Onchocerca parasites and Wolbachia endosymbionts: evaluation of a spectrum of antibiotic types for activity against Onchocerca gutturosa in vitro

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Abstract

Background: The filarial parasites of major importance in humans contain the symbiotic bacterium Wolbachia and recent studies have shown that targeting of these bacteria with antibiotics results in a reduction in worm viability, development, embryogenesis, and survival. Doxycycline has been effective in human trials, but there is a need to develop drugs that can be given for shorter periods and to pregnant women and children. The World Health Organisation-approved assay to screen for anti-filarial activity in vitro uses male Onchocerca gutturosa, with effects being determined by worm motility and viability as measured by reduction of MTT to MTT formazan. Here we have used this system to screen antibiotics for anti-fil arial activity. In addition we have determined the contribution of Wolbachia depletion to the MTT assay.

Methods: Adult male O. gutturosa were cultured on a monkey kidney cell (LLCMK 2) feeder layer in 24-well plates with antibiotics and antibiotic combinations (6 to 10 worms per group). The macrofilaricide CGP 6140 (Amocarzine) was used as a positive control. Worm viability was assessed by two methods, (i) motility levels and (ii) MTT/formazan colorimetry. Worm motility was scored on a scale of 0 (immotile) to 10 (maximum) every 5 days up to 40 days. On day 40 worm viability was evaluated by MTT/formazan colorimetry, and results were expressed as a mean percentage reduction compared with untreated control values at day 40. To determine the contribution of Wolbachia to the MTT assay, the MTT formazan formation of an insect cell-line (C6/36) with or without insect Wolbachia infection and treated or untreated with tetracycline was compared.

Results: Antibiotics with known anti-Wolbachia activity were efficacious in this system. Rifampicin (5 × 10⁻⁴M) was the most effective anti-mycobacterial agent; clofazimine (1.25 × 10⁻³M and 3.13 × 10⁻⁴M) produced a gradual reduction in motility and by 40 days had reduced worm viability. The other anti-mycobacterial drugs tested had limited or no activity. Doxycycline (5 × 10⁻⁵M) was filaricidal, but minocycline was more effective and at a lower concentration (5 × 10⁻⁵M and 1.25 × 10⁻⁵M). Inactive compounds included erythromycin, oxytetracycline, trimethoprim and...
Although antibiotics have a valuable activity against filarial nematodes, the long treatment regimens that are required present logistical problems for mass drug administration (MDA). Another obstacle to MDA is the contraindication of tetracyclines in pregnant women and children under the age of eight years. Therefore, alternative treatment options that target Wolbachia but circumvent these problems would be advantageous. For more than 18 years, an in vitro drug screen for identifying potential macrofilaricidal activity has used adult male O. gutturosa [21-23], with assessment of efficacy being made by observing worm motility and inhibition of MTT formazan formation [24,25]. In the present work we have extended this system and developed a long-term assay, which has enabled us to screen many of the commonly used antibiotics for macrofilaricidal activity, both individually and in combinations. We have also determined the contribution of Wolbachia to the MTT assay.

Methods

In vitro drug screen

Adult male O. gutturosa were dissected from the nuchal ligament connective tissues obtained from naturally infected cattle in Kumasi, Ghana, as previously described [6]. They were maintained individually in the wells of a 24-well plate containing 1.8 ml of Minimum Essential Medium with 10% heat inactivated calf serum and a monkey kidney cell (LLCMK2) feeder layer [26] at 36.5 °C with 5% CO₂ for 24 to 48 hours until the addition of drugs. The medium of all wells included the antibiotics penicillin (200 U/ml) and streptomycin (200 µg/ml), which have no anti-Wolbachia activity [27], and the anti-mycotic agent amphotericin B (50 µg/ml). Drugs were prepared as previously described [6] in medium and each drug concentration was tested against six to ten worms, maintained as above. Medium (with or without drug) was replaced every 5 days. The compounds tested included a range of test antibiotics (Tables 1, 2 and 3), both individually and in combination. The amoscanate derivative CGP 6140 (Amocarzine) was used as a positive control since it is macrofilaricidal against Onchocerca parasites [28], reviewed by [29].

Worm viability was measured by the motility levels and MTT colorimetry. Motility scores were assessed on an inverted microscope on a scale of 0 (immotile) to 10...
(maximum) [22] at regular intervals up to 40 days. The biochemical evaluation of worm viability was carried out by MTT/formazan colorimetry on day 40. In this assay, the yellow compound MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is reduced by the mitochondrial enzyme succinate dehydrogenase of living tissues to produce the blue precipitate MTT formazan [24,25]. Single intact worms were placed in each well of a 48-well plate (Falcon, UK) containing 0.5 ml of 0.5 mg/ml MTT (Sigma, UK) in phosphate buffered saline, and incubated for 30 minutes at 37°C. The worms were then transferred to separate wells of a 96 well plate, each containing 200 µl of dimethyl sulphoxide to solubilize the formazan. After one hour the plate was gently agitated to disperse the colour evenly and the absorbance value (optical density) of the resulting formazan solution was determined at 490 nm on an ELISA reader. Inhibition of formazan formation is correlated with worm damage or death. The motility and MTT assay results were expressed as a mean percentage reduction compared with untreated control values at day 40. Comparisons of test groups to untreated controls for both motility levels and MTT colorimetry on day 40 were carried out using a 2-sample t-test.

**Table 1: Summary of long-term trial 1: parasite mean motility scores and viability (MTT colorimetry)**

| Compound/drug and conc. | Mean motility scores over a period of 40 days | Motility | MTT |
|-------------------------|-----------------------------------------------|----------|-----|
|                         | DAY 1 5 10 15 20 25 30 35 40 % reduction on day 40 (P) % inhibition on day 40 (P) |
| Control                 | 8.0 7.4 7.1 7.0 5.3 5.5 6.3 6.4 4.9 | 100.0 (0.005) | 83.3 (0.098) |
| CGP 6140 1.25 × 10⁻⁵M (positive control) | 0.5 0.8 0.0 0.5 0.0 0.0 0.0 0.0 0.0 | 100.0 (0.002) | 89.6 (0.033) |
| Rifampicin 5 × 10⁻⁵M     | 7.7 6.8 6.7 6.8 3.2 1.7 0.2 0.2 0.0 | 100.0 (0.002) | 84.1 (0.042) |
| Rifampicin 1.25 × 10⁻⁵M  | 7.0 7.0 7.3 5.7 6.5 5.2 5.7 5.0 5.6 | 0.0 (0.647) | 0.0 (0.798) |
| Minocycline 5 × 10⁻⁵M    | 8.0 6.7 7.3 5.7 0.0 0.0 0.0 0.0 0.0 | 100.0 (0.002) | 89.6 (0.033) |
| Minocycline 1.25 × 10⁻⁵M | 7.8 7.5 6.7 5.7 4.3 2.5 4.8 1.7 0.0 | 100.0 (0.002) | 93.7 (0.026) |
| Doxycycline 5 × 10⁻⁵M    | 8.3 8.0 8.0 5.7 0.3 0.0 0.0 0.0 0.0 | 100.0 (0.002) | 93.0 (0.027) |
| Doxycycline 1.25 × 10⁻⁵M | 7.3 6.5 6.3 4.8 6.3 6.3 6.2 6.2 5.5 | 0.0 (0.699) | 0.0 (0.801) |
| Ethambutol 5 × 10⁻¹M     | 6.8 6.8 6.5 6.7 4.0 4.8 4.3 5.0 2.7 | 44.9 (0.170) | 73.0 (0.108) |
| Dapsone 5 × 10⁻⁴M        | 7.3 7.3 7.7 5.0 6.8 7.0 7.2 7.3 6.5 | 0.0 (0.250) | 0.0 (0.798) |
| Pyrazinamide 5 × 10⁻³M   | 7.7 7.7 7.2 3.5 5.5 5.5 5.7 5.2 3.7 | 24.5 (0.503) | 4.8 (0.949) |

The effects of exposure to a range of antibiotics on the viability of Onchocerca gutturosa adult males in long-term (40 day) in vitro culture. Viability was assessed by measuring worm motility scores throughout the trial and by MTT colorimetry on day 40.

**Table 2: Summary of long-term trial 2: parasite mean motility scores and viability (MTT colorimetry)**

| Compound/drug and conc. | Mean motility scores over a period of 40 days | Motility | MTT |
|-------------------------|-----------------------------------------------|----------|-----|
|                         | DAY 1 5 10 15 20 25 30 35 40 % reduction on day 40 (P) % inhibition on day 40 (P) |
| Control                 | 7.6 7.2 7.1 6.8 5.9 4.9 5.8 6.1 6.3 | 100.0 (ND) | 87.2 (ND) |
| CGP 6140 1.25 × 10⁻⁵M (positive control) | 1.3 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 | 100.0 (ND) | 83.3 (ND) |
| Norfloxacin 5 × 10⁻⁵M    | 6.9 7.4 7.1 2.4 2.5 2.6 1.4 1.0 2.5 | 60.3 (ND) | 90.7 (ND) |
| Ciprofloxacin 5 × 10⁻⁵M  | 6.9 7.9 7.1 3.6 4.1 3.0 2.3 2.5 3.0 | 52.4 (0.080) | 73.3 (0.040) |
| Vancomycin 5 × 10⁻⁴M     | 6.4 7.4 6.5 3.6 3.6 2.8 2.4 1.6 2.1 | 66.7 (0.009) | 83.7 (0.022) |
| Gentamicin 5 × 10⁻⁴M     | 7.6 5.5 6.3 2.8 2.6 0.8 1.5 1.0 1.6 | 74.6 (0.005) | 83.7 (0.017) |
| Ceftriaxone 5 × 10⁻⁴M    | 7.1 6.8 7.0 2.4 2.0 0.9 0.6 0.6 0.7 | 88.9 (0.001) | 90.7 (0.011) |
| Triclosan 5 × 10⁻⁴M      | 6.9 4.9 0.4 0.1 0.0 0.0 0.0 0.0 0.0 | 100.0 (ND) | 98.8 (ND) |
| Cerulenin 5 × 10⁻³M      | 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0 | 100.0 (ND) | 100.0 (ND) |

a. Day 40 results based on 1 worm; 5 worms lost to microbial contamination
b. Day 40 results based on 2 worms; 4 worms lost to microbial contamination
c. Denotes compounds that were toxic to the monkey kidney cell feeder layer

The effects of exposure to a range of antibiotics on the viability of Onchocerca gutturosa adult males in long-term (40 day) in vitro culture. Viability was assessed by measuring worm motility scores throughout the trial and by MTT colorimetry on day 40.
Table 3: Summary of long-term trial 3: parasite mean motility scores and viability (MTT colorimetry)

| Compound/drug and conc.       | Mean motility scores over a period of 40 days | % reduction on day 40 (P) | % inhibition on day 40 (P) |
|-------------------------------|---------------------------------------------|---------------------------|---------------------------|
| Control                       | 8.4 8.3 7.6 7.5 7.8 7.7 7.6 7.2 7.2         |                           |                           |
| CGP 6140 1.25 × 10⁻⁵M (positive control) | 2.5 0.3 0.0 0.0 0.0 0.0 0.0 0.0 0.0         | 100.0 (<0.001)            | 67.4 (0.0032)             |
| Rifampicin 5 × 10⁻⁵M          | 8.0 6.6 2.1 0.4 0.4 0.0 0.0 0.0 0.0          | 100.0 (<0.001)            | 67.4 (<0.001)             |
| Erythromycin 5 × 10⁻⁵M       | 8.4 8.5 8.0 7.6 7.7 7.6 7.4 7.5 7.4         | 0.0 (0.539)               | 0.0 (0.210)               |
| Oxytetracycline 5 × 10⁻⁵M    | 8.0 8.1 7.7 7.5 7.6 7.5 7.1 6.4 6.9         | 4.2 (0.360)               | 0.0 (0.995)               |
| Isoniazid 5 × 10⁻⁶M          | 8.2 8.2 7.7 8.0 7.7 7.3 6.8 5.7 5.2         | 27.8 (0.010)              | 35.5 (0.074)              |
| Isoniazid 5 × 10⁻⁵M + Rifampicin 5 × 10⁻⁵M | 7.8 8.2 5.0 4.0 2.7 0.8 0.0 0.0 0.0 | 100.0 (<0.001)           | 71.7 (0.002)              |
| Trimethoprim 5 × 10⁻⁵M       | 8.4 8.4 7.9 7.6 7.6 8.0 7.5 7.0 7.0         | 2.8 (0.549)               | 0.0 (0.319)               |
| Sulphamethoxazole 5 × 10⁻⁵M  | 8.1 8.3 8.4 7.6 7.6 8.0 7.5 7.3 7.1         | 1.4 (0.848)               | 0.0 (0.998)               |
| Trimeth. 5 × 10⁻⁴M + Sulphameth. 5 × 10⁻⁵M | 8.5 8.4 8.0 7.5 6.8 7.5 7.1 7.4 6.9 | 4.2 (0.449)               | 0.0 (0.828)               |
| Clofazimine 1.25 × 10⁻⁵M     | 7.7 6.5 5.2 3.5 4.3 3.5 3.2 0.8 0.0         | 100.0 (<0.001)            | 77.4 (<0.001)             |
| Clofazimine 3.13 × 10⁻⁴M     | 8.2 7.0 4.5 5.5 5.0 4.8 3.4 1.2 0.8         | 88.9 (<0.001)             | 53.3 (0.028)              |

The effects of exposure to a range of antibiotics and antibiotic combinations on the viability of Onchocerca gutturosa adult males in long-term (40 day) in vitro culture. Viability was assessed by measuring worm motility scores throughout the trial and by MTT colorimetry on day 40.

Results

In vitro drug screen

The motility scores and percentage inhibitions of MTT reduction by all the tested compounds are shown in Tables 1, 2 and 3. The positive control, CGP 6140, had a very rapid onset of action, with reductions in worm motility even on the first day after initiation of treatment. No worm movement was observed after day 15, and by day 40 there was a 67.4 to 83.3 inhibition in formazan formation compared to untreated controls. In contrast, the average motility score of the untreated control worms in each experiment ranged from 4.9 to 7.2 at day 40 (Tables 1, 2 and 3).

Several anti-mycobacterial drugs were tested. Of these, rifampicin (5 × 10⁻⁵M) was the most effective, completely inhibiting motility by day 40 (Table 1 and Fig. 1) or day 25 (Table 3). Clofazimine (1.25 × 10⁻⁵M and 3.13 × 10⁻⁶M) gradually affected worm motility so that, by day 40, there were 100% and 88.9% reductions in motility and 77.4 and 53.3% inhibition of MTT reduction, with the higher and lower concentrations, respectively (Table 3 and Fig. 4). Ethambutol (5 × 10⁻⁵M) was less effective, giving 44.9% and 73% reductions in motility and MTT formation, respectively (Table 1 and Fig. 5). However, the other agents with activity against Mycobacterium species showed limited (pyrazinamide, isoniazid) or no (dapsone) activity against the worms (Tables 1 and 3). Also, the addition of isoniazid (5 × 10⁻⁵M) to rifampicin (5 × 10⁻⁵M) did not improve the efficacy of the latter (Table 3).

Doxycycline was effective (100% reduction in motility by day 25, 93% inhibition of formazan formation at day 40) at a concentration of 5 × 10⁻⁵M, but showed no activity at
1.25 × 10⁻⁵M (Fig. 3). However, minocycline was filaricidal at both of these concentrations (Fig. 2). Intermediate activity was shown by norfloxacin, ciprofloxacin, vancomycin and gentamycin (all at a concentration of 5 × 10⁻⁵M). The drugs that did not have a filaricidal effect at 5 × 10⁻⁵M included erythromycin, oxytetracycline, trimethoprim and sulphamethoxazole (these two either alone or in combination).

Two compounds, triclosan and cerulenin, were toxic to the monkey kidney cell feeder layer, so it was not possible to conclude if they had an anti-Onchocerca effect, since a viable cell layer is essential to the long-term survival of the worms.

**Contribution of Wolbachia to the MTT reduction assay**

C6/36 cells with and without *W. pipientis* infection were incubated in medium or medium plus tetracycline for two or four weeks before being analysed by the MTT reduction assay. This assay showed that infection with *Wolbachia* did contribute to extra metabolic activity in the cells, since C6/36 Wp had significantly higher absorbance readings than C6/36 (P = 0.017 after two weeks’ incubation; P = 0.000 after four weeks’ incubation, Fig. 6). However, in C6/36 Wp treated with tetracycline for two to four weeks the absorbance was no different from C6/36 with tetracycline (P = 0.269 at two weeks; P = 0.475 at four weeks). At neither time-point did uninfected C6/36 treated with tet-
racycline show a significant difference from those not treated ($P = 0.958$ at two weeks; $P = 0.543$ at four weeks, Fig. 6), indicating that the tetracycline itself had no effect in reducing the metabolic activity.

**Discussion**

The results presented here confirm that *O. gutturosa* males provide a suitable in vitro screen for slow-acting antibiotic drugs with macrofilaricidal activity. Male *O. gutturosa* are smaller than *O. volvulus* males but contain an almost identical *Wolbachia*/*nematode* ratio (H.F. McGarry and M.J. Taylor, unpublished observation) indicating that they are a suitable model for human onchocerciasis.

The gradual reduction in motility (as with clofazimine) or a delay before effects were seen (rifampcin, doxycycline, minocycline) is consistent with activity against the endosymbionts, in contrast to the direct and rapid effects on worm viability of the positive control, Amocarzine. Interestingly, with rifampcin, doxycycline, minocycline and ethambutol, the most rapid decline in worm motility occurred between 15 and 20 days of treatment. This period of treatment may be sufficient to inhibit *Wolbachia*-dependent processes, such as inhibition of protein synthesis (which is the mode of action of tetracyclines), with a resultant deterioration in nematode health before

![Figure 4](image)

**Figure 4**

*Mean motility score of *O. gutturosa* adult males in vitro exposed to clofazimine.* Control, ○ clofazimine $1.25 \times 10^{-5}$M, ■ clofazimine $3.13 \times 10^{-6}$M, ● CGP 6140 $1.25 \times 10^{-5}$M, □ (positive control). (Data from Table 3).

![Figure 5](image)

**Figure 5**

*Mean motility score of *O. gutturosa* adult males in vitro exposed to ethambutol.* Control, ○ ethambutol $5 \times 10^{-5}$M, ● CGP 6140 $1.25 \times 10^{-5}$M, □ (positive control). (Data from Table 1).

![Figure 6](image)

**Figure 6**

*Wolbachia* contribute to metabolic activity as measured by the MTT reduction assay. C6/36 and C6/36 Wp were incubated in medium or medium with tetracycline 20 µg/ml for four weeks before the MTT reduction assay was performed. Bars represent means of five repeats each of 250,000 cells (± S.D.). Values that are significantly lower than those of C6/36 Wp are denoted by * ($P = 0.000$) and ** ($P = 0.03$).
a decline in bacterial numbers is observable. As such, this O. gutturosa system is likely to be more sensitive than one that utilises Wolbachia-infected mosquito cell lines, which relies on direct observation for the presence or absence of bacteria [27,32].

Currently, doxycycline (200 mg) administered daily for three weeks is the shortest effective regimen that has been tried against a human filarial infection; when followed by standard anti-filarial chemotherapy, this treatment resulted in prolonged reductions in microfilaraemia and in Wolbachia numbers in the microfilariae, but was not macrofilaricidal [20] and is similar to the timeframe after which effects were observable in vitro.

Rifampicin has previously been shown to be very effective against Wolbachia [6,27,32,33], which was confirmed in this in vitro antifilarial assay. Rifampicin interferes with nucleic acid synthesis by combining with and inhibiting the RNA-polymerase of bacteria and is bactericidal. The anti-leprosy agent clofazimine also demonstrated good activity against O. gutturosa, and is also bactericidal. Anti-tuberculosis and -leprosy therapies may have benefits at the population level by reducing the prevalence of filariasis [34]. However, the other drugs tested that are used against Mycobacterium species (ethambutol, dapsone, pyrazinamide, isoniazid) showed little anti-Onchocerca activity.

The tetracycline doxycycline was filaricidal in this drug screen, confirming previous findings that it has anti-Wolbachia effects [27,33,35]. This drug has also been used in human filarial infections, in which it has resulted in a prolonged loss of microfilariae and lack of embryogenesis [10,11,14], and has recently been shown to be macrofilaricidal against W. bancrofti [13]. In the present work minocycline was even more effective than doxycycline and warrants further investigation in vivo. This result agrees with our unpublished work (S. Townson) that minocycline is more active than doxycycline against O. lienalis microfilariae in mice. Minocycline has the advantage over doxycycline of inducing less phototoxicity. However, oxytetracycline showed no anti-Wolbachia activity, in contrast to previous reports [6,9,32]; it is not clear why there were contradictory findings. The lack of activity by other compounds (erythromycin, trimethoprim and sulphamethoxazole) was consistent with previous findings [27,36].

Filarial nematodes cultured in vitro often succumb to drug treatments more readily than they do in vivo. The system described here provides a relatively easy and inexpensive way to perform a primary or secondary screen of compounds for anti-filarial/Wolbachia activity, which would then be followed by in vivo experiments. Antibiotics are slow-acting against filarial nematodes, as seen in the present study and in field trials (for example, [12,13]); an advantage of the male O. gutturosa culture system is that it can be maintained for at least 40 days. In the present study the effects of the compounds has not been directly related to their activity against Wolbachia and it is possible that they also had direct anti-nematodal activity. Studies on the dynamics of the loss of the bacteria from worms are currently underway using quantitative polymerase chain reaction and/or immunohistology in the screening system.

The presence of Wolbachia made a significant contribution to the MTT assay in C6/36 cells as would be predicted from the presence of succinate dehydrogenase as determined by genomic annotation. Thus partial reduction in formazan formation in MTT assays could reflect either the loss of bacteria and retention of worm viability or vice versa. Should an in vitro screen for specific activity against either the bacteria or worm be required then alternative markers of viability may need to be developed [37].

**Conclusion**

This study has demonstrated that the in vitro screen for macrofilaricidal activity, which uses the culture of male O. gutturosa, can be successfully extended and is also valid for the screening of antibiotic compounds with potential anti-Wolbachia/filarial activity. This system can be used for long-term screening, in this case for 40 days. Rifampicin and doxycycline were two of the most active antibiotics tested in this screen, in agreement with previous findings. However, a new finding was that minocycline was more quickly and completely effective than either of these compounds. It was also found that Wolbachia contribute to the MTT formazan formation which is used as a marker of filarial worm viability, suggesting that bacteria contribute directly to the metabolic activity of the nematode and that it may be necessary to reassess alternative indicators of worm and or bacterial viability.

**Abbreviations**

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MDA, mass drug administration.

**Competing interests**

The author(s) declare that they have no competing interests.

**Authors’ contributions**

STo designed the antibiotic screen experiments, analysed the results and advised on the manuscript preparation. STo and STA performed the antibiotic screen experiments. HFM analysed results, performed statistical analysis and prepared the manuscript. GLE performed the C6/36 MTT analyses. MJT designed the C6/36 MTT assay experiments,
analysed the results and advised on the manuscript preparation.

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