PARASITIC DISEASES

Preclinical development of an oral anti-Wolbachia macrolide drug for the treatment of lymphatic filariasis and onchocerciasis

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There is an urgent global need for a safe macrofilaricidal drug to accelerate elimination of the neglected tropical diseases onchocerciasis and lymphatic filariasis. From an anti-infective compound library, the macroline veterinary antibiotic, tylosin A, was identified as a hit against Wolbachia. This bacterial endosymbiont is required for filarial worm viability and fertility and is a validated target for macrofilaricidal drugs. Medicinal chemistry was undertaken to develop tylosin A analogs with improved oral bioavailability. Two analogs, A-1535469 and A-1535469, were selected. Their efficacy was tested against the gold-standard second-generation tetracycline antibiotics, doxycycline and minocycline, in mouse and gerbil infection models of lymphatic filariasis (Brugia malayi and Litomosoides sigmodontis) and onchocerciasis (Onchocerca ochengi). A 1- or 2-week course of oral A-1535469 or A-1574083 provided >90% Wolbachia depletion from nematodes in infected animals, resulting in a block in embryogenesis and depletion of microfilarial worm loads. The two analogs delivered comparative or superior efficacy compared to a 3- to 4-week course of doxycycline or minocycline. A-1574083 (now called ABBV-4083) was selected for further preclinical testing. Cardiovascular studies in dogs and toxicology studies in rats and dogs revealed no adverse effects at doses (50 mg/kg) that achieved plasma concentrations >10-fold above the efficacious concentration. A-1574083 (ABBV-4083) shows potential as an anti-Wolbachia macrolide with an efficacy, pharmacology, and safety profile that is compatible with a short-term oral drug course for treating lymphatic filariasis and onchocerciasis.

INTRODUCTION

Control programs for neglected tropical diseases caused by the filarial parasites responsible for lymphatic filariasis and onchocerciasis have been operating for more than 40 years, with notable successes in the Americas and West Africa (1). Nevertheless, it is recognized that the transition from control to elimination will require alternative strategies and approaches (1, 2). In particular, global efforts to eliminate filarial diseases are hampered by the lack of a drug that can kill the adult nematode, relying instead on multiple rounds of mass drug administration with agents that target the transmission of microfilariae (the larval stages found in either blood or skin) (2, 3). The success of existing strategies in Central Africa is also impeded by the risk of severe adverse events in areas coendemic for infection with the related filarial nematode, Loa loa (2, 3).

The anti-Wolbachia (A-WOL) consortium was formed to exploit a unique aspect of filarial parasite biology, which is their reliance on the presence of the bacterial endosymbiont Wolbachia for fertility and viability (4). Clinical studies using doxycycline have demonstrated that depletion of Wolbachia within filarial parasites arrests embryogenesis, prevents larval development, clears microfilarial stages from blood or skin, mediates transmission blockade and prevention of onchocerciasis pathology, and leads to a slow but innocuous death of adult parasites over 12 to 24 months (3, 5, 6). However, doxycycline is contraindicated in children and pregnant women and requires 4 to 6 weeks of daily therapy to sufficiently deplete the Wolbachia population to produce a macrofilaricidal effect. Therefore, agents with more favorable safety profiles and shorter duration of therapy are needed (4). To date, more than 2 million compounds have been screened for anti-Wolbachia activity in an insect cell line (7, 8).

RESULTS

Here, we screened an anti-infective compound library and identified the 16-membered macrolide antibiotic tylosin A (Fig. 1) as a potent hit. This drug had an in vitro effective concentration providing 50% effect (EC50) of 30 nM against Wolbachia in the C6/36 (wAlbB)-infected insect cell line and 66 nM against Wolbachia in microfilariae of Brugia malayi (Table 1). Tylosin A, the principal component of a widely used veterinary antibiotic produced by fermentation, was the only macrolide in the screening set to demonstrate anti-Wolbachia activity; other commercially available 16-membered macrolides were devoid of activity (8). We tested the efficacy of tylosin A in two mouse models of lymphatic
Selective activation of the mycarosyl sugar was accomplished through formation of a cyclic tin complex; this was reacted with diethylcarbamoyl chloride to give compound 3 \([R=\text{C}(\text{O})\text{NET}_2]\) or with 4-fluorobenzylic chloride to give compound \(3\) \([R=\text{Bn}(4\text{-F})]\). Warming in methanol liberated the 2-ester protective group to provide A-1535469 \([\text{compound} \ 4, \ R=\text{C}(\text{O})\text{NET}_2]\) or A-1574083 \([\text{compound} \ 4, \ R=\text{Bn}(4\text{-F})]\). Both A-1535469 and A-1574083 were tested for efficacy against the bacterial endosymbiont Wolbachia both in insect cells and microfilariae in vitro and in worms infecting mice and gerbils in vivo.

Table 1. Anti-Wolbachia activity of tylosin A analogs in vitro.

<table>
<thead>
<tr>
<th></th>
<th>Anti-Wolbachia activity EC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insect cells</td>
</tr>
<tr>
<td>A-1535469</td>
<td>1.3</td>
</tr>
<tr>
<td>A-1574083</td>
<td>0.02</td>
</tr>
<tr>
<td>Tylosin A</td>
<td>30</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>20</td>
</tr>
</tbody>
</table>

Fig. 1. Synthesis of tylosin A analogs. Tylosin A (the parent compound 1) was first protected on the reactive 2'-hydroxyl group through acetylation to give compound 2. Selective activation of the mycarosyl sugar was accomplished through formation of a cyclic tin complex; this was reacted with diethylcarbamoyl chloride to give compound 3 \([R=\text{C}(\text{O})\text{NET}_2]\) or with 4-fluorobenzylic chloride to give compound 3 \([R=\text{Bn}(4\text{-F})]\). Warming in methanol liberated the 2'-ester protective group to provide A-1535469 \([\text{compound} \ 4, \ R=\text{C}(\text{O})\text{NET}_2]\) or A-1574083 \([\text{compound} \ 4, \ R=\text{Bn}(4\text{-F})]\). Both A-1535469 and A-1574083 were tested for efficacy against the bacterial endosymbiont Wolbachia both in insect cells and microfilariae in vitro and in worms infecting mice and gerbils in vivo.

Filariasis (9, 10). In the first model, interleukin-4 receptor alpha-deficient (IL-4Rα−/−) BALB/c immunodeficient mice were infected with third-stage larvae (L3) of B. malayi, a human lymphatic filarial pathogen (9). In the second model, BALB/c mice were infected with L3 larvae of Litomosoides sigmodontis, which naturally infects cotton rats (10). In the B. malayi model, infectious stage larvae were isolated from mosquitoes and, after inoculation into the immunodeficient mice, were allowed to develop into adults that produced microfilarial stages contained within the peritoneal cavity of the host animals. In the L. sigmodontis natural infection model, larvae migrated to the pleural cavity of mice via the lymphatics, heart, and lungs and became microfilariae-producing adults. Worm infections were established by exposing BALB/c mice to blood feeding by L. sigmodontis–infected Ornithonyssus bacoti mites. In both animal models, drugs were delivered orally or parenterally (via intraperitoneal injection) immediately after infection, thus targeting larval developmental stages. In initial screening, the impact on the Wolbachia load was assessed at either 2 (B. malayi) or 5 weeks (L. sigmodontis) after infection, corresponding to the mid-L4 larval stage or early adult stage, respectively. In both animal models, anti-Wolbachia efficacy was demonstrated when tylosin A was delivered parenterally but not orally (Fig. 2A and fig. S1). Pharmacokinetic (PK) studies in mice confirmed that the lack of anti-Wolbachia activity of oral tylosin A was the result of poor oral bioavailability (table S1). Worm burdens in the mice were not affected by either tylosin analogs or doxycycline after 2 weeks of treatment (table S2).

We hypothesized that low intestinal permeability limited the oral bioavailability of tylosin A due to its extensive network of hydrogen-bond donors, specifically the five free hydroxyl groups in the molecule. To address this challenge, we focused medicinal chemistry efforts primarily on the derivatization of these hydroxyl moieties, recognizing that such modifications might compromise activity against Wolbachia. From studies on ~150 new tylosin analog macrofilaricide (TylAMac) compounds, we determined that derivatives of the 4'-OH (on the mycarose sugar) provided a dual benefit of improved bioavailability and enhanced potency against Wolbachia. Table 1 shows the in vitro anti-Wolbachia potency (EC50 values in insect cells and cultured B. malayi microfilariae) for two extensively characterized semisynthetic lead compounds (A-1535469 and A-1574083) compared to tylosin A and doxycycline. The increased potency exhibited by these TylAMac analogs appeared to be specific for Wolbachia. Their spectrum of activity across a panel of other common bacterial species was similar to that of tylosin A (table S3) and erythromycin. The efficacy of A-1535469 and A-1574083 against B. malayi developing larvae (L3 to L4 stage) in susceptible IL-4Rα−/− BALB/c immunodeficient mice was compared to that of tylosin A and a human equivalent dose of doxycycline (fig. S2) (9, 11). Fourteen-day oral dosing with A-1535469 at ≥25 mg/kg delivered superior efficacy versus doxycycline (50 mg/kg) [Kruskal–Wallis one-way analysis of variance (ANOVA) statistic = 90.7, \(P < 0.0001\); Fig. 2A]. Fourteen-day oral dosing with A-1535469 resulted in an anti-Wolbachia efficacy equivalent to that achieved with 14 daily injections of tylosin A (50 mg/kg) (98.6% versus >99.9%, respectively; Fig. 2A). When halving the dose duration to 7 days, A-1535469 (≥25 mg/kg) provided superior activity versus 14 days of oral doxycycline (A-1535469 >99.7% median reduction in Wolbachia versus 98.1% for doxycycline; Fig. 2A). Wolbachia depletion
**Fig. 2. In vivo efficacy of two oral tylosin A analogs against Wolbachia in several animal models of nematode infection.** The efficacy of two tylosin A analogs (A-1535469 and A-1574083) against Wolbachia in mice infected with *B. malayi*, *L. sigmodontis*, or *O. ochengi* is shown. Efficacy of the two drugs was tested against Wolbachia in (A) *B. malayi* L3/L4 larvae infecting IL-4Rα and A-1574083) against in several animal models of nematode infection. Wolbachia

The efficacy of two tylosin A analogs (A-1535469, A-1574083) against in several animal models of nematode infection. Wolbachia
Our aim was to develop an anti-*Wolbachia* compound that could be given as an oral short-course treatment effective against adult lymphatic worms and *Onchocerca* filarial worms. To that end, A-1535469 and A-1574083 were tested in several in vivo mouse and gerbil models infected with adult-stage *B. malayi* or *L. sigmodontis* or implanted with male *Onchocerca ochengi* (Fig. 2, B and F, and fig. S2) (9-11). Both C-C chemokine receptor 3 (CCR3)–deficient (CCR3−/−) mice or severe combined immunodeficient (SCID) mice were used, in which chronic infections of *B. malayi* adult worms producing microfilariae could be maintained (9, 11).

Gerbil models were used in addition to mouse models for testing A-1574083 due to improved PKs in gerbils, which enabled higher in vivo plasma concentrations of drug to be obtained. When targeting prefecund *B. malayi* adult worms in vivo with A-1535469 in the CCR3−/− BALB/c mouse infection model (Fig. 2B), all oral treatments given daily (50 to 250 mg/kg) for 14 days provided efficacy that was comparable to a 4-week bioequivalent human dose of minocycline (12) at 25 mg/kg given twice daily. Minocycline was used as a more potent tetracycline antibiotic because it was shown to be superior to doxycycline in depleting *Wolbachia* from female *Onchocerca volvulus* in a pilot clinical study of infected individuals (13). Treatment of CCR3−/− immunodeficient mice with A-1535469 (250 mg/kg) for 7 days provided 98.3% median reduction in *Wolbachia* in adult female *B. malayi* worms (compared to 91.4% after 28 days of minocycline treatment). This threshold indicated the potential for curative efficacy for lymphatic filariasis in humans (14, 15). Treatment with A-1574083 (50 mg/kg) for 14 days in a gerbil model of *B. malayi* infection showed targeting of the fecund stage of *B. malayi* worms. This treatment regimen resulted in a 99.6% median reduction in *Wolbachia* and was superior to a 21-day (100 mg/kg) dose of doxycycline (98.5% median reduction with doxycycline; Fig. 2C). Administration of a 14-day reduced (10 mg/kg) dose of A-1574083 also resulted in >99% depletion of *Wolbachia* that was comparable to that of a 21-day doxycycline regimen (Fig. 2C). In a parallel study of *L. sigmodontis* infection in a gerbil model, dosing was delayed until after the onset of patency (i.e., when circulating microfilariae were detectable in peripheral blood). A dose of A-1574083 (100 mg/kg) given for 14 days resulted in 99.7% mean reduction in *Wolbachia* compared to 0% median reduction in *Wolbachia* after doxycycline treatment (Fig. 2D). The drug treatments did not affect adult worm burdens in these two gerbil models (tables S4 to S6).

Because of the high efficacy achieved in mouse and gerbil models of lymphatic filariasis infection, we next investigated whether A-1535469 and A-1574083 showed anti-*Wolbachia* activity in mouse and gerbil models of *Onchocerca* infection. Preclinical models of onchocerciasis...
were generated by implanting adult male *O. ochengi* parasites isolated from naturally infected cattle (11, 16) into immunodeficient mice or outbred gerbils. Both A-1535469 (250 mg/kg) and A-1574083 (75 mg/kg) given for 14 days mediated a 99.4 and 98.8% median reduction in *Wolbachia*, respectively, in immunodeficient mice implanted with *Onchocerca* (Fig. 2E). This was comparable to the efficacy of a 28-day course of minocycline (25 mg/kg), resulting in a 99.2% median reduction in *Wolbachia* (Fig. 2E). When *Wolbachia*-infected gerbils were dosed at 150 mg/kg of A-1574083 for 7 days, there was a 95.7% depletion in *Wolbachia* compared to 98.0% for doxycycline (Fig. 2F). *Onchocerca* male worm burdens were not affected by drug treatments (tables S7 and S8).

We next assessed whether depletion of *Wolbachia* by A-1535469 or A-1574083 also resulted in reduced production of microfilariae in *B. malayi* and *L. sigmodontis* infection models (Fig. 3). Dosing experiments were undertaken in immunodeficient mice or gerbils infected with *B. malayi* or *L. sigmodontis*, with washout periods of up to 22 weeks after dosing to measure the impact of these two tylosin A analogs on parasite fecundity (fig. S2). In immunodeficient mice with fecund *B. malayi* infection, released microfilariae accrued in the peritoneal cavity and typically reached a steady-state burden of \( \geq 10^5 \) per animal. Twenty-four weeks after a 14-day dosing regimen with A-1535469 (250 mg/kg), there was no difference in adult worm burden compared with vehicle control (table S9). However, 24 weeks after treatment, four of nine mice showed no peritoneal microfilariae burden compared with vehicle control (table S9).

**Table 2. Plasma concentrations of tylosin A analogs after single oral dosing in mice and gerbils.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Species</th>
<th>Dose (mg/kg)</th>
<th>( \text{AUC} )ss (ng∙hr/ml) (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1535469</td>
<td>BALB/c mice</td>
<td>30</td>
<td>7800 (1620)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>36100 (2910)</td>
</tr>
<tr>
<td>A-1574083</td>
<td>BALB/c mice</td>
<td>25</td>
<td>17.9 (0.85)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>271 (23.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75</td>
<td>2153 (1089)</td>
</tr>
<tr>
<td>Gerbils</td>
<td></td>
<td>8.92</td>
<td>60.9 (15.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47</td>
<td>3646 (619)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>82.2</td>
<td>5169 (478)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>149</td>
<td>9200 (276)</td>
</tr>
</tbody>
</table>

**Table 3. Activity of A-1574083 (ABBV-4083) in preclinical safety assays.**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride channel (GABA-gated) (agonist radioligand)</td>
<td>( K_i ) 7.5 ( \mu )M</td>
</tr>
<tr>
<td>NK1 (human) (agonist radioligand)</td>
<td>( K_i ) 0.1 ( \mu )M</td>
</tr>
<tr>
<td>NK1 (human) (agonist effect)</td>
<td>( K_i ) 0.46 ( \mu )M</td>
</tr>
<tr>
<td>Motilin (human) (agonist radioligand)</td>
<td>( IC_{50} ) &gt; 100 ( \mu )M</td>
</tr>
<tr>
<td>ERG (human) (QPatch, human embryonic kidney--293 cells)</td>
<td>( IC_{50} ) &gt; 28 ( \mu )M</td>
</tr>
<tr>
<td>MiniAmes test (Salmonella TA98/TA100)</td>
<td>Negative</td>
</tr>
<tr>
<td>In vitro micronucleus test (V79 hamster)</td>
<td>Negative</td>
</tr>
<tr>
<td>Anesthetized dog cardiovascular effects (intravenous infusion)</td>
<td>Minimal effects* at 12.4 ( \mu )g/ml</td>
</tr>
<tr>
<td>28-day toxicology study in Sprague-Dawley rats</td>
<td>NOAEL: 300 mg/kg per day</td>
</tr>
<tr>
<td>28-day toxicology study in beagle dogs</td>
<td>Plasma ( \text{AUC} )ss 7.4 ( \mu )g∙hour/ml</td>
</tr>
</tbody>
</table>

*Monitored characteristics: mean arterial pressure, heart rate, systemic vascular resistance, cardiac output, cardiac contractility, QT interval corrected using Van de Water formula, and interval from P to R on electrocardiogram.

All gerbils showed peritoneal microfilariae (Fig. 3B), and the median peritoneal microfilarial burden (4.2 \( \times \) 10^5) was similar to that of animals given vehicle control (10.9 \( \times \) 10^5). After a further 6 weeks (12 weeks after treatment), peritoneal microfilarial burdens were sustained in the vehicle group with six of six gerbils, showing a median microfilarial burden of 0.15 \( \times \) 10^5. No microfilarial burden was detected in lavages of infected gerbils dosed with A-1574083 (Fig. 3B).

Patent *L. sigmodontis* infection in gerbils seeds the blood with microfilariae, hence establishing persistent microfilaraemia akin to that of patients infected with lymphatic filariasis. We undertook a longitudinal assessment of *L. sigmodontis* microfilaraemia after dosing with A-1574083 (100 mg/kg once daily) or doxycycline (40 mg/kg twice daily) for 14 days. Baseline microfilaraemias were similar between gerbils treated with A-1574083, doxycycline, or untreated controls (Fig. 3C). At 10 weeks after treatment, *L. sigmodontis* microfilaraemia declined in gerbils treated with A-1574083 or doxycycline compared to untreated animals. However, in A-1574083–treated gerbils, the microfilaraemia continued to decline from 11 to 16 weeks after treatment, and the gerbils showed no microfilariae in the blood at week 15 after dosing (Fig. 3C). The effect of A-1574083 on circulating microfilariae was superior to that of doxycycline over the same time period (Fig. 3C) and correlated with the improved clearance of *Wolbachia* by A-1574083 (Fig. 2D). At 16 weeks after dosing, a similar number of adult *L. sigmodontis* worms were retrieved across all groups (table S6). Microscopic evaluation after homogenization of remaining *L. sigmodontis* female worms confirmed a decline in the number of intrauterine embryonic stages (morulae, coiled microfilariae, and stretched microfilariae) after 14 days of A-1574083 treatment compared to untreated controls (Fig. 3D).
Table 2 shows the plasma concentrations [area under the curve (AUC)] for A-1535469 and A-1574083 after a single oral dose in uninfected BALB/c mice and gerbils. Although A-1574083 showed poor bioavailability in this strain of mouse, the efficacy of this drug against Wolbachia in vivo was similar to that of A-1535469. Combining data from PK and efficacy studies, we created an exploratory PK/PD (pharmacodynamic) model (fig. S3). We analyzed plasma concentrations of drug in both larval and adult B. malayi–infected animals dosed for 14 days with A-1574083 or A-1535469. We determined that the maintenance of unbound plasma concentrations of drug above the in vitro EC50 (defined using Wolbachia in insect cells) for more than 12 hours/day produced a >90% decline in Wolbachia from nema-
todes in infected mice (fig. S3).

After extensive comparison of the two lead compounds, A-1574083 (now called ABBV-4083) was selected for evaluation as a preclinical drug candidate on the basis of its greater potency together with acceptable PKs and safety in mouse and gerbil worm infection models. More limited development of A-1535469 has been undertaken as a backup strategy. A-1574083 demonstrated interactions with only 2 of 77 mammalian receptors, ion channels, enzymes, and transporter assays [neurokinin NK1 receptor and the γ-aminobutyric acid (GABA)–gated chloride channel at a concentration of 10 μM] assessed in vitro (Table 3). The inhibitory concentration providing 50% inhibition (IC50) for the human ether-a-go-go-related gene encoding a potassium channel (associated with cardiovascular toxicities) was >28 μM. A-1574083 exhibited no binding at 100 μM to the gastrointestinal motilin receptor, an off-target receptor for some macrolide antibiotics (Table 3) (17). A-1574083 tested negative in the Mini-Ames mutation assay and in the micronucleus assay (Table 3). Cardiovascular studies completed in dogs and 28-day toxicity studies completed in rats and dogs showed that A-1574083 plasma concentrations at the no adverse effect levels (NOAELs) were above the plasma concentrations obtained with efficacious doses in mouse and gerbil infection models (Table 3).

The L. loa filarial parasite lacks Wolbachia, precluding an anti-Wolbachia–based mechanism of action against this parasitic worm. To investigate potential off-target effects, we undertook in vitro counter screening against L. loa microfilariae using continuous exposure to 0.032 to 100 μM with an estimated IC50 of 23.3 μM (fig. S4). The IC50 for L. loa motility inhibition was >100-fold above the estimated peak unbound plasma concentration of A-1574083 (unbound Cmax = 0.18 μM) at the efficacious dose (50 mg/kg) in gerbils. This indicated a wide separation in the concentration of A-1574083 needed for anti-Wolbachia activity and direct microfilaricidal effects on L. loa microfilariae.

**DISCUSSION**

The discovery and development of anti-Wolbachia tylosin analog macrofilaricides yielded the candidate molecule A-1574083 (ABBV-4083). A-1574083 demonstrated potent anti-Wolbachia activity in animal models, including nematode-infected immunodeficient mice and gerbils. This activity was comparable or superior to “gold-standard” second-generation tetracycline antibiotics. A-1574083 showed robust anti-Wolbachia activity with a short treatment time frame of 7 to 14 days compared to second-generation tetracycline antibiotics with a treatment time frame of 21 to 28 days. This suggests that the A-1574083 analog may provide clinical benefits with a short dosing regimen. A-1574083 also displayed a promising preclinical safety profile.

Curative activity after effective Wolbachia depletion is evident 18 months after treatment in patients infected with nematodes (13, 18). A more immediate manifestation of Wolbachia depletion from adult female filarial reproductive tissue, observed both in rodent models and in clinical trials (13, 18), is the arrest of worm embryogenesis 2 to 6 months after treatment. This subsequently leads to a slow waning of mature microfilariae from the blood or skin and ultimately clearance of microfilarial infection in lymphatic filariasis and onchocerciasis within a period of 12 to 22 months after treatment (19-22). This important effect of anti-Wolbachia treatment mediates transmission-blocking efficacy and prevents further skin and eye disease attributable to O. volvulus microfilariae infection. Here, we demonstrate the anti-Wolbachia activity of A-1574083 and the sustained disruptive effects of this treatment on adult filarial embryogenesis. This led to a blockade in the release of microfilariae and gradual complete clearance of circulating microfilariae in B. malayi and L. sigmodontis rodent models of worm infection.

The risk of potentially life-threatening serious adverse events in patients with onchocerciasis who are coinfected with the related filarial worm L. loa (23) is a major issue hindering adherence to the rapid-acting microfilaricidal drug, ivermectin. New macrofilaricidal agents need to be safe to administer to coinfected patients at risk of severe adverse reactions to ivermectin. On the basis of its anti-Wolbachia activity, A-1574083 was predicted to be safe for treating individuals coinfected with Onchocerca and L. loa, with initial in vitro testing indicating no direct L. loa cytolality at concentrations far exceeding those required for depletion of Wolbachia.

Our study has several limitations. Although robust Wolbachia depletion was demonstrated for several nematode species including Onchocerca, the rodent models of filarial infection harbor adult worms in sites (e.g., the peritoneal cavity) that are highly accessible to drug entry from the blood. In contrast, in human infection, O. volvulus adults are present in subcutaneous and deep-tissue nodules. Although these nodules are well vascularized, drug uptake into O. volvulus nodules may not be as efficient as into the adult worms in rodent models. The dependence of efficacy on drug dose versus duration of dosing is also not well defined. Although higher doses offer the potential for shorter dose duration, the detailed relationship between drug dose and dose duration is not well understood. This crucial relationship needs to be elucidated if this drug regimen is to satisfy specific target product profiles for point-of-care treatment and mass administration under difficult field conditions.

**MATERIAL AND METHODS**

**Study design**

The research objective of our study was to develop a short-course oral antibiotic that was effective against nematodes infected with the bacterial endosymbiont Wolbachia, which could be advanced into clinical testing as a new treatment for lymphatic filariasis and onchocerciasis. Phenotypic in vitro screening of a small-molecule library against insect Wolbachia was done in a blinded manner and subsequently decoded to identify a potent “hit” antibiotic, tylosin A. Confirmatory phenotypic screening against nematode Wolbachia, medicinal chemistry, iterative in vitro screening, and rodent (mouse and gerbil) PKs of tylosin A oral analogs were performed in a nonblinded manner. Two tylosin A analogs with improved in vitro potency and acceptable
rodent PK characteristics were advanced into a series of rodent efficacy studies. Rodents were randomized into drug groups. Scientists were involved in drug treatments, and parasitological and molecular read-outs were blinded to the treatment group, with the exception of the *L. sigmodontis* drug screens, which were done in a nonblinded manner. Details of animal age and sex are provided below. Group numbers of rodents used in in vivo experiments are provided below and in the figure legends.

**Synthesis of tylosin A analogs**

A-1535469 and A-1574083 were synthesized from tylosin A using a three-step procedure (Fig. 1). Briefly, the 2'-OH (mucaminose) of tylosin was protected (acetic anhydride) as the corresponding acetate, followed by selective activation of the 3' - and 4'-OH groups through the formation of a cyclic tin complex (using dibutyltin oxide) and then by derivatization of the 4'-OH (mycarose) with either diethylcarbamoyl chloride or p-fluorobenzyl bromide/Caesium fluoride. Subsequent deprotection of the 2'-acetate (refluxing methanol) provided A-1535469 or A-1574083.

**In vitro cell-based screen for anti-Wolbachia activity**

Compounds were screened for anti-Wolbachia activity in vitro in the A-WOL-validated *Wolbachia*-infected *Aedes albopictus* (C6/36 wAlbB) 7-day cell-based screen. This screen used a 384-well format assay with high-content imaging (Operetta), as previously described (7).

**Animals**

Inbred male SCID CB.17 (BALB/c congeneric) mice were purchased from Charles River Laboratories, UK. Inbred IL-4Rα−/− or CCR3−/− BALB/c breeding pairs were purchased from Jackson laboratory, USA. Outbred *Meriones unguiculatus* gerbil breeding pairs were purchased from Charles River Laboratories, Europe. Rodents were maintained and bred under specific pathogen-free conditions at the University of Liverpool Biological Services Unit or in individually ventilated cages at the Research Foundation for Tropical Medicine and The Environment, Buea, Cameroon. All experiments were approved by the internal Institutional Animal Care and Use Committee.

**Screening of anti-Wolbachia activity in nematodes in vitro**

Compounds were further scrutinized for anti-Wolbachia activity in vitro in a microfilarial assay. Microfilariae were obtained by peritoneal lavage of gerbils (*M. unguiculatus*) harboring a patent infection of *B. malayi* as previously described (11). Microfilariae were purified and resuspended in complete medium consisting of RPMI (Invitrogen) supplemented with 10% fetal bovine serum (Fisher Scientific), 1% penicillin/streptomycin, and 1% amphotericin B (Life Technologies). Microfilariae were plated at 8000 microfilariae per well in a 96-well plate (100 μl per well). Compounds to be tested [10-mm stock in 100% dimethyl sulfoxide (DMSO)] were diluted in complete medium, and 100 μl was added to the appropriate wells to give a dose-response curve. Five replicates were used for each compound, and each plate contained doxycycline (5 μM) and DMSO controls. The assay plates were incubated at 37°C for 6 days in 5% CO2, after which DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). Quantitative polymerase chain reaction (qPCR) was performed to obtain the ratio of *wsp* to *gst* gene copy number, as previously described (11).

**Screening of anti-Wolbachia activity in nematode-infected animals**

Infectious stage *B. malayi* L3 larvae were propagated, as previously described (11). Male mice 6 to 12 weeks of age or male gerbils 8 to 16 weeks of age were infected with *B. malayi* L3 larvae intraperitoneally (50 or 100 L3 larvae per mouse; 400 L3 larvae per gerbil), and infections were maintained for a maximum of 40 weeks. Mobile *B. malayi* microfilariae were recovered by peritoneal lavage at necropsy and were enumerated by microscopy. Viable adult male *O. ochengi* nematodes were aseptically isolated from naturally parasitized cattle, as previously described (11). Between 10 to 20 male *Onchocerca* were surgically implanted into the peritoneal cavity of male SCID CB.17 mice or female gerbils, under anesthesia, as previously described (11).

For *L. sigmodontis* infection, 6- to 8-week-old female gerbils or 6- to 8-week-old female Balb/c mice were exposed to *O. bacoti* mites containing the infective stage L3 larvae of *L. sigmodontis*, as previously described (24). The same batch of mite-containing bedding was used to infect all animals of one experiment. Treatment of mice with tylosin A analogs or antibiotics was initiated 1 day after infection, and necropsies were performed 35 days after infection. Treatment of gerbils with tylosin A analogs or antibiotics started 12 weeks after infection, and necropsies of gerbils occurred 16 weeks after the start of treatment. *L. sigmodontis* worms were isolated from the thoracic cavity and peritoneum and were quantified, as previously described (24). For microfilarial counts in gerbils, 10 μl of peripheral blood was taken from the saphenous vein in weekly intervals starting at 11 weeks after infection and diluted in 300 μl of Hinkelmann solution (0.5% eosin Y, 0.5% phenol, and 0.185% formaldehyde in aqua dest). After centrifugation at 400g for 5 min, the supernatant was discarded, and the pellet was resuspended and completely transferred to a microscope slide to quantify microfilariae using a microscope, as previously described (25). After necropsy of gerbils, remaining intact female worms...
were used in equal numbers for either embryograms or Wolbachia quantification. For embryograms, single female adult worms were homogenized in 20% Hinkelman/80% phosphate-buffered saline (PBS), diluted 1:10 in PBS, and enumerated via microscopy. The analyzed embryonal stages included eggs, morulae, pretzel microfilariae, and stretched microfilariae.

**Treatment of infected animals with tylosin A analogs and antibiotics**

Infected animals were randomly assigned into dose groups with \( n = 3 \) to 7 for larval-stage testing and \( n = 4 \) to 9 for adult-stage testing. Randomization was done by stratification based on ID number (\( B. \ malayi \) larval and precuculent adult infections), baseline ultrasonography estimates of worm burden (\( B. \ malayi \) infection of SCID mice and gerbils) using a semi-quantifiable scoring system (26), baseline microfilariae (\( L. \ sigmodontis \) infection of gerbils), or order of implantation and source of male worms (\( O. \ ochengi \) implantation into SCID mice or gerbils).

Tylosin A (Sigma-Aldrich) or the tylosin A analogs, A-1535469 or A-1574083, were dissolved in PEG300/propylene glycol/H\(_2\)O (55:30:15). Minocycline hycolate and doxycycline hyclate (Sigma-Aldrich) were dissolved in water. Animals were weighed, and weight-corrected volumes of dosing solution (100 \( \mu \)l per 25 g) were administered by oral gavage or intraperitoneal injection of tylosin A. Experimentors involved in dosing were blinded to the treatment group except for \( L. \ sigmodontis \) studies.

**Molecular assays**

For synchronous \( B. \ malayi \) infections, determination of Wolbachia single copy wsp gene quantity was undertaken by qPCR, as previously described (11). In rodent screens where mature adult worm age varied, wsp quantity per worm was normalized to filarial nematode gst using \( Brugia\)- or \( Onchocerca\)-specific primers (11). Between 5 and 20 individual worms were assayed, derived from dose groups of between three and nine animals. Experiments using \( L. \ sigmodontis \) quantified Wolbachia \( fsZ \) and used \( L. \ sigmodontis \) \( actin \) for normalization, as previously described (27).

**Testing bacterial susceptibility**

Broth microdilution test methods were conducted according to the Clinical Laboratory Standards Institute guidelines (28) to determine the susceptibility of different bacterial strains to the tylosin A analogs and comparators. Concurrent quality control testing was performed to ensure proper test conditions and procedures. The quality control strains included Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 29213, and Streptococcus pneumoniae ATCC 49619. All quality control results were within published ranges (29).

**Testing \( L. \ loa \) in vitro**

\( L. \ loa \) microfilariae were extracted from baboon peripheral blood and placed in culture in vitro, as previously described (30). Between 20 and 30 microfilariae per well were seeded onto a 96-well plate containing test compounds prepared in DMSO to a final concentration of 1% in culture medium (10% FBS–supplemented Dulbecco’s modified Eagle’s medium). All compounds were tested in triplicate, and daily motility was scored as follows: 0 (immotile/dead), 1 (very slow, only tip movement), 2 (break in activity, sluggish), or 3 (fully motile, continuously active) on day 2 compared to a DMSO-only control. Operators were blinded to treatment groups. Ethical and administrative clearances for the use of baboons in this study were obtained from the Ministry of Scientific Research and Innovation of Cameroon (Research permit no. 028/MIN-RESI/B00/C00/C10/C12) and Research Foundation for Tropical Diseases and the Environment (REFOTDE) Institutional Animal Ethics Committee (no. 2009/032). The animal procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee at the National Institutes of Health (USA) and University of Georgia, Athens, USA, as previously described (31).

**Plasma drug concentrations in mice and gerbils**

Oral drug doses were administered by gavage to BALB/c mice (Charles River Laboratories, USA) or Mongolian gerbils (Charles River Laboratories, Beijing). Serial blood samples collected into EDTA anticoagulant for plasma concentration analysis were obtained from each animal after dosing. EDTA preserved plasma samples were extracted by protein precipitation with acetonitrile fortified with internal standards. The supernatant was injected into a high-performance liquid chromatography–mass spectroscopy (MS)/MS system for separation and quantitation. Detection was accomplished using a triple-quadruple mass spectrometer operated either in electrospray or in an atmospheric chemical ionization mode. The area under the plasma concentration time curve (AUC) was calculated using the linear trapezoidal rule.

**Statistical analysis**

Percentage reduction in Wolbachia in microfilariae after in vivo drug treatments was normalized to median vehicle control concentrations derived from the same experimental infection and screen. Where repeat experimental data were available, data were pooled after normalization. Grouped continuous variables were tested for normal distribution by D’Agostino-Pearson omnibus normality tests. Continuous variables failing normal distribution tests were log\(_{10}\)-transformed and retested. Continuous variables satisfying the assumptions of normal distribution were examined by one-way ANOVA with Dunnett’s multiple tests post hoc. Variables not satisfying the assumption of normality were compared by Mann-Whitney (two variables) or Kruskal-Wallis test with Dunnett’s multiple tests post hoc (\( >2 \) variables). Post hoc testing scrutinized inferior, noninferior, or superior anti-Wolbachia activity compared with doxycycline or minocycline controls or assessed changes in microfilarial loads compared with vehicle controls or doxycycline/minocycline controls. Significance levels are indicated as *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \), and ****\( P < 0.0001 \). All statistics were undertaken using GraphPad Prism v.6 software.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Efficacy of tylosin A in Litomosoides larval model after intraperitoneal or oral dosing.

Fig. S2. Study design schematics for efficacy models.

Table S1. PK parameters of tylosin A after intraperitoneal and oral dosing in BALB/c mice.

Table S2. \( B. \ malayi \) larval worm burden 2 weeks after dosing with doxycycline, tylosin A, or A-1553469, or A-1574083 in IL-4R\(^{-/-}\) BALB/c mice.

Table S3. Spectrum of TylAMac analogs and tylosin A against selected bacteria.

Table S4. Percentage reduction in \( L. \ sigmodontis \) adult worm burdens 6 weeks after dosing with minocycline, tylosin A, or A-1535469 in CCR3\(^{-/-}\) BALB/c mice.

Table S5. Plasma drug concentrations in mice and gerbils.

Table S6. Plasma drug concentrations in mice and gerbils.
REFERENCES AND NOTES


**Data and materials availability:** All data associated with this study are available in the main text or the Supplementary Materials. This study was performed under a partnership agreement between AbbVie Inc. and the Liverpool School of Tropical Medicine, which has confidentiality provisions. Compounds were provided to the University of Bonn through the A-WOL consortium. ABBV-4083 and related compounds are available from AbbVie Inc. under a material transfer agreement through submission of a proposal to AbbVie Inc.’s investigator-initiated studies portal: www.abbbiemedicalresearch.com/external/AV_SPT_SiteLoginPage and www.abbbvie.com/partnerships/additional-collaboration-opportunities/investigator-initiated-studies-iis.html.

Submitted 17 May 2018
Accepted 19 October 2018
Published 13 March 2019

10.1126/scitranslmed.aau2086

Preclinical development of an oral anti-Wolbachia macrolide drug for the treatment of lymphatic filariasis and onchocerciasis


Sci Transl Med 11, eaau2086.
DOI: 10.1126/scitranslmed.aau2086

Nematodes go A-WOL

New drugs are urgently needed for treating the neglected tropical diseases, onchocerciasis and lymphatic filariasis. As part of the A-WOL consortium, Taylor et al. launched a drug discovery program that identified macrolide antibiotic molecules that were capable of eliminating the bacterial endosymbiont, Wolbachia, which is necessary for the viability and fertility of filarial worms. Two macrolide compounds cleared Wolbachia from filarial nematodes in animal models of lymphatic filariasis and onchocerciasis. The authors showed that the tylosin A analog, A-1574083 (ABBV-4083), had superior efficacy compared to tetracycline antibiotics for clearing Wolbachia in mouse and gerbil models of filarial infection. The safety and pharmacology profiles of A-1574083 have enabled this compound to be moved forward into clinical testing.