AWZ1066S, a highly specific anti-*Wolbachia* drug candidate for a short-course treatment of filariasis

W. David Hong[a,b], Farid Benayoud[d], Gemma L. Nixon[a], Louise Ford[b], Kelly L. Johnston[b], Rachel H. Clare[b], Andrew Cassidy[b], Darren A. N. Cook[b], Amy Siu[d], Motohiro Shiotani[e], Peter J. H. Webborn[f1], Stefan Kavanagh[f], Ghaith Aljayyoussi[b], Emma Murphy[b], Andrew Steven[b], John Archer[b], Dominique Struever[g], Stefan J. Frohberger[g], Alexandra Ehrens[g], Marc P. Hübner[g], Achim Hoerauf[g], Adam P. Roberts[b], Alasdair T. M. Hubbard[b], Edward W. Tate[h], Remigiusz A Serwa[h], Suet C. Leung[a,b], Li Qie[a], Neil G. Berry[a], Fabian Gusovsky[d], Janet Hemingway[c2],Joseph D. Turner[b], Mark J. Taylor[b], Stephen A. Ward[b2] and Paul M. O'Neill[b2]

a. Department of Chemistry, University of Liverpool, Liverpool, L69 7ZD, UK; b. Research Centre for Drugs and Diagnostics, Department of Parasitology, Liverpool School of Tropical Medicine, Liverpool, L3 5QA, UK; c. Department of International Health, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK; d. Eisai AiM Institute, Eisai Inc. 4 Corporate Drive, Andover, MA 01810 U.S.A.; e. Drug Safety, Eisai Co., Ltd., Tsukuba 300-2635, Japan; f. Drug Safety & Metabolism, IMED Biotech Unit, AstraZeneca, Cambridge, CB2 0AA, UK; g. Institute for Medical Microbiology, Immunology and Parasitology, University Hospital Bonn, 53127, Germany; h. Department of Chemistry, Imperial College London, Exhibition Road, South Kensington Campus, London SW7 2AZ, UK.

1. Current address is PJHW Consultancy Ltd., Willowmoor School Lane, Macclesfield, Cheshire, U.K., SK11 9HD.

2. To whom corresponding may be addressed. E-mail: Janet.Hemingway@lstmed.ac.uk, pmoneill@liverpool.ac.uk or Steve.Ward@lstmed.ac.uk

Abstract

Onchocerciasis and lymphatic filariasis are two neglected tropical diseases (NTDs) that together affect ~157 million people and inflict severe disability. Both diseases are caused by

parasitic filarial nematodes with elimination efforts constrained by lack of a safe drug that can kill the adult filaria (macrofilaricide). Previous proof-of-concept human trials have demonstrated that depleting >90% of the essential nematode endosymbiont bacterium, *Wolbachia*, using antibiotics, can lead to permanent sterilisation of adult female parasites and a safe macrofilaricidal outcome. AWZ1066S is a highly specific anti-*Wolbachia* candidate selected through a lead optimisation programme focused on balancing efficacy, safety and drug metabolism/pharmacokinetic (DMPK) features of a thienopyrimidine/quinazoline scaffold derived from phenotypic screening. AWZ1066S shows superior efficacy to existing anti-*Wolbachia* therapies in validated preclinical models of infection and has DMPK characteristics that are compatible with a short therapeutic regimen of seven days or less. This candidate molecule is well-positioned for onward development and has the potential to make a significant impact to communities affected by filariasis.

Significance

Onchocerciasis (river blindness) and lymphatic filariasis (elephantiasis) are neglected tropical diseases that cause severe disability and affect more than 157 million people globally. The current control efforts are hindered by the lack of a safe macrofilaricidal drug that can eliminate the parasitic adult nematodes safely. A clinically validated approach for delivering macrofilaricidal activity is to target the *Wolbachia* bacterial endosymbiont of the causative nematodes. This first-in-class, highly potent and specific anti-*Wolbachia* pre-clinical candidate molecule, AWZ1066S has the potential to significantly impact current global onchocerciasis and lymphatic filariasis elimination programmes and to reduce elimination time frames from decades to years.

Introduction

Onchocerciasis (river blindness) and lymphatic filariasis (elephantiasis) continue to inflict serious public health problems throughout tropical communities, globally affecting more than 38 million and 120 million people, respectively. These two NTDs are caused by parasitic filarial nematodes *Onchocerca volvulus* (onchocerciasis), *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori* (lyphatic filariasis).(1) Global programmes for control and elimination have been developed, but existing approaches target only microfilaria and require prolonged delivery with high treatment coverage to break the transmission cycle of the long-lived adult worm.(2, 3) This inability of current drugs to kill adult parasites has highlighted the need for new macrofilaricidal drugs.

Wolbachia are essential for multiple components of filarial nematode biology including larval growth, development, embryogenesis and, ultimately, survival of many filarial nematodes, including the causative parasites of onchocerciasis and lyphatic filariasis, making this symbiont a validated chemotherapeutic target.(4, 5) Although the macrofilaricidal activity of doxycycline through depletion of *Wolbachia* has been proven clinically (6-12), protracted treatment regimens (≥28 days) and contraindications restrict its widespread implementation.(13, 14)

Results

Lead optimization led to the selection of AWZ1066S as preclinical candidate

To identify novel anti-*Wolbachia* chemotypes, we screened 10,000 compounds selected from the BioFocus Soft-Focus library using a phenotypic cell-based screen incorporating a *Wolbachia*-infected *Aedes albopictus* cell line [C6/36 (wAlbB)] as reported previously.(15) From this screen we identified and confirmed 50 anti-*Wolbachia* active hits (0.5% hit rate) that clustered into six unique chemotypes. Based on an evaluation of drug-like properties and potency, we selected a series of thienopyrimidines for medicinal chemistry optimisation. Through a highly integrated academic-industrial partnership, multi-parameter lead

optimisation was performed with over 300 analogues synthesised and assessed for both anti-*Wolbachia* activity and DMPK properties (LogD, aqueous solubility, plasma protein binding, microsomal and hepatocyte stability) *in vitro*.(16) In this process, we transformed the thienopyrimidine core of the starting hit molecule into quinazoline **AWB158** and eventually azaquinazoline **AWZ1066**, resolving the metabolic weakness associated with the original scaffold whilst improving potency. Optimisation of the 2-position of the azaquinazoline core led to AWZ1066 which was selected as a front runner (Fig 1a, see SI Appendix Figure S1 for detailed structural-activity relationship analyses of this series). AWZ1066 was active against *Wolbachia* in a cell-based assay(17) with an EC_{50} of 2.6 ± 0.5 nM (Fig 1b). In an orthogonal secondary *in vitro* assay utilising microfilariae (mf) of the human parasite *Brugia malayi*, AWZ1066 reduced *Wolbachia* within the mf with an EC_{50} of 150 nM whilst had no effect of the viability and motility of the mf at up to the top testing concentration of 5 µM.

Specific structural features of AWZ1066, such as the lipophilic electron withdrawing CF_3 group in the 4-position side-chain, the nitrogen at the 8-position of the azaquinazoline core and the methyl group in the 2-position of the morpholinyl side-chain contribute significantly to potency in both assays (SI Appendix Figure S1). Matched pair analyses with or without these individual structural features demonstrated a 10 – 100 fold potency loss. AWZ1066 has two enantiomers, namely AWZ1066S and AWZ1066R, demonstrating 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) (Fig 1c). The predicted cost of goods for AWZ1066 and its enantiomers are considered to be low based on two simple sequential amination steps from readily available 2,4-dichloropyrido[2,3-d]pyrimidine (SI Appendix Figure S1). This will support cost efficient large-scale synthesis.

Extensive comparative analysis of the two enantiomers led to the selection of the (*S*)-enantiomer, AWZ1066S, as the preclinical candidate based on superiority in terms of *in vitro* potency, *in vivo* efficacy and safety pharmacology (*vide infra*).

Pharmacokinetics, pharmacodynamics and safety profiles of AWZ1066S match anti-*Wolbachia* macrofilaricide Target Candidate Profile (TCP)

A key criterion for anti-*Wolbachia* macrofilaricides is the need for oral drug delivery, necessitating good aqueous solubility and metabolic stability. AWZ1066S meets the desired physicochemical properties with acceptable solubility in both PBS buffer (238 µM) and fasted state simulated intestinal fluid (FaSSIF) (0.56 mg/ml), moderate human plasma protein binding (91%) and ready formulation as a salt (pKa = 5.6) (Fig 1d). The metabolic turnover of AWZ1066S by both liver microsomes and hepatocytes across a range of different species, including mouse, rat, dog, monkey and human is low and negatively correlated with the species body size (SI Appendix Table S3). High metabolic stability was also observed in the *in vivo* mouse PK profile (total clearance = 0.47 L/hr/kg and $T_{1/2}$ = 3.5 hours) when dosed intravenously. The permeability of AWZ1066S in a LLC-PK1 cell assay is good (P_{app} = 7.4 X 10^6 cm/s) and despite being a mammalian P-gp transporter substrate (P-gp mediated efflux ratio = 7.8), AWZ1066S showed good to excellent oral bioavailability (range 54 – 91 %) across a range of dosages up to 250 mg/kg in the mouse (SI Appendix Table S4). AWZ1066S is a weak CYP2C9 inhibitor (IC_{50} = 9.7 µM) and a weak CYP3A4 inducer (IC_{50} = 37 µM) but does not show any time-dependent inhibition across 5 major human CYP450 isoforms (CYP1A, CYP2C9, CYP2C19, CYP2D6, CYP3A).

One of the major limitations of doxycycline, the current “gold standard” anti-*Wolbachia* macrofilaricide in the treatment of filariasis, is its prolonged treatment period of 4 – 6 weeks to deliver a cure.(6, 9, 10, 12) The community consensus is that novel macrofilaricide treatments need to be implementable in seven days or less.(5, 14) To assess if AWZ1066S could meet this demand, determined as a threshold reduction of *Wolbachia* >90% in seven

days, efficacy was evaluated in two independent animal models of filarial infection. As an initial screen, we used the adult *Brugia malayi* SCID mouse model.(14) In this model, human bioequivalent exposures to 100 mg/day doxycycline require 6 weeks to mediate >90% *Wolbachia* depletion within female *B. malayi* adult parasites, whilst the more potent second generation tetracycline, minocycline, still requires 4 weeks exposure bioequivalent to 100 mg/day dosing to mediate similar efficacy.(18, 19) AWZ1066S (100 mg/kg) reduced *Wolbachia* by 98% after only seven days orally administrated treatment (compared to vehicle group) (Kruskal Wallis statistic 50.7, P<0.0001 Fig 2a). Furthermore, the release of mf was completely prevented in 9/10 mice treated with AWZ1066S over the 6 weeks of observation post treatment (SI Appendix Table S5). We further investigated the preclinical efficacy of 7-day AWZ1066S dosing in a second rodent filarial infection system (*Litomosoides sigmodontis* gerbil model(20)). After seven days of twice-daily treatment of AWZ1066S at either 100 or 50 mg/kg orally, *Wolbachia* load was reduced by >99% compared to control untreated animals, 18 weeks post-treatment (Kruskal Wallis statistic 43.5, P<0.0001, Fig 2b). In this model, mf produced by female adult worms circulate in the blood and thus peripheral microfilaremias can be tracked longitudinally post-treatment. After AWZ1066S treatment the peripheral blood microfilaremia began to decline from 6 weeks post treatment. A state of amicrofilaremia (absence of mf in blood) was evident from 14 weeks post-treatment in the 7-day AWZ1066S treatment groups, a state that was sustained until the end of the experiment at 18 weeks post-treatment. Thus, treatment with AWZ1066S for seven days leads to sterilisation, gradual depletion of mf with the assumption, based on extensive clinical trial data with doxycycline, that this will result in a 'slow' killing of adult worms alongside an improved safety profile. Based on PK/PD modelling(21) of the available data, using human PK simulations, we predict that >90% of a patient population will achieve a 90% or higher *Wolbachia* reduction (considered fully efficacious clinically(8, 10, 22)) within seven days using an oral dose of 10 mg per kg body weight of AWZ1066S in human (Fig 2d, SI Appendix Table S6).

AWZ1066S was subsequently tested for safety pharmacology characteristics. AWZ1066S showed very low cytotoxicity in multiple *in vitro* toxicology assays (SI Appendix Table S7). AWZ1066S was also tested against a panel of 25 well characterised human protein assays(23) (CEREP panel) *in vitro* to assess secondary pharmacology. The CEREP panel screen results indicated low promiscuity across the panel (SI Appendix Table S8). AWZ1066S was negative in both the Ames test and the *in vivo* micronucleus assay in the mouse (SI Appendix Table S9). AWZ1066 was also evaluated for cardiovascular safety both *in vitro* (hERG and Nav1.5) and *in vivo* (anesthetised and conscious rats, SI Appendix Table S10) demonstrating a low risk. In a preliminary 7-day dose range finding (DRF) study in the rat (SI Appendix Table S11), the no observed adverse effect level (NOAEL) for AWZ1066 was determined to be >300 mg/kg.

Discussion

The antibacterial activity profile of AWZ1066S was examined against a panel of clinically relevant bacterial strains, including gram-positive and gram-negative species and strains with known drug resistance profiles. The data indicated that AWZ1066S is highly specific for *Wolbachia* (SI Appendix Table S12). This high specificity of AWZ1066S against *Wolbachia* is a significant advantage as it would predict minimal impact on the gut microbiota, lowering the risk of dysbiosis and the selection and emergence of resistance following administration to patients, in comparison to other anti-*Wolbachia* based broad spectrum antibiotic treatments.

In addition to the ability to elicit efficacy after seven days of treatment, AWZ1066 also has a faster kill rate compared to other known antibiotics tested against *Wolbachia in vitro*. In a time-kill assay modified from the standard mf assay, a panel of anti-*Wolbachia* drugs(24) including moxifloxacin, rifampicin, minocycline, doxycycline and AWZ1066S were screened over a range of exposure intervals (1, 2 and 6 days). The results show AWZ1066S can achieve maximum reduction of *Wolbachia* just after one day of drug exposure compared to the other antibiotics tested (1 – 6 days of exposure, Figure 3a). These results further support

the potential of AWZ1066S to deliver treatment time frames <7 days. Furthermore, differential time to kill profiles suggest a novel mode of action for the AWZ1066S class compared to antibiotics that work *via* inhibition of aspects of protein synthesis. Genetic approaches to identify the molecular target(s) of AWZ1066S have been thwarted by the intractability of this intracellular bacterium. However, using a proteomic approach with photoreactive chemical probes we have identified a number of credible *Wolbachia* and host proteins that are being evaluated as potential drug targets of AWZ1066S (Fig 3b and c, SI Dataset S1).

In conclusion, starting with a hit chemotype from a modest scale whole cell screen, a medicinal chemistry led scaffold hopping and multi-parameter optimisation programme has delivered the first novel synthetic candidate molecule, AWZ1066S that is highly specific against *Wolbachia*. Seven-day oral dosing regimens with AWZ1066S is capable of depleting *Wolbachia* >90% with sustained sterilisation of mf production in two independent filarial infection models. This candidate selected molecule meets all Target Candidate Profile criteria for an anti-*Wolbachia* macrofilaricidal drug and has entered formal preclinical evaluation. AWZ1066S has the potential to deliver a novel anti-filarial therapy that could be deployed in target populations in a sub-seven-day dosing regimen. The compound provides a unique opportunity to make a significant contribution to communities affected by filariasis especially if the predicted macrofilaricidal effect can be confirmed in clinical trials.

Materials and Methods

The chemical characterization of AWB158, AWZ1066S (and its HCl salt), and Photoreactive probe 1 & 2

(Please see Supplementary Information for the synthesis of AWB158, AWZ1066S (and its HCl salt), and Photoreactive probe 1 & 2.)

***N*^{2}-isopropyl-*N*^{4}-(2-(trifluoromethyl)benzyl)quinazoline-2,4-diamine (AWB158).** ^{1}H NMR (400 MHz, MeOD) δ 8.23 (d, *J* = 8.0 Hz, 1H), 7.89 – 7.81 (m, 1H), 7.78 (d, *J* = 7.8 Hz, 1H),

7.61 (t, *J* = 7.5 Hz, 1H), 7.56 – 7.42 (m, 4H), 5.11 (s, 2H), 4.18 – 4.06 (m, 1H), 1.13 (br, 6H); HRMS (ES) $C_{19}H_{20}N_4F_3$ $[M+H]^+$ requires 361.1640, found 361.1648; purity by HPLC 97.2%, R_t = 9.255 min.

(*S*)-2-(3-methylmorpholino)-*N*-((2-(trifluoromethyl)pyridin-3-yl)methyl)pyrido[2,3-d]pyrimidin-4-amine (AWZ1066S). ^{1}H NMR (400 MHz, DMSO) δ 8.98 (t, *J* = 5.1 Hz, 1H), 8.70 (d, *J* = 4.4 Hz, 1H), 8.60 (d, *J* = 4.3 Hz, 1H), 8.50 (d, *J* = 8.0 Hz, 1H), 7.95 (d, *J* = 7.9 Hz, 1H), 7.64 (dd, *J* = 8.0, 4.6 Hz, 1H), 7.15 (dd, *J* = 8.0, 4.5 Hz, 1H), 4.94 (dd, *J* = 16.3, 4.7 Hz, 1H), 4.79 (dd, *J* = 16.3, 4.8 Hz, 1H), 4.45 (m, 1H), 4.21 (d, *J* = 13.1 Hz, 1H), 3.82 (d, *J* = 11.1 Hz, 1H), 3.58 (d, *J* = 11.3 Hz, 1H), 3.44 (d, *J* = 11.3 Hz, 1H), 3.32 – 3.23 (m, 1H), 2.99 (td, *J* = 13.2, 3.7 Hz, 1H), 0.87 (br, 3H); HRMS (ES) $C_{19}H_{19}N_6OF_3$ $[M+H]^+$ requires 405.1645, found 405.1641; purity by HPLC 97.8%, R_t = 7.147 min. **AWZ1066S HCl salt** ^{1}H NMR (400 MHz, DMSO) δ 10.49 (s, 1H), 9.24 (d, *J* = 7.8 Hz, 1H), 9.02 – 8.69 (m, 1H), 8.64 (d, *J* = 4.4 Hz, 1H), 8.05 (d, *J* = 7.9 Hz, 1H), 7.67 (dd, *J* = 7.9, 4.6 Hz, 1H), 7.52 (dd, *J* = 7.8, 5.6 Hz, 1H), 4.97 (dd, *J* = 15.9, 4.4 Hz, 1H), 4.88 (dd, *J* = 15.9, 3.9 Hz, 1H), 4.50 (m, 1H), 4.26 (d, *J* = 13.1 Hz, 1H), 3.88 (d, *J* = 10.1 Hz, 1H), 3.62 (t, *J* = 17.2 Hz, 1H), 3.55 – 3.13 (m, 4H), 0.98 (br, 3H).

2-(1-(4-(((2-(trifluoromethyl)pyridin-3-yl)methyl)amino)pyrido[2,3-d]pyrimidin-2-yl)piperidin-4-yl)ethyl (2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethyl)carbamate (Photoreactive probe 1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.75 (d, *J* = 4.3 Hz, 1H), 8.61 (d, *J* = 4.4 Hz, 1H), 7.94 (d, *J* = 7.7 Hz, 1H), 7.86 (d, *J* = 7.9 Hz, 1H), 7.44 (dd, *J* = 7.9, 4.7 Hz, 1H), 6.98 (dd, *J* = 7.9, 4.5 Hz, 1H), 6.10 (t, *J* = 5.4 Hz, 1H), 5.02 (d, *J* = 5.6 Hz, 2H), 4.90 – 4.73 (m, 2H), 4.11 (t, *J* = 6.2 Hz, 2H), 3.05 (dd, *J* = 12.0, 5.7 Hz, 2H), 2.83 (t, *J* = 12.2 Hz, 2H), 2.10 – 1.88 (m, 2H), 1.80 – 1.62 (m, 7H), 1.59 – 1.47 (m, 4H), 1.10 (br, 2H); HRMS (ES) $C_{29}H_{33}N_9O_2F_3$ $[M+H]^+$ requires 596.2704, found 596.2709; purity by HPLC 90.3%, R_t = 8.888 min.

2-(1-(4-(((2-(trifluoromethyl)pyridin-3-yl)methyl)amino)pyrido[2,3-d]pyrimidin-2-yl)piperidin-4-yl)ethyl (1-oxo-1-((4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzyl)amino)pent-4-yn-2-yl)carbamate (Photoreactive probe 2). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.75 (d, *J* = 4.3 Hz, 1H), 8.61 (d, *J* = 4.3 Hz, 1H), 8.04 – 7.86 (m, 2H), 7.44 (dd, *J* = 7.9, 4.6 Hz, 1H), 7.31 (d, *J* = 8.3 Hz, 2H), 7.14 (d, *J* = 8.1 Hz, 2H), 6.99 (dd, *J* = 8.0, 4.5 Hz, 1H), 6.75 (br, 1H), 6.27 (br, 1H), 5.65 (br, 1H), 5.02 (d, *J* = 5.4 Hz, 2H), 4.91 – 4.72 (m, 2H), 4.67 – 4.44 (m, 2H), 4.41 – 4.27 (m, 1H), 4.19 – 4.06 (m, 2H), 3.64 (s, 1H), 2.94 – 2.73 (m, 3H), 2.65 (ddd, *J* = 17.0, 6.9, 2.6 Hz, 1H), 2.09 (t, *J* = 2.6 Hz, 1H), 1.78 – 1.66 (m, 2H), 1.59 – 1.41 (m, 2H), 1.14 – 0.96 (m, 2H); HRMS (ES) $C_{36}H_{35}N_{10}O_3F_6$ $[M+H]^+$ requires 769.2792, found 769.2796; purity by HPLC 91.0%, R_t = 9.561 min.

In vitro anti-*Wolbachia* cell based screening

Anti-*Wolbachia* activity was assessed in the A·WOL high content imaging (HCI) assayas described previously.(17) In brief this utilised a mosquito (*Aedes albopictus*) derived cell line (C6/36), stably infected with *Wolbachia pipientis* (*w*AlbB) with a read out of the percentage of the cells infected with *Wolbachia*. The EC_{50} values reported were the means of at least 5 individual experiments.

In vitro anti-*Wolbachia Brugia malayi* microfilaria (mf) assay and time-kill assay

Brugia malayi mf were obtained from patently infected *Meriones unguiculatus* gerbils as described previously.(25) A series dilution of compounds (5 concentrations) were incubated with 8,000 mf/well (5 replicates), in RPMI supplemented with 10% FBS, 1% Penicillin Streptomycin and 1% Amphotericin B. After 6 days incubation (37 ºC, 5% CO_2) motility was assessed by microscopy and DNA extracted for quantitative PCR (qPCR) analysis to obtain ratios of *Wolbachia* Surface Protein copy number (*wsp*) to Glutathione S-Transferase copy number (*gst*) as previously described.(26) Time kill assays were completed using the same method with the exception of a wash step on the indicated day. The drug concentration in the time-kill assay was 10 times of EC_{50}. There were five replicates per time point per drug

treatment. The EC_{50} values or *Wolbachia* reduction percentages reported were from one individual experiment.

In vitro drug metabolism/pharmacokinetic (DMPK) assays

The DMPK properties data described above were measured once through a high throughput platform provided by AstraZeneca U.K. The methods of the five assays, including $LogD_{7.4}$, aqueous solubility, plasma protein binding, microsome and hepatocyte clearance measurements has been reported previously.(27, 28)

In vivo anti-*Wolbachia* pharmacodynamic studies.

Animals; for *Brugia malayi* studies, inbred male SCID CB.17 mice and outbred *M. unguiculatus* gerbil breeding pairs were purchased from Charles River, Europe. Rodents were maintained/bred in SPF conditions at the University of Liverpool Biological Services Unit. All experiments were approved by animal welfare ethics committees of the University of Liverpool and LSTM. Studies were conducted in accordance with Home Office legislation. Experiments with *L. sigmodontis* were performed at the Institute for Medical Microbiology, Immunology and Parasitology, University Hospital Bonn in accordance to the European Union animal welfare guidelines and all protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Cologne, Germany (AZ 84-02.04.2015.A507). Female *M. unguiculatus* gerbils were obtained from Janvier labs (Saint-Berthevin, France) and housed at the animal facility of the Institute for Medical Microbiology, Immunology and Parasitology in individually ventilated cages on a 12h light/dark cycle with food and water ad libitum.

Filarial experimental infections; infectious stage *B. malayi* L3 were propagated as previously described.(14) Male mice 6-12 weeks of age (n=5-9) or male gerbils (n=6) 8-16 weeks of age were infected with *B. malayi* L3 i.p. (50 or 100xL3 mouse, 400xL3, gerbil). Mouse experimental infections were maintained for 13 weeks whilst gerbil infections were maintained for maximum of 12 months. Motile *B. malayi* parasites were recovered by peritoneal lavage at necropsy and enumerated by microscopy. Motile mf from gerbil

infections were periodically isolated by intra-peritoneal catheter drain under anaesthesia with recovery as previously described.(14)

For *L. sigmodontis* infection, 6-8 week old female gerbils were exposed to *Ornithonyssus bacoti* mites containing infective-stage *L. sigmodontis* L3. Treatment of microfilaraemic gerbils started 13 weeks following infection. Necropsies occurred 18 weeks following start of treatment. At necropsy, *L. sigmodontis* worms were isolated from the serous cavities and were quantified as previously described.(29) For mf counts, 10µl of peripheral blood were taken from the saphenous vein in bi-weekly intervals starting at 12 weeks post infection and diluted in 300µl of Hinkelmann solution (0.5% Eosin Y, 0.5% Phenol, 0.185% Formaldehyde in aqua dest). After centrifugation at 400g for 5 minutes, the supernatant was discarded; the pellet was resuspended in 1ml and transferred to a 1ml Sedgewick Rafter Counting Chamber to quantify total mf using a microscope.

Drug dosing; infected animals were randomly assigned into dose groups (n = 5 - 9). AWZ1066S (or it HCl salt) was dissolved in PEG300/Propylene Glycol/H_20 (55/25/20). Minocycline- and doxycycline-hyclate (Sigma-Aldrich) were dissolved in water. Animals were weighed and weight-corrected volumes of dosing solution administered by oral gavage (mouse: 100 µl per 25 g body weight; gerbil: 2.5 ml/kg).

Molecular assays; for *B. malayi* infections, determination of *Wolbachia* single-copy *wsp* gene quantity was undertaken by qPCR as previously described(14, 18) whereas, for *L. sigmodontis, Wolbachia* was determined by FtsZ qPCR as previously described.(30) Between 5 - 20 individual worms were assayed, derived from dose groups of 3 - 9 animals.

Statistical analysis; continuous variables (*Wolbachia* loads, adult worm burdens, mf burdens) were tested for normal distribution by D'Agostino & Pearson omnibus normality tests pre- and post-Log^{10} transformation. Variables did not satisfy the assumption of normality and were compared by two-tailed Kruskal-Wallis Tests with Dunn's Multiple Tests, post-hoc. Post-hoc testing scrutinized drug groups compared with vehicle or untreated

controls. Significance levels are indicated $P<0.05$*, $P<0.01$** $P<0.001$*** $P<0.0001$****. All statistics were undertaken using GraphPad Prism v6 software.

PK/PD predictions of AWZ1066S activity in clinical settings; the predicted human pharmacokinetic parameters (SI Appendix Table S6) were used to predict the activity of AWZ1066S when administered in humans for 1 week using the simulator tool in Pmetrics™.(31) As previously described(21) we used a PK/PD model which links predicted human pharmacokinetic parameters with the observed *in vitro* activity of the drug against intracellular *Wolbachia*. 1000 subjects were simulated and % of subjects achieving >90% *Wolbachia* reduction at each week was recorded and data presented as a cumulative number of patients who achieve >90% *Wolbachia* load reduction.

Preliminary target pulldown using photoreactive probes with *Wolbachia* infected cells.

Please see Supplementary Information for experimental details of sample preparation and pull-down for proteomics, and LC-MS/MS analysis of proteomics samples and data processing.

Figures

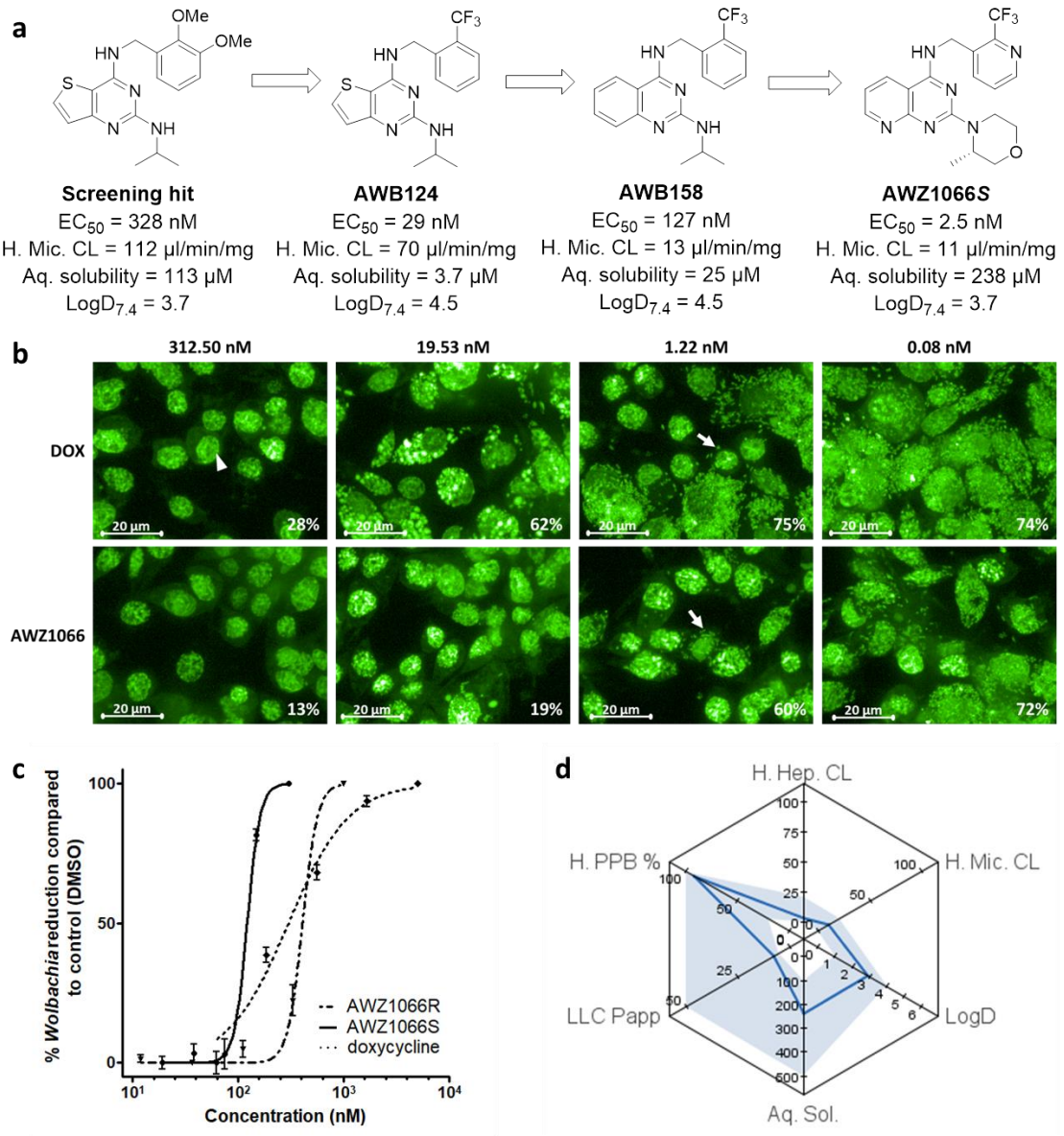

Figure 1 Medicinal chemistry optimisation to identify AWZ1066S including *in vitro* potency and DMPK properties. (**a**) Progression of the thienopyrimidine screening hit to the azaquinazoline candidate AWZ1066S through multi-parameter optimisation to improve potency and DMPK properties. (**b**) AWZ1066 treatment causes a dose-dependent reduction of *Wolbachia* from *Wolbachia*-infected cells. Images are visual representations of cells treated with doxycycline (top panel) and AWZ1066 (bottom panel), at various concentrations. In the cell-based assay C6/36 (*w*AlbB) cells are incubated with compounds, then stained with SYTO11 stain, which stains cell nuclei (large, bright areas, arrowhead) and *Wolbachia* (small, bright punctate staining, arrow) green. Automated capture and image analysis is

conducted by the Operetta and Harmony software (6 fields per well, two wells per concentration), which generates 'percentage of infected cells' values (shown in white, typical vehicle-treated cells have 70-80% infection, while doxycycline at maximum effect has 10-15% infection) that are used to calculate the effective concentration of 50% inhibition of *Wolbachia* (EC_{50}, doxycycline = 20 nM and AWZ1066 = 2.6 nM). (**c**) Anti-*Wolbachia* activity of AWZ1066S in the mf assay (EC_{50} = 121 nM) in comparison to the (*R*)-isomer AWZ1066R (EC_{50} = 408 nM). Doxycycline was used as positive control (EC_{50} = 300 nM). Data are expressed as mean ± s.d. of five replicates for each concentration. (**d**) *In vitro* DMPK related data of AWZ1066S presented in a radar plot. Six axes represent: human hepatocytes clearance (µl $min^{-1}1X10^6$ cells $^{-1}$) (H. Hep. CL), human microsome clearance (µl min^{-1} mg^{-1}) (H. Mic. CL), $LogD_{7.4}$ (LogD), aqueous solubility in pH7.4 PBS buffer (µM) (Aq. sol.), permeability in LLC-PK1 cell assay (1 X 10^6 cm/s) (LLC Papp) and human plasma protein binding (%) (H. PPB). The light blue shaded area indicates the desired DMPK properties target ranges for oral administration.

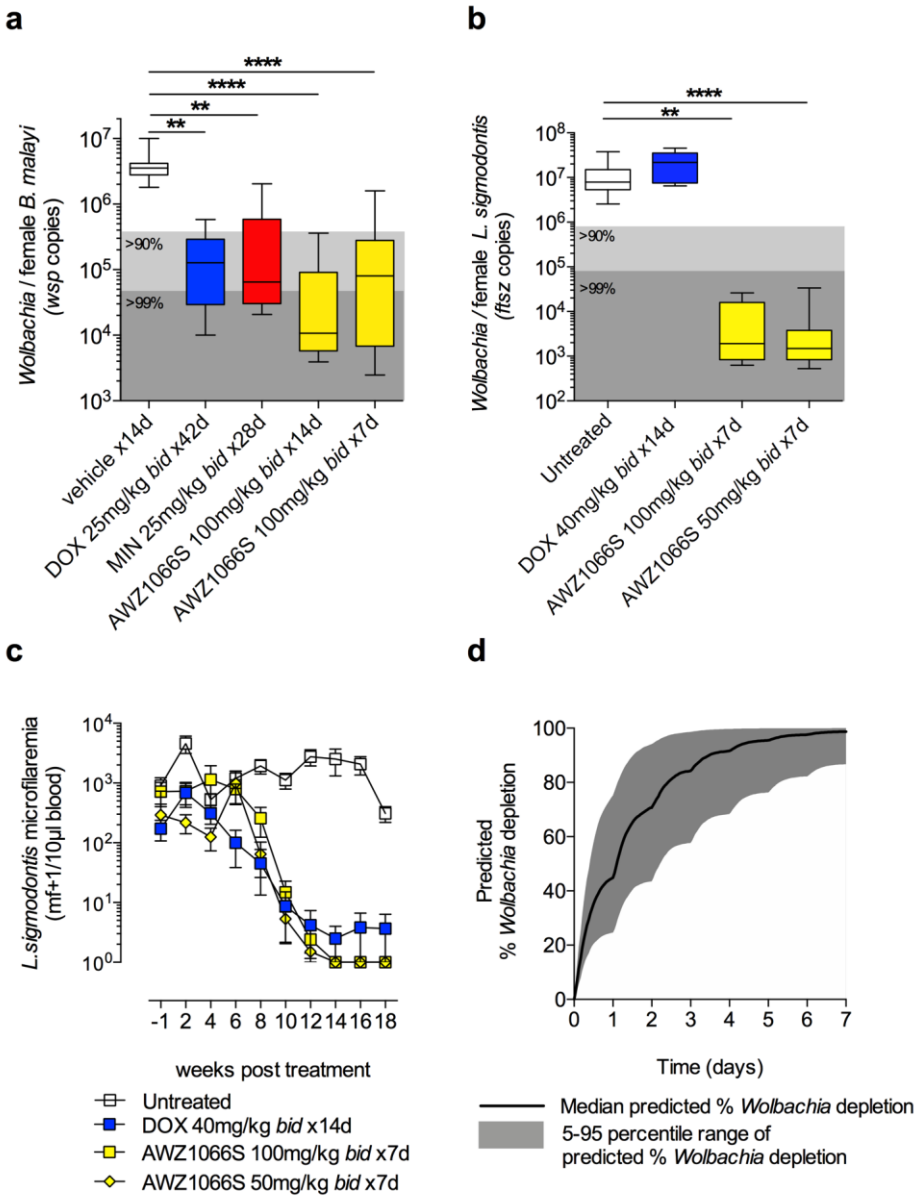

Figure 2 *In vivo* anti-*Wolbachia* efficacy of AWZ1066S. Anti-*Wolbachia* efficacy against female adult stage *B. malayi* in SCID mice (**a**) or *L. sigmodontis* in gerbils (**b**) after doxycycline (DOX) minocycline (MIN) or AWZ1066S oral treatments at indicated doses. Tail and whisker plots are min, 25th, median, 75th and max *Wolbachia wsp* or *ftsz* copy number/female worm in samples of 5-24 worms derived from groups of 4-9 animals from an individual experiment. Significant differences compared with vechicle/untreated are indicated ****P<0.0001, ***P<0.001, **P<0.01 (Kruskal-Wallis with Dunn's tests for inter-group *Wolbachia* variation). (**c**) Longitudinal effects on circulating *L. sigmodontis* microfilaremias -1

to 18 weeks after DOX or AWZ1066S oral treatments at indicated doses in gerbils. (**d**) Result of PK/PD montecarlo prediction of clinical activity of AWZ1066S when dosed at 600mg given predicted PK properties in man and established *in vitro* PD properties against *Wolbachia*.

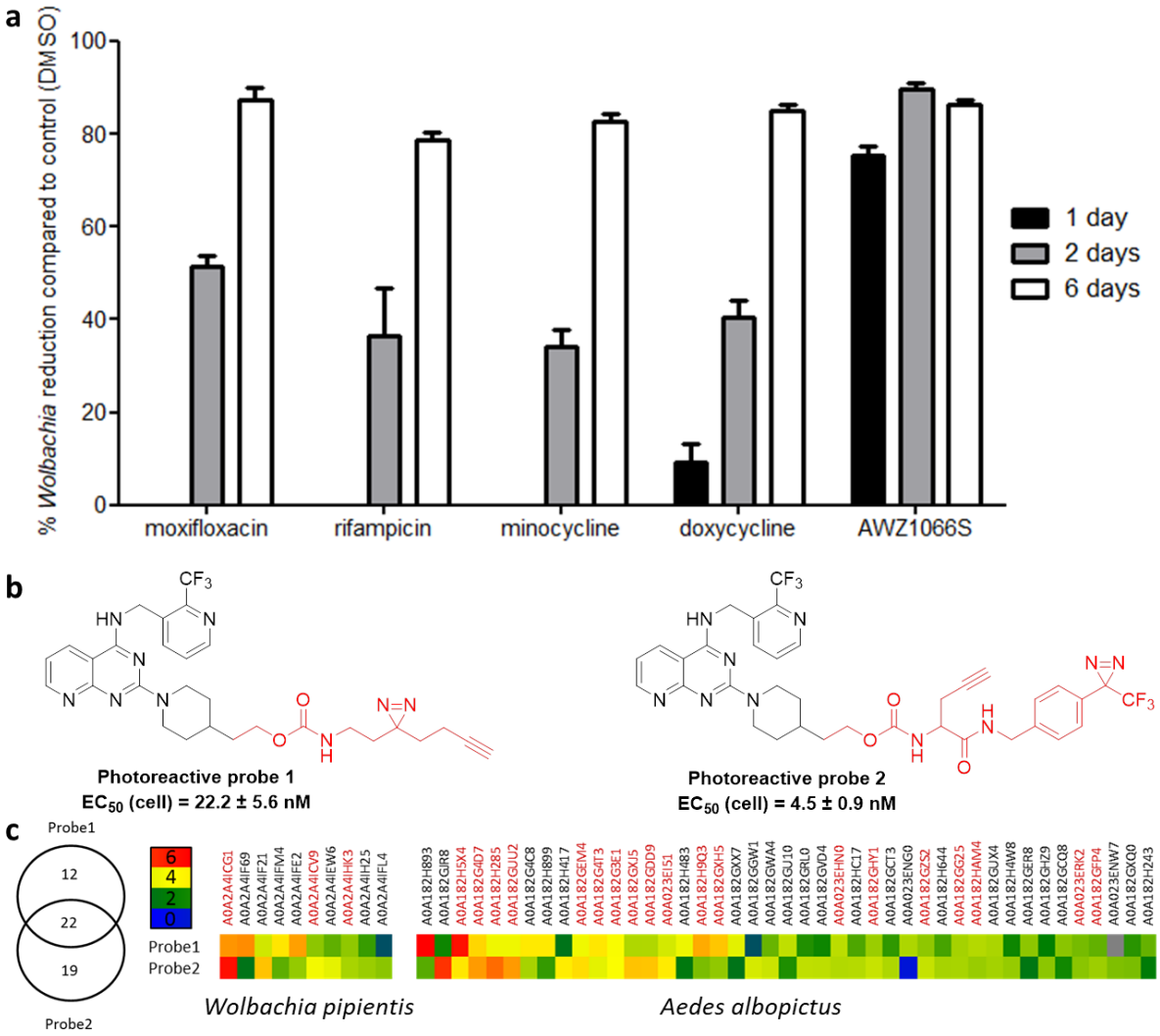

Figure 3 Unique mode-of-action of AWZ1066S was indicated by its fast kill-rate and was investigated through a proteomic target identification approach using two photoreactive chemical probes. (**a**) The *Wolbachia* depletion rates of AWZ1066S measured for three different treatment lengths (1, 2 and 6 days) in the *Brugia malayi* mf time-kill assay. Doxycycline, minocycline, moxifloxacin and rifampicin were used as positive controls (only doxycycline for day 1). Data are expressed as mean ± s.d. of five replicates for each time

point. (**b**) Two photoreactive chemical probes used for target identification of AWZ1066S in *Wolbachia*. Structures (the bifunctional, photoreactive and clickable, side-chains are highlighted in red) and their anti-*Wolbachia* activity in the cell assay (n = 3). (**c**) 53 Putative protein targets of photoreactive probes 1 and 2 assigned (as showed in the Venn diagram) based on statistical testing (right-sided t-test, permutation based FDR=0.001, S0=3) of LFQ intensities measured in samples derived from probe-treated cells and control, DMSO-treated cells (n = 3). Protein targets presented in the left-hand side block are from *Wolbachia pipientis* and in the right-hand side block are from *Aedes albopictus*. Numbers within the heatmap legend represent Log2 LFQ t-test differences. Protein IDs above the heatmap given in red indicate 22 proteins identified as common targets of both probes.

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Author contributions

W.D.H., M.P.H., A.P.R., E.W.T., F.G., M.J.T., J.D.T., J.H., S.A.W. and P.M.O. designed research; W.D.H., F.B., K.L.J., R.H.C., A.C., D.A.N.C., A.Siu, M.S., G.A., E.M. A.S., J.A., D.B., A.E., S.J.F., M.P.H., A.T.M.H., R.A.S. performed research; W.D.H., F.B., S.C.L., L.Q. contributed new reagents; W.D.H., G.L.N., L.F., K.L.J., R.H.C., P.J.H.W., S.K., M.P.H., D.B., A.H., A.P.R., E.W.T, N.G.B., F.G., J.D.T., M.J.T., S.A.W. and P.M.O. analysed data and W.D.H., J.D.T., M.J.T, J.H., S.A.W. and P.M.O. wrote the paper.

Data availability statement:

The authors declare that the data supporting the findings in this manuscript are available within the paper and its supplementary information files. The raw data related to the studies described in this manuscript are available from the corresponding author upon reasonable request.

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