a Pregnancy categories (US-FDA): A = Adequate and well-controlled studies have failed to demonstrate a risk to the foetus in the first trimester of pregnancy (and there is no evidence of risk in later trimesters); B = Animal reproduction studies have failed to demonstrate a risk to the foetus and there are no adequate and well-controlled studies in pregnant women; C = Animal reproduction studies have shown an adverse effect on the foetus and there are no adequate and well-controlled studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks; D = There is positive evidence of human foetal risk based on adverse reaction data from investigational or marketing experience or studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks.

or compounds currently only used in livestock were also deprioritised.

3.4. The effect of a selection of hits on the motility and *Wolbachia* load of filarial worms *in vitro*

Due to the promising results obtained in the *in vitro* cell-based assay screen some compounds were assessed more fully using *in vitro* nematode assays for proof-of-concept in our screening strategy. Further examination of active antibiotic classes that emerged as hits in the *in vitro* cell-based screening assay was conducted using an *O. gutturosa* adult nematode screen. A dose response assay using fourfold dilutions from 12.5 to 0.195 μM was used. All antibiotics tested showed only marginal or no effect on motility (data not shown) indicating that there was no direct toxicity against the nematode. The fluoroquinolones ciprofloxacin and moxifloxacin as well as the rifamycin rifapentine greatly reduced the *Wolbachia* load at all concentrations tested (Table 3). While doxycycline also reduced the *Wolbachia* load, the response across the different concentrations was more variable than the other compounds. Overall, no dose responses were observed for any of the compounds tested, suggesting a ceiling effect of the concentrations used. Taken together, these *in vitro* worm experiments demonstrate good translation of hits from the *in vitro* insect cell based assay to nematode *Wolbachia* within their natural hosts.

3.5. The effect of prioritised hits on the length and *Wolbachia* load of *L. sigmodontis* *in vivo*

Due to their *in vitro* activity the prioritised hits were tested in the *L. sigmodontis*-mouse model (Hoerauf et al., 1999, 2000; Volkmann et al., 2003), using intra-peritoneal dosing. A total of 15 *in vitro* hit compounds were screened using doses calculated based on approved human doses and compared to doxycycline (Table 4). This included ciprofloxacin, which, although had been deprioritised due to previous work, was retested given its activity in the *in vitro* *O. gutturosa* screen. Dose responses were also performed on a selection of these compounds (Table 4).

Four compounds, methacycline, minocycline, rifapentine and sparfloxacin, significantly reduced the worm length and the *Wolbachia* load at the standard doses tested ($P < 0.05$) (Table 4). Methacycline treatment resulted in significant reductions in both worm length and *Wolbachia* load at both 50 and 10 MKD doses. Interestingly, when treated with methacycline at 50 MKD the

reduction in *Wolbachia* load was significantly greater than from worms recovered from mice treated with the same dose of doxycycline ($P < 0.05$). In contrast, loratadine had no effect on either worm length or *Wolbachia* load as measured by *Wolbachia* *ftsZ* copies at any dose tested. Ciprofloxacin also did not reduce worm length or *Wolbachia* load (Table 4), confirming a previous report using this model (Hoerauf et al., 2000), while sparfloxacin did produce a significant reduction in both ($P < 0.01$). Further experiments using other members of this class have shown that levofloxacin is inactive, while moxifloxacin is active (data not shown), demonstrating diversity within this class. As part of the drive to reduce the duration of treatment for anti-*Wolbachia* therapy, a reduction in doses and treatment durations have also been investigated using this model. While the reduced regimen tested for sparfloxacin did not significantly affect worm length or *Wolbachia* numbers, a reduced minocycline dose and treatment duration (25 MKD for 10 days) significantly reduced the *Wolbachia* load ($P < 0.0001$) and this reduction was also significantly greater than *Wolbachia* reduction following the equivalent doxycycline treatment ($P < 0.05$) (Table 4).

4. Discussion

Here we describe the development of an *in vitro* *Wolbachia* screening assay and the subsequent use of this assay to screen the complete human pharmacopoeia, with a view to drug repurposing for filariasis. Repurposing or repositioning of drugs provides a less risky route to drug discovery given that candidates will already have well-known safety and pharmacokinetic profiles (Ashburn and Thor, 2004; Tobinick, 2009; Mucke, 2010; Grimberg and Mehlotra, 2011).

This study identified 121 compounds with *in vitro* activity against *Wolbachia*, 69 of which satisfied our hit criteria. These hits included, as expected, numerous anti-infective compounds (35%). These included drugs from classes known to show some efficacy against *Wolbachia*, namely the tetracyclines, rifamycins and fluoroquinolones (Hoerauf et al., 2000; Townson et al., 2000; Hermans et al., 2001; Rao et al., 2002; Fenollar et al., 2003; Volkmann et al., 2003; Townson et al., 2006). Interestingly, many were non anti-infective compounds (65%) encompassing several different drug classes, thereby pointing towards potentially novel mechanisms of action. Although mechanisms of action have not been investigated here, the number of non anti-infective compounds that demonstrated activity against *Wolbachia* *in vitro* offers several interesting avenues to pursue. As well as the possibility that these compounds are acting on the bacteria directly, a perturbation of the complex interplay between the *Wolbachia* and their host cells must also be considered. Indeed, interfering with the *Wolbachia*-host relationship through chemotherapy may be just as effective as targeting the bacteria themselves. The involvement of *Wolbachia* in the maintenance of host homeostasis has been referred to in previous studies, especially in relation to oxidative stress regulation (Brennan et al., 2008; Kremer et al., 2012). Antioxidants were among the compounds active against *Wolbachia* in this study and this class is currently being mined further to inform the potential repurposing and repositioning of these drugs. Autophagy, a conserved intracellular defence mechanism, has recently been demonstrated to play a key role in controlling *Wolbachia* populations (Voronin et al., 2012) and therefore components of the pathways involved in this mechanism may be the targets of some of non anti-infective compounds that were hits. This aspect of the screening outcomes is also currently being investigated. Furthermore, the presence of these hits offers the potential for combining drugs, such as antibiotics and non-antibiotics for synergistic effect (Ejim et al., 2011).

Table 3
Comparison of different classes of antibiotic on *O. gutturosa* *Wolbachia* loads.

Compound	Class	Concentration (μM)	Reduction in <i>Wolbachia</i> (<i>wsp:gst</i> log drop from vehicle controls)
Doxycycline	Tetracycline	12.5	0.80
		3.125	0.21
		0.781	0.14
		0.195	0.77
Ciprofloxacin	Fluoroquinolone	12.5	0.67
		3.125	0.79
		0.781	0.80
		0.195	0.39
Moxifloxacin	Fluoroquinolone	12.5	0.97
		3.125	0.83
		0.781	1.00
		0.195	1.03
Rifapentine	Rifamycin	12.5	1.04
		3.125	0.77
		0.781	0.78
		0.195	0.99

Table 4Testing of several prioritised hits in the *L. sigmodontis* *in vivo* model. The *in vitro* cell based *Wolbachia* reductions and cytotoxicity values are shown for comparison.

Compound	Characteristics	16S log drop <i>in vitro</i>	Cytotoxicity (%)	<i>in vivo</i> dose(s) (MKD ^a)	Ls length reduction <i>in vivo</i> (%)	wLs ftsz log drop <i>in vivo</i>
Doxycycline	Anti-biotic	1.6	0	25, ^b 50	78.3, 79.4	2.2, 4.7
Methacycline hydrochloride	Anti-biotic	1.8	0	10, 50	73.4, 80.5	3.0, 5.7
Minocycline	Anti-biotic	1.6	0	25 ^b	81.7	3.78
Paromomycin sulfate	Anti-biotic	1.7	0	25	0	1.5
Rifapentine	Anti-TB	1.7	20	50	68.3	3.0
Indomethacin	Non-steroidal anti-inflammatory	1.7	0	15	0	1.0
Abacavir sulfate	Anti-viral	1	0	200	0	N.D.
Sparfloxacin	Anti-biotic	1	0	25, ^b 130	28.6, 77.4	0.6, 5.7
Ciprofloxacin	Anti-biotic	2	0	100	17.56	0.46
Docusate calcium	Laxative	0.8	0	200	20.0	1.0
Loratadine	Allergy medication	0.8	20.7	0.3, 1, 3	0, 0, 0	0.2, 0, 0.35
Ethoxzolamide	Diuretic	0.7	19	200	0	0
Isoniazid	Anti-TB	0.5	0.3	25	0	1.0
Curcumin	Hepatoprotective agent	0.5	0	100	0	N.D.
Nilutamide	Androgen receptor blocker	0.5	0.01	5	12.6	2.3
Diacerein	Osteoarthritis drug	0.5	0	100	0	1.3

^a MKD = mg/kg/day.^b 25 MKD given for 10 days.

As expected with any screening strategy, many of the compounds found to be hits *in vitro* failed at the *in vivo* model stage. Minocycline, methacycline, rifapentine and sparfloxacin demonstrated activity in the *L. sigmodontis* mouse screening assay. The drop-outs can be explained in a variety of ways. Firstly there may be differences in drug susceptibility between insect and nematode *Wolbachia*, but, more likely, they may also reflect issues of penetration across the nematode cuticle or bioavailability within the mouse model. As the compounds within the CRX library were registered drugs, the *in vitro* nematode screens described here were not a key decision-making checkpoint, as they were for the screening of other focused and diversity libraries containing novel chemical entities. The prioritised hits progressed directly into the primary *in vivo* screen, thus making it impossible to distinguish between issues of penetration or bioavailability. The lack of activity of loratadine on both *L. sigmodontis* length and *Wolbachia* load can potentially be explained by the dosage used. Generally, the recommended human dosages of anti-histamines are very low and, given that this dose of loratadine was used to calculate doses for this experiment, this could be a factor in its lack of activity. Alternatively, the relatively weaker activity of loratadine in the cell assay in comparison to the anti-bacterial hits may make it difficult to translate to the *in vivo* situation. Certainly, as the A-WOL screening process has been developed and improved, now utilising a high content imaging platform (Clare et al., *in press*), potency has become a more important driver of *in vivo* experiments.

Pharmacological factors may also explain the more surprising differences observed between closely-related drugs within the same class. The fluoroquinolone antibiotics sparfloxacin and ciprofloxacin were demonstrated to be active *in vitro*. Ciprofloxacin has previously been shown to have either no activity (Hermans et al., 2001) or modest activity (Fenollar et al., 2003) in other cell-based *Wolbachia* assays, and the fact that this activity extended to the *in vitro* nematode screen in our screening strategy, suggests that the optimisation of our cell-based screening assay has increased the detection of active compounds. Despite this, of the two fluoroquinolone antibiotics tested, only sparfloxacin was active *in vivo*, thus demonstrating that pharmacological parameters must differ between members of the class in our screens. Further studies in the *L. sigmodontis* model conducted recently have extended this knowledge of diversity within the fluoroquinolone class by demonstrating that moxifloxacin is active, thereby confirming our result

in the *O. gutturosa* *in vitro* model, yet levofloxacin is inactive (S. Specht, unpublished observations). The inconsistency in the activity of ciprofloxacin throughout previous work and the absence of studies using other fluoroquinolones has meant that this class has largely been overlooked as a potential source of novel anti-*Wolbachia* compounds. DNA gyrase can now be considered as a valid chemotherapeutic target of *Wolbachia*. The *L. sigmodontis* model demonstrated increased potency of minocycline over doxycycline *in vivo*, adding weight to a previous observation made using nematodes *in vitro* (Townson et al., 2006). Minocycline is considered to be more lipid-soluble than doxycycline (Barza et al., 1975) and this may therefore lead to higher concentrations of the drug reaching the appropriate tissues, such as the nematode hypodermal cords, in which the *Wolbachia* reside. Work is currently ongoing to determine whether any increased potency observed across the models with the fluoroquinolones and tetracyclines translates into reduced treatment duration: a potentially important improvement when considering implementation of an anti-*Wolbachia* treatment for mass drug administration programs. Further outcomes based on double and triple combinations are also progressing through the screening strategy.

The development of the 96-well *in vitro* cell-based assay described here has, in itself, been a major development in the study of the biology and chemotherapy of *Wolbachia*. *Wolbachia* are obligate intracellular bacteria and previous cell-based screening had used either flasks (Hermans et al., 2001) or 24-well plates (Fenollar et al., 2003) therefore deeming large-scale screening studies unachievable within the five-year project. The robustness of this assay has already allowed, prior to the publication of this report, other studies to provide further insights into *Wolbachia* biology (Johnston et al., 2010; Schiefer et al., 2012; Voronin et al., 2012). Furthermore, this screening assay has since been further optimised and up-scaled (Clare et al., *in press*) to the extent that the A-WOL consortium has now screened tens of thousands of compounds from both focused and diversity compound libraries for anti-*Wolbachia* activity, a selection of which are moving down the screening funnel. The funnel, too, has been further optimised to streamline the A-WOL screening strategy and thus maximise hit discovery (Johnston et al., 2014).

These experiments not only provided a proof of concept of our cell-based assay and screening platform but also identified potential lead candidates that are better than the gold standard

doxycycline in reducing *Wolbachia* load *in vivo*. A-WOL is currently testing in clinical trials whether refined regimes of registered anti-*Wolbachia* drugs can translate into improved regimes for macrofilaricidal therapy of onchocerciasis and lymphatic filariasis.

Conflict of interest

The authors declared that there is no conflict of interest.

Acknowledgements

The A-WOL consortium is supported by a grant from the Bill and Melinda Gates Foundation awarded to the Liverpool School of Tropical Medicine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpddr.2014.09.001>.

References

- Arumugam, S., Pfarr, K.M., Hoerauf, A., 2008. Infection of the intermediate mite host with *Wolbachia*-depleted *Litomosoides sigmodontis* microfilariae: impaired L1 to L3 development and subsequent sex-ratio distortion in adult worms. *Int. J. Parasitol.* 38, 981–987.
- Ashburn, T.T., Thor, K.B., 2004. Drug repositioning: identifying and developing new uses for existing drugs. *Nat. Rev. Drug Discov.* 3, 673–683.
- Barza, M., Brown, R.B., Shanks, C., Gamble, C., Weinstein, L., 1975. Relation between lipophilicity and pharmacological behavior of minocycline, doxycycline, tetracycline, and oxytetracycline in dogs. *Antimicrob. Agents Chemother.* 8, 713–720.
- Bockarie, M.J., Deb, R.M., 2010. Elimination of lymphatic filariasis: do we have the drugs to complete the job? *Curr. Opin. Infect. Dis.* 23, 617–620.
- Brennan, L.J., Keddie, B.A., Braig, H.R., Harris, H.L., 2008. The endosymbiont *Wolbachia pipientis* induces the expression of host antioxidant proteins in an *Aedes albopictus* cell line. *PLoS One* 3, e2083.
- Chu, B.K., Hooper, P.J., Bradley, M.H., McFarland, D.A., Ottesen, E.A., 2010. The economic benefits resulting from the first 8 years of the global programme to eliminate lymphatic filariasis (2000–2007). *PLoS Negl. Trop. Dis.* 4, e708.
- Clare, R., Cook, D.A., Johnston, K.L., Ford, L., Ward, S.A., Taylor, M.J., in press. Development and validation of a high throughput anti-*Wolbachia* whole cell screen: a route to macrofilaricidal drugs against onchocerciasis and lymphatic filariasis. *J. Biomol. Screen.*
- Coffeng, L.E., Stolk, W.A., Zoure, H.G., Veerman, J.L., Agblewou, K.B., Murdoch, M.E., Noma, M., Fobi, G., Richardus, J.H., Bundy, D.A., Habbema, D., de Vlas, S.J., Amazigo, U.V., 2013. African programme for onchocerciasis control 1995–2015: model-estimated health impact and cost. *PLoS Negl. Trop. Dis.* 7, e2032.
- Ejim, L., Farha, M.A., Falconer, S.B., Wildenhain, J., Coombes, B.K., Tyers, M., Brown, E.D., Wright, G.D., 2011. Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. *Nat. Chem. Biol.* 7, 348–350.
- Fenollar, F., Maurin, M., Raoult, D., 2003. *Wolbachia pipientis* growth kinetics and susceptibilities to 13 antibiotics determined by immunofluorescence staining and real-time PCR. *Antimicrob. Agents Chemother.* 47, 1665–1671.
- Gardon, J., Gardon-Wendel, N., Demanga, N., Kamgno, J., Chippaux, J.P., Boussinesq, M., 1997. Serious reactions after mass treatment of onchocerciasis with ivermectin in an area endemic for *Loa loa* infection. *Lancet* 350, 18–22.
- Grimberg, B.T., Mehlotra, R.K., 2011. Expanding the antimalarial drug arsenal—now, but how? *Pharmaceuticals (Basel)* 4, 681–712.
- Hermans, P.G., Hart, C.A., Trees, A.J., 2001. *In vitro* activity of antimicrobial agents against the endosymbiont *Wolbachia pipientis*. *J. Antimicrob. Chemother.* 47, 659–663.
- Hoerauf, A., 2008. Filariasis: new drugs and new opportunities for lymphatic filariasis and onchocerciasis. *Curr. Opin. Infect. Dis.* 21, 673–681.
- Hoerauf, A., Nissen-Pahle, K., Schmetz, C., Henkle-Dührsen, K., Blaxter, M.L., Buttner, D.W., Gallin, M.Y., Al-Qaoud, K.M., Lucius, R., Fleischer, B., 1999. Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. *J. Clin. Invest.* 103, 11–18.
- Hoerauf, A., Volkmann, L., Nissen-Paehle, K., Schmetz, C., Autenrieth, I., Buttner, D.W., Fleischer, B., 2000. Targeting of *Wolbachia* endobacteria in *Litomosoides sigmodontis*: comparison of tetracyclines with chloramphenicol, macrolides and ciprofloxacin. *Trop. Med. Int. Health* 5, 275–279.
- Johnston, K.L., Taylor, M.J., 2007. *Wolbachia* in filarial parasites: targets for filarial infection and disease control. *Curr. Infect. Dis. Rep.* 9, 55–59.
- Johnston, K.L., Wu, B., Guimaraes, A., Ford, L., Slatko, B.E., Taylor, M.J., 2010. Lipoprotein biosynthesis as a target for anti-*Wolbachia* treatment of filarial nematodes. *Parasit. Vectors* 3, 99.
- Johnston, K.L., Ford, L., Taylor, M.J., 2014. Overcoming the challenges of drug discovery for neglected tropical diseases: the A-WOL experience. *J. Biomol. Screen.* 19, 335–343.
- Kremer, N., Charif, D., Henri, H., Gavory, F., Wincker, P., Mavingui, P., Vavre, F., 2012. Influence of *Wolbachia* on host gene expression in an obligatory symbiosis. *BMC Microbiol.* 12 (Suppl 1), S7.
- Liu, L.X., Weller, P.F., 1996. Antiparasitic drugs. *N. Engl. J. Med.* 334, 1178–1184.
- Mackenzie, C.D., Homeida, M.M., Hopkins, A.D., Lawrence, J.C., 2012. Elimination of onchocerciasis from Africa: possible? *Trends Parasitol.* 28, 16–22.
- Makepeace, B.L., Rodgers, L., Trees, A.J., 2006. Rate of elimination of *Wolbachia pipientis* by doxycycline *in vitro* increases following drug withdrawal. *Antimicrob. Agents Chemother.* 50, 922–927.
- McGarry, H.F., Pfarr, K., Egerton, G., Hoerauf, A., Akue, J.P., Enyong, P., Wanji, S., Klager, S.L., Bianco, A.E., Beeching, N.J., Taylor, M.J., 2003. Evidence against *Wolbachia* symbiosis in *Loa loa*. *Filaria J.* 2, 9.
- Mucke, H.A.M., 2010. Drug repositioning: extracting value from prior R&D investments. *Insight Pharma Rep.* <www.insightpharmareports.com>.
- Pritchard, R.K., Basanez, M.G., Boatman, B.A., McCarthy, J.S., Garcia, H.H., Yang, G.J., Sripa, B., Lustigman, S., 2012. A research agenda for helminth diseases of humans: intervention for control and elimination. *PLoS Negl. Trop. Dis.* 6, e1549.
- Rao, R.U., Moussa, H., Weil, G.J., 2002. *Brugia malayi*: effects of antibacterial agents on larval viability and development *in vitro*. *Exp. Parasitol.* 101, 77–81.
- Richard-Lenoble, D., Chandenier, J., Gaxotte, P., 2003. Ivermectin and filariasis. *Fundam. Clin. Pharmacol.* 17, 199–203.
- Schiefer, A., Schmitz, A., Schaberle, T.F., Specht, S., Lammer, C., Johnston, K.L., Vassilyev, D.G., Konig, G.M., Hoerauf, A., Pfarr, K., 2012. Coralopyronin a specifically targets and depletes essential obligate *Wolbachia* endobacteria from filarial nematodes *in vivo*. *J. Infect. Dis.* 206, 249–257.
- Smits, H.L., 2009. Prospects for the control of neglected tropical diseases by mass drug administration. *Expert Rev. Anti Infect. Ther.* 7, 37–56.
- Strübing, U., Lucius, R., Hoerauf, A., Pfarr, K.M., 2010. Mitochondrial genes for heme-dependent respiratory chain complexes are up-regulated after depletion of *Wolbachia* from filarial nematodes. *Int. J. Parasitol.* 40, 1193–1202.
- Tamarozzi, F., Tendongfor, N., Enyong, P.A., Esum, M., Faragher, B., Wanji, S., Taylor, M.J., 2012. Long term impact of large scale community-directed delivery of doxycycline for the treatment of onchocerciasis. *Parasit. Vectors* 5, 53.
- Taylor, M.J., Hoerauf, A., Bockarie, M., 2010. Lymphatic filariasis and onchocerciasis. *Lancet* 376, 1175–1185.
- Taylor, M.J., Hoerauf, A., Townson, S., Slatko, B.E., Ward, S.A., 2014. Anti-*Wolbachia* drug discovery and development: safe macrofilaricides for onchocerciasis and lymphatic filariasis. *Parasitol.* 141, 119–127.
- Tobinick, E.L., 2009. The value of drug repositioning in the current pharmaceutical market. *Drug News Perspect.* 22, 119–125.
- Townson, S., Hutton, D., Siemianska, J., Hollick, L., Scanlon, T., Tagboto, S.K., Taylor, M.J., 2000. Antibiotics and *Wolbachia* in filarial nematodes: antifilarial activity of rifampicin, oxytetracycline and chloramphenicol against *Onchocerca gutturosa*, *Onchocerca lienalis* and *Brugia pahangi*. *Ann. Trop. Med. Parasitol.* 94, 801–816.
- Townson, S., Tagboto, S., McGarry, H.F., Egerton, G.L., Taylor, M.J., 2006. *Onchocerca* parasites and *Wolbachia* endosymbionts: evaluation of a spectrum of antibiotic types for activity against *Onchocerca gutturosa* *in vitro*. *Filaria J.* 5, 4.
- Townson, S., Ramirez, B., Fakorede, F., Mouries, M.A., Nwaka, S., 2007. Challenges in drug discovery for novel antifilarials. *Expert Opin. Drug Discov.* 2, S63–73.
- Turner, J.D., Langley, R.S., Johnston, K.L., Egerton, G., Wanji, S., Taylor, M.J., 2006. *Wolbachia* endosymbiotic bacteria of *Brugia malayi* mediate macrophage tolerance to TLR- and CD40-specific stimuli in a MyD88/TLR2-dependent manner. *J. Immunol.* 177, 1240–1249.
- Turner, J.D., Tendongfor, N., Esum, M., Johnston, K.L., Langley, R.S., Ford, L., Faragher, B., Specht, S., Mand, S., Hoerauf, A., Enyong, P., Wanji, S., Taylor, M.J., 2010. Macrofilaricidal activity after doxycycline only treatment of *Onchocerca volvulus* in an area of *Loa loa* co-endemicity: a randomized controlled trial. *PLoS Negl. Trop. Dis.* 4, e660.
- Volkmann, L., Fischer, K., Taylor, M., Hoerauf, A., 2003. Antibiotic therapy in murine filariasis (*Litomosoides sigmodontis*): comparative effects of doxycycline and rifampicin on *Wolbachia* and filarial viability. *Trop. Med. Int. Health* 8, 392–401.
- Voronin, D., Cook, D.A., Steven, A., Taylor, M.J., 2012. Autophagy regulates *Wolbachia* populations across diverse symbiotic associations. *Proc. Natl. Acad. Sci. USA* 109, E1638–1646.
- Wanji, S., Tendongfor, N., Nji, T., Esum, M., Che, J.N., Nkwescheu, A., Alassa, F., Kamnang, G., Enyong, P.A., Taylor, M.J., Hoerauf, A., Taylor, D.W., 2009. Community-directed delivery of doxycycline for the treatment of onchocerciasis in areas of co-endemicity with loiasis in Cameroon. *Parasit. Vectors* 2, 39.
- WHO, 2007. Global plan to combat neglected tropical diseases 2008–2015. <http://whqlibdoc.who.int/hq/2007/WHO_CDS_NTD_2007.2003_eng.pdf>.
- Zhang, J.H., Chung, T.D., Oldenburg, K.R., 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* 4, 67–73.