Identification and prioritization of novel anti-Wolbachia chemotypes from screening a 10,000-compound diversity library

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Lymphatic filariasis and onchocerciasis are two important neglected tropical diseases (NTDs) that cause severe disability. Control efforts are hindered by the lack of a safe macrofilaricidal drug. Targeting the Wolbachia bacterial endosymbionts in these parasites with doxycycline leads to a macrofilaricidal outcome, but protracted treatment regimens and contraindications restrict its widespread implementation. The Anti-Wolbachia consortium aims to develop improved anti-Wolbachia drugs to overcome these barriers. We describe the first screening of a large, diverse compound library against Wolbachia. This whole-organism screen, streamlined to reduce bottlenecks, produced a hit rate of 0.5%. Chemoinformatic analysis of the top 50 hits led to the identification of six structurally diverse chemotypes, the disclosure of which could offer interesting avenues of investigation to other researchers active in this field. An example of hit-to-lead optimization is described to further demonstrate the potential of developing these high-quality hit series as safe, efficacious, and selective anti-Wolbachia macrofilaricides.

INTRODUCTION
The Wolbachia endosymbiont of filarial nematodes is a validated chemotherapeutic target for the treatment of lymphatic filariasis (1–7) and onchocerciasis (8–11). These debilitating neglected tropical diseases (NTDs) affect more than 150 million people, and although control programs have made great strides in reducing prevalence in endemic countries (12, 13), progress toward elimination is hampered by the lack of a safe drug capable of killing adult worms (14, 15). Targeting the Wolbachia bacteria within filarial nematodes using antibiotics offers the desired macrofilaricidal activity because the nematodes rely on their endosymbionts for their development, fecundity, and survival (16). Furthermore, an anti-Wolbachia mode of action provides an excellent safety profile due to the protracted death of adult worms. Such a mechanism has no effect on the Wolbachia-negative Loa loa parasites that can cause serious adverse events in some coinfected individuals treated with the standard anti-filarial drug ivermectin (17). Doxycycline has been proven effective as an anti-Wolbachia macrofilaricidal agent, but its long course of treatment (4 to 6 weeks) and contraindications in pregnancy and young children restrict its widespread use. The Anti-Wolbachia (A-WOL) consortium was formed with the aim of finding a new anti-Wolbachia treatment with a shorter treatment duration (ideally less than 7 days) and utility in pregnant women and children (18, 19).

The A-WOL consortium has a drug discovery and development pipeline that has been refined over time to optimize the translation of compounds from preclinical models to the clinic (18). The initial step in the pipeline was to screen compounds using a cell-based screen incorporating a Wolbachia-infected Aedes albopictus cell line [C6/36 (wAlbB)]. This validated screen was used to screen the complete human pharmacopoeia (20) and a variety of focused antimicrobial libraries (18). Although hits have been discovered from these screens, some of which have progressed further down the pipeline, the diversity in the chemical space covered thus far has been fairly limited. To explore greater chemical diversity, with the aim of driving the discovery of novel compounds with activity against Wolbachia, we chemoinformatically selected 10,000 compounds from the BioFocus SoftFocus small-molecule libraries. The rationale and successes of the SoftFocus screening approach have been described elsewhere (21), whereas hits from these libraries against malaria (22–28) and tuberculosis (29) have been identified and published previously. Here, we describe the first large diverse screen to be conducted against a validated chemotherapeutic target of filarial nematodes and report on six structurally diverse active chemotypes, one of which was optimized to provide a quality lead compound with a potential for further refinement toward a clinical candidate.

RESULTS
Assay development
To optimize our existing validated screening assay (20) for increasing throughput and capacity, we made a number of adaptations that are detailed in Supplementary Materials and Methods. Briefly, all aspects of the screening workflow were scrutinized in terms of scalability by conducting pilot experiments using a small selection of BioFocus compound plates. The main improvements made were to the cell toxicity readout and the quantitative polymerase chain reaction (qPCR) reagents, both of which reduced the screening time without affecting assay robustness. All amendments to the published protocol were validated using the analysis of Z and Z’ statistical parameters, and the subsequent primary screening of the library was completed in less than 1 year, an order of magnitude increase in our screening efficiency.

Hits from primary screen
The 10,000 compounds from the BioFocus SoftFocus libraries were screened against Wolbachia using the validated A-WOL cell-based assay (Fig. 1). We incubated C6/36 (wAlbB) cells with compounds for 9 days and performed qPCR on a Wolbachia single copy gene to analyze reductions in Wolbachia load. Doxycycline and vehicle [dimethyl sulfoxide
(DMSO)] were used as internal controls on each plate and direct cellular toxicity was assessed through the scoring of confluence using microscopy (Fig. 1). Hits were defined as those compounds that reduced the *Wolbachia* 16S copy number by 1 log or greater (≥1 log drop), without evidence of cytotoxicity to the *A. albopictus* cell line (a reduction in cell confluence of <50%, as scored by microscopy). These stringent criteria were implemented to best optimize our ability to triage hits for follow-up, given the size of the library and the throughput of our assay. Initial screening using a concentration of 2.5 mg/ml resulted in 174 primary hits (Fig. 2). Overall, the proportion of compounds producing direct toxicity to the cell line was 3.6%. Hits were rescreened at the same concentration, resulting in 112 reconfirmed hits (confirmation rate of 64%).

**Hit validation**

Of the 112 confirmed hits, 71 were available to buy as dry stocks and to be used in the validation process. As the original compound plates were provided as 10-mg/ml stocks, the final molar concentrations used in the primary screen fell within the range of 3.97 to 14.75 mM, with a mean of 6.65 μM and a median of 6.68 μM. We conducted the validation testing using a standard concentration of 5 μM on two separate occasions. Toxicity was quantified on replicate plates using the CellTiter-Glo assay. Hits were confirmed as those compounds that reduced the *Wolbachia* 16S copy number by 0.5 log or greater while maintaining a CellTiter-Glo readout of 70% or greater than the vehicle-treated control wells. In this way, 50 hits were identified and progressed into chemoinformatic analysis (Fig. 2).

**Chemoinformatic analysis**

Chemoinformatic techniques can assist the drug discovery process by viewing, organizing, and understanding relationships between measured biological data and molecular properties (30) to make quicker, more accurate decisions. Using these chemoinformatics techniques, the 50 validated hits were profiled using the parameters listed in table S1 to identify the optimal chemotypes to enter a hit-to-lead phase. An ideal chemotype chosen for hit-to-lead development would be potent, display some initial structure-activity relationship (SAR) and

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**Fig. 1. Primary screening workflow.** The graphics and text demonstrate a typical screening run involving a batch of four compound plates (216 compounds), with each plate representing one color. Compounds, as well as vehicle (DMSO) and doxycycline controls (shown as hatched areas), were used in triplicate. Following a 9-day incubation, plates were scored for cytotoxicity using microscopy, followed by cell lysis and DNA extraction. qPCR targeting the 16S rRNA gene of *Wolbachia* was conducted, and data were subjected to quality control using Z’ statistical analysis. *Wolbachia* 16S log reductions (“log drop”) were calculated per compound using in-plate DMSO controls, and those that were considered hits moved into confirmation screening. A more detailed description of this workflow is presented in Supplementary Materials and Methods.
ideally be lead/drug-like in pharmacokinetic, physiochemical, and structural parameters. Full details of the filtering procedure are included in Supplementary Materials and Methods.

**Cluster analysis**
Cluster analysis on the 50 hit compounds using molecular fingerprints (31) and their representation within chemical space using principal component analysis (PCA) (32) revealed six main clusters, with the other compounds being identified as singletons (Fig. 3). This clustering in chemical space was confirmed by inspection of the maximal common substructure of the clustered hits.

Following approaches from the analysis performed on the outputs from the antimalarial high-throughput screening completed by GlaxoSmithKline (33), we have used a procedure to assign a “selection score” to each compound, with the maximum score being 30. We aimed to identify the best compounds and series, and to this end, we focused on compound potency, lipophilicity, number of compounds in the cluster, and molecular weight (see Supplementary Materials and Methods for details on how the scores were assigned to each compound’s properties). This analysis revealed that the six clusters identified all contained multiple compounds above our score threshold of 15 (Fig. 3).

The chemically distinct clusters displayed an initial SAR (that is, they were not singletons) and had micromolar to nanomolar potency and acceptable lipophilicity with low molecular weights. The six hit series are summarized in Table 1 and include Thienopyrimidines.
Table 1. Cluster analysis of the 50 hits together with the core structures of the six clusters identified.

| Cluster no., name and generic structure | Number of active/inactive compounds | Average log drop of actives ± SD |
|----------------------------------------|------------------------------------|---------------------------------|
| 1. Thienopyrimidine                    | 11/143                             | 0.73 ± 0.41                     |
| ![Thienopyrimidine](image)             |                                    |                                 |
| 2. Imidazo[4,5-c]pyridine              | 5/202                              | 0.89 ± 0.40                     |
| ![Imidazo[4,5-c]pyridine](image)       |                                    |                                 |
| 3. Oxazepinone                         | 7/329                              | 0.96 ± 0.46                     |
| ![Oxazepinone](image)                  |                                    |                                 |
| 4. Imidazo[1,2-a]pyridine              | 6/849                              | 0.94 ± 0.35                     |
| ![Imidazo[1,2-a]pyridine](image)       |                                    |                                 |
| 5. Pyrrolopyridine                     | 14/371                             | 0.57 ± 0.29                     |
| ![Pyrrolopyridine](image)              |                                    |                                 |
| 6. Oxazole imidazolidinone             | 2/126                              | 1.50 ± 0.04                     |
| ![Oxazole imidazolidinone](image)      |                                    |                                 |

(series 1), Imidazo[4,5-c]pyridines (series 2), Oxazepinones (series 3), Imidazo[1,2-a]pyridines (series 4), Pyrrolopyridines (series 5), and Oxazole imidazolidinones (series 6). Pareto analysis was performed to identify compound clusters with the best overall profile (34), considering the compounds’ potency, in silico toxicity, and ADME (absorption, distribution, metabolism, and excretion) properties. Pareto optimization allows simultaneous optimization of many properties (for example, biological and physiochemical) through multiparameter optimization (35). This approach allows trade-offs between sometimes competing objectives to be made, resulting in a better compound profile overall. This approach is in contrast to historical drug discovery that focused on potency and led to many late-stage failures due to poor ADMET (absorption, distribution, metabolism, excretion, and toxicity) and/or physiochemical properties (36). Our analysis ranked all six series equally highly, giving us
confidence in the quality of the hit series for subsequent lead optimization (see Supplementary Materials and Methods).

During the screening and analysis of this compound set, we were in the process of developing a high-content imaging (Operetta, PerkinElmer) assay (37). This assay enables dose-response studies to be conducted and median effective concentration (EC50) values to be estimated (data not obtainable using the qPCR technique). We used this Operetta-based assay to titrate the activity of selected hits and generate EC50 values. The EC50 range produced by the six hit chemotypes was 112 to 5681 nM (Table 2). At the same time, some of the selected hits were also assessed for their drug metabolism and pharmacokinetic (DMPK) properties, including lipophilicity, aqueous solubility, and metabolic stability in vitro.

In the Thienopyrimidine series (series 1), representative compound 1 has good potency, low logD, and a molecular weight of 358. The metabolic stability is poor, but this could be rectified by the manipulation of metabolically susceptible groups within the starting point (dimethoxy ring system and benzylic linker). An additional attractive feature of this template is the modular nature of the chemistry for template syntheses, where simple S$_{Ar}$ reactions of 2,4-dichloro thienopyridine with suit

Table 2. Measured EC$_{50}$ value and DMPK properties for representatives from six identified hit clusters. MW, molecular weight.

| Chemical class/series name | Representative structure | BioFocus code | Measured EC$_{50}$ ± SD (nM) | LogD$_{7,4}$ | Solubility (µM)* | Human microsome clearance (µl/min/mg) | Rat hepatocytes clearance (µl/min/1×10$^6$ cells) | Selection score† |
|-----------------------------|--------------------------|---------------|-----------------------------|-------------|-----------------|--------------------------------------|-----------------------------------------------|-----------------|
| Thienopyrimidine (1)         | ![Thienopyrimidine](image) | 790_4020_0354 | 328 ± 55                    | 3.7         | 4.1             | 2                           | 2                             | 113             |
| Imidazo[4,5-c]pyridine (2)   | ![Imidazo[4,5-c]pyridine](image) | 729_2047_0052_0263 | 292.4 ± 12                 | 4.1         | 2               | 2                             | 2                             | 83              |
| Oxazepinone (3)              | ![Oxazepinone](image)     | 853_6001_0174  | 292.4 ± 12                 | 4.1         | 2               | 2                             | 2                             | 83              |
| Imidazo[1,2-a]pyrimidine (4) | ![Imidazo[1,2-a]pyrimidine](image) | 310_5632_5837_8158_0236 | 448.5 ± 75                 | 3.7         | 4.1             | 2                             | 2                             | 4.1             |
| Pyrrolopyridine (5)          | ![Pyrrolopyridine](image) | 809_2679_5837_0109 | 548 ± 564                  | 3.7         | 4.1             | 2                             | 2                             | 44             |
| Oxazole imidazolidinone (6)  | ![Oxazole imidazolidinone](image) | 894_7990_4021 | 5681 ± 756                 | 3.7         | 4.1             | 2                             | 2                             | 4.1             |

*Acceptable and desired ranges of measured parameters: EC$_{50}$ <1000 nM (acceptable) and <100 nM (desired); logD$_{7,4}$ <5 (acceptable) and <3 (desired); aqueous solubility, >20 µM (acceptable) and >50 µM (desired); human microsome and rat hepatocytes clearance, <70 µl min$^{-1}$ mg$^{-1}$ and <70 µl min$^{-1}$ per 1×10$^6$ cells (acceptable) and <20 µl min$^{-1}$ mg$^{-1}$ and <20 µl min$^{-1}$ per 1×10$^6$ cells (desired). †Note that selection score was based solely on potency, logP, the number of compounds in cluster, and molecular weight, as discussed in the main text.
strong electron-withdrawing group in the 4-position of the benzylic side chain, we could significantly improve the anti-Wolbachia activity and metabolic stability. **AWB124** was established as an early lead in the Thienopyrimidine series, with relatively low molecular weight, excellent potency, significantly improved DMPK properties, and good safety window (Fig. 4). Further optimization was focused on the trifluorophenyl ring side chain of **AWB124** and modification/truncation of the side chain at the 2-position of the thienopyrimidine ring. We found that the 2-position N-isopropylamine is not essential for anti-Wolbachia activity, and by conversion of the trifluorophenyl ring into the corresponding pyridyl ring system, the resulting molecule **AWG117** was obtained. **AWG117** expressed nanomolar anti-Wolbachia activity, had significantly lower lipophilicity (logD7.4 = 2.6) compared to **AWB124** (logD7.4 = 4.5), and had much improved microsomal stability and aqueous solubility. Cytotoxicity evaluation in THP-1 cells indicated low cytotoxicity with a very good in vitro therapeutic index. This lead compound is now primed for additional lead optimization toward a candidate molecule.

**DISCUSSION**

Here, we describe the first large-scale diversity screen against Wolbachia, a validated target of the filarial nematodes responsible for the NTDs lymphatic filariasis and onchocerciasis. We identified and validated 50 hits that could be clustered into six unique chemotypes with the potential for onward drug development. This is the first diversity screen ever performed against these NTDs and one that has already delivered chemical novelty and diversity into what is currently a relatively narrow and mechanistically restricted drug portfolio.

The drug discovery landscape for helminth diseases in general has been moribund for a long time, with most drugs for nematode infections of humans coming via the much less neglected veterinary market (38). There is an urgent need for an effective macrofilaricide to treat lymphatic filariasis and onchocerciasis, and proof-of-concept clinical trials (1–11) have demonstrated that targeting Wolbachia provides this action with an excellent safety profile. The success of the A-WOL approach has been validated because compounds discovered as active in the primary cell-based screen (20) have progressed through the A-WOL screening funnel and a selection (including minocycline, rifampicin, and the tylosin analog TylAMac) have been either tested in clinical trials (39, 40) or selected for clinical candidate development (www.dndi.org/diseases-projects/portfolio/macro-filaricide-2). These repurposed anti-infective compounds were sourced either from currently registered drugs on the market (20) or from focused anti-infective libraries provided by pharmaceutical companies working on drug discovery programs for other pathogens (18, 19). Therefore, the six structurally diverse chemotypes described here represent the first foray into the unknown for A-WOL drug discovery and, hence, offer exciting avenues of drug discovery research.

We have conducted the first large-scale diverse screening campaign against Wolbachia and validated 50 hits. A range of molecular properties were assessed to see whether they correlated with delivering the one log drop in bacterial load desired of a hit compound. Analysis of molecular weight, CLogP, number of rotatable bonds, number of hydrogen bond donors and acceptors, and polar surface area for both the 9950 inactive molecules and the 50 validated hits demonstrated that there is no clear trend for any of these molecular parameters in determining biological activity (fig. S1), thereby underscoring the benefits of examining diverse compound sets, with a broad range of molecular characteristics, against phenotype in the search for novel active compounds.

Through chemoinformatic and cluster analysis, six novel chemotypes were identified and assessed for various drug-related properties in silico. In addition, the most potent representatives determined by
their measured EC_{50} values from each series were also assessed for their DMPK properties in vitro to evaluate their suitability for further development in the pipeline. We have demonstrated the tractability of one of the series by performing hit-to-lead optimization through the use of well-considered medicinal chemistry optimization strategies focused on enhancing metabolic stability.

Although the top hits exemplified in Table 2 are not presented in any previously published literature, the core of five series (series 1 to 5) belongs to a group of privileged pharmacophores that can target multiple biologically significant proteins, which are relevant to a range of diseases. Thiienopyrimidines (series 1), imidazo[4,5-c]pyridines (series 2), oxazepinones (series 3), and pyrrolopyridines (series 5) have been identified as inhibitors of a range of kinases (41–43) or modulators of various heterotrimeric guanine nucleotide–binding protein–coupled receptor (GPCR) proteins (44, 45) in the literature. In addition, noticebly, thiienopyrimidines (series 1) have been reported to have antimalarial activities (46), and the oxazepinone core in series 3 and the imidazo[1,2-a]pyridine core in series 4 have appeared in the experimental drugs TAK-475 (squalene synthase inhibitor) (47) and Q203 (antituberculosis agent) (48), respectively. However, the substituent patterns of the hit series identified in this campaign are distinctly different from any known molecules, and we do not speculate that the protein targets of these anti-Wolbachia hits are related to any of those reported in the literature. Oxazole imidazolidinones (series 6) have very limited representation in the literature and can be considered as a completely novel chemotype for anti-infective activity.

All the available evidence indicates that the six novel chemotypes presented here have great potential to be developed further as anti-Wolbachia macrofiliarial drugs for the treatment of lymphatic filariasis and onchocerciasis. The disclosure of these starting points should also enable other researchers active in this field to initiate their own medicinal chemistry optimization programs akin to those seen in the malaria and tuberculosis drug discovery fields (49, 50).

**MATERIALS AND METHODS**

**Compounds**

The collection of 10,000 compounds was provided by BioFocus. Although most of the compounds present were derived from the SoftFocus libraries, some came from ThemePair (approximately 900), FieldFocus (approximately 1000), and other (approximately 1200) libraries. Overall, the collection encompassed kinase, ion channel, GPCR, protease, protein–protein interaction, helix mimetic, and nucleoside-targeted libraries. Compounds were provided as DMSO stocks in 96-well compound storage plates at a concentration of 10 mg mL^{-1} and were stored at −20°C until use. Dry stocks of hits were purchased from eMolecules.

The synthesis of AWB124 and AWG117 are described in Supplementary Material and Methods and schemes S1 and S2, and the chemical characterization of both compounds is listed below. N^{4}-(2-(trifluoromethyl)benzyl)thieno[3,2-d]pyrimidine-2,4-diamine (AWB124): ¹H nuclear magnetic resonance (NMR) (400 MHz, CDCl₃) δ [parts per million (ppm)] 7.68 (d, J = 7.8 Hz, 1H), 7.61 (d, J = 7.8 Hz, 1H), 7.55 (d, J = 5.3 Hz, 1H), 7.50 (t, J = 7.6 Hz, 1H), 7.38 (t, J = 7.6 Hz, 1H), 7.11 (d, J = 5.3 Hz, 1H), 5.09 (br. s, 1H), 5.01 (d, J = 5.7 Hz, 2H), 4.79 (br. s, 1H), 4.20 to 4.06 (m, 1H), 1.18 (d, J = 6.5 Hz, 6H). ¹³C NMR (101 MHz, CDCl₃) δ (ppm) 160.87, 157.68, 137.84, 132.58, 131.40, 131.02, 130.02, 128.52, 127.75, 126.39, 124.96, 124.29, 106.24, 43.39, 41.42, 23.44. High-resolution mass spectrometry (HRMS) (CI)

C_{17}H_{18}F_{3}N_{4}S [M+H]^{+} 367.1196. For elemental (CHN) analysis, C_{17}H_{17}F_{3}N_{4}S required the following: C, 55.73%; H, 4.68%; N, 15.29%; found C, 55.64%; H, 4.71%; N, 15.33%.

N-(2-(trifluoromethyl)pyridin-3-yl)methylthieno[3,2-d]pyrimidine-4-amine (AWG117): ¹H NMR (400 MHz, CDCl₃) δ (ppm) 8.58 (s, 1H), 8.54 (d, J = 4.3 Hz, 1H), 7.99 (d, J = 7.9 Hz, 1H), 7.68 (d, J = 5.4 Hz, 1H), 7.39 (dd, J = 10.1, 5.0 Hz, 2H), 5.41 (d, J = 5.2 Hz, 1H), 5.05 (d, J = 6.1 Hz, 2H). ¹³C NMR (101 MHz, CDCl₃) δ (ppm) 160.21, 159.91, 156.94, 154.91, 147.89, 138.88, 133.22, 131.37, 126.64, 125.53, 123.96, 115.26, 40.17. HRMS (ES+) C_{130}H_{5}_{3}N_{4}S[M+H]^{+} 311.0575. For elemental (CHN) analysis, C_{130}H_{5}_{3}N_{4}S required the following: C, 50.32%; H, 2.92%; N 18.06%; found C, 50.41%; H, 2.88%; N, 18.10%.

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

An A. albopictus cell line C6/36 (American Type Culture Collection number CRL-1660) stably infected with Wolbachia pipientis wAlbB [C6/36 (wAlbB)] was routinely cultured in Leibovitz’s L-15 medium containing 2 mM l-glutamine, 1% nonessential amino acids, 2% tryptose phosphate broth (Sigma-Aldrich), and 5% heat-inactivated fetal calf serum (Cambrex Bio Science) at 26°C (51). A C6/36 (wAlbB) cell-based assay developed to screen drugs/compounds active against Wolbachia in vitro was used as previously described (52), with a few modifications. Briefly, 24 hours before use, compound plates were thawed for at least 2 hours at room temperature, with rocking. Plates were centrifuged briefly (less than 30 s) to remove drops from the underside of the seal. Daughter plates were prepared from the master plates by transferring 10 μL into new plates, and these were stored at 4°C until the following day. C6/36 (wAlbB) cells, subcultured 24 hours previously, were seeded at 10,000 cells per well in 96-well flat-bottom sterile culture plates. Compounds from the daughter plates were diluted to the appropriate working concentration in culture medium and added to test wells, and cells were cultured in a total volume of 200 μL at 26°C for 9 days. The final concentration of each compound used was 2.5 μg mL⁻¹. Vehicle (DMSO)–treated medium and doxycycline at a concentration of 8 μM were used as negative and positive controls, respectively, and were included on each test plate. Compounds and controls were added in triplicate within plates (that is, compounds from one plate were spread over three reciprocal cell plates, with 18 compounds per plate). At the end of the screening assay, cytotoxicity was scored using microscopy (medium toxicity was determined as approximately 50% or greater reduction in cell confluence in comparison to the DMSO control, and high toxicity was determined as approximately 90% or greater reduction in confluence), and samples were collected by washing adherent cells once in sterile Dulbecco’s phosphate-buffered saline (Sigma-Aldrich) and adding 150 μL of Wizard SV Lysis Buffer (Promega) for genomic DNA (gDNA) extraction.

**DNA isolation and qPCR**

gDNA 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 of water. Quantification of the ribosomal gene W. pipientis 16S was performed as previously described (53), with modifications. Briefly, qPCR was performed on a C1000 thermocycler with a CFX384 Real-Time System (Bio-Rad Laboratories Ltd) under the following conditions: 95°C for 3 min, 40 cycles of 95°C for 10 s, and 55°C for 30 s. qPCRs were performed in 10 μL of SsoFast EvaGreen (Bio-Rad) reactions containing 2 μL of gDNA, 0.4 μM concentration of each primer in 1× SsoFast EvaGreen Supermix (Bio-Rad). Quantification was calculated by reference to a linear standard curve
of log_{10}-diluted (5 × 10^6 to 5 × 10^0) full-length amplicons synthesized as single-stranded oligonucleotides (Sigma-Genosys).

**Quality control**

The quality of individual assays was assessed by calculating Z and Z' statistical parameters using the data generated by control wells (54). Data from assays that did not achieve Z' factors of 0.4 or above were rejected, and the compounds were retested. A typical primary screening run is shown in Fig. 1 and described in further detail in Supplementary Materials and Methods.

**Hit confirmation**

Compounds that reduced the *Wolbachia* 16S copy number by 1 log or greater were cherry-picked from the daughter plates and tested again to confirm the activity. Those that again achieved a log reduction in *Wolbachia* 16S copy numbers of 1 log or greater were considered confirmed hits.

**Validation with dry stocks**

Compounds were purchased from eMolecules and dissolved in DMSO before use. Working dilutions were prepared using appropriate molar concentrations, and cell-based assays, with a qPCR readout, were conducted using the methods described above. Each compound was tested at a standard concentration of 5 μM. Duplicate plates were prepared and used to quantify cytotoxicity in a 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 CellTiter-Glo luminescence readout against the vehicle-control wells.

**Chemoinformatics**

Compounds were provided as SMILES (simplified molecular-input line-entry system) strings, and ADMET predictions were made using Chemoinformatics scheme S1. Synthetic route for AWB124.

**Supplementary materials**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/9/eaa01551/DC1

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Acknowledgments: We want to thank the DMPK group (led by P. Webborn) in AstraZeneca UK for providing the in vitro measurement of DMPK properties, including logD7.4, aqueous solubility, human plasma protein binding, mouse microsome clearance, and rat hepatocyte clearance of hit and lead molecules. Funding: The A·WOL consortium is supported by a grant from the Bill and Melinda Gates Foundation awarded to the Liverpool School of Tropical Medicine (grant numbers 39284 and OPP1054324). Author contributions: KLJ, D.A.N.C., N.G.B., W.D.H., P.M.O., S.A.W., and M.J.T. wrote the manuscript. N.G.B., P.M.O., S.A.W., and M.J.T. conceived the work. KLJ and D.A.N.C. performed the assay development. R.H.C., M.G., D.A.N.C., K.L.J., and L.F. performed or directed the in vitro screening work. N.G.B. performed the chemoinformatic analysis. W.D.H. and G.L.N. performed or directed the hit-lead chemistry work. Competing interests: Some compounds described in this manuscript, namely AWB124 and AWG117, are included in a UK patent application filed by the Liverpool School of Tropical Medicine and the University of Liverpool (application no. 1700814.5, filed 17 January 2017). The authors declare that they have no other competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Citation: K. L. Johnston, D. A. N. Cook, N. G. Berry, W. D. Hong, R. H. Clare, M. Goddard, L. Ford, G. L. Nixon, P. M. O'Neill, S. A. Ward, M. J. Taylor, Identification and prioritization of novel anti-Wolbachia chemotypes from screening a 10,000-compound diversity library. Sci. Adv. 3, eaao1551 (2017).