Safe, effective, and inexpensive clearance of mycoplasma contamination from cultures of apicomplexan parasites with Sparfloxacin.

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ABSTRACT

Most commercial products cannot be used for clearance of mycoplasma contamination from cultures of apicomplexan parasites due to the parasites' dependence on the apicoplast, an essential organelle with DNA replication and translation machinery of cyanobacterial origin. The lone exception, Mycoplasma Removal Agent (MRA), is relatively expensive and some mycoplasma strains have shown resistance to clearance with MRA. Here we report that the fluoroquinolone antibiotic Sparfloxacin is a safe, effective, and inexpensive alternative for treatment of mycoplasma contamination in cultures of apicomplexan parasites. Sparfloxacin cleared both MRA-sensitive and MRA-resistant mycoplasma species from *P. falciparu*m cultures at 1 and 4 µg/mL, respectively. Furthermore, we show that cultures of three different apicomplexan parasites can be maintained at concentrations of Sparfloxacin required to clear mycoplasma without resulting in substantial deleterious effects on parasite growth.

INTRODUCTION

Contamination with mycoplasma species (class Mollicutes) is a common problem in continuous cell culture and found in 15-35% of cell lines [1-3], including co-cultures of apicomplexan parasites and their host cells. Dozens of mycobacterial species have been found as part of the human microbiota, including skin and hair, but only a small number have been linked to human disease. With a 0.15-0.3 micron diameter, mycoplasmas are some of the smallest known cellular organisms and can pass through the standard 0.2 micron filters standardly used in culture media preparation. Contamination of cell cultures with mycoplasma is easily missed for several reasons, including their small size, poor staining due to the absence of a cell wall, and failure to change culture turbidity or pH [4]. Unlike contamination with most other bacteria or fungi, the presence of mycoplasma rarely results in the collapse of the cell culture but can nonetheless substantially alter the properties and behavior of infected cell lines, making it a potentially important confounder in experimental comparisons.

Mycoplasma species are broadly resistant to many classes of commonly used antibiotics, including beta-lactams, glycopeptides, fosfomycin, polymixins, sulfonamides, rifampicin, first-generation quinolones, and trimethoprim, but generally remain susceptible to some tetracyclines, macrolides, and second-generation quinolones [5]. These form the basis of commercial products aimed at eradicating mycoplasma contamination from cell cultures, such as Bayer's Ciprobay and Promocell's Biomyc-3 (both the fluoroquinolone ciprofloxacin), Promocells's Biomyc-1/2 and Roche's BM-Cyclin (both are a combination of the macrolide tiamulin and the tetracycline minocycline), Invivogen's Plasmocin (a combination of a proprietary fluoroquinolone and a proprietary macrolide), Bayer's Baytril (the fluoroquinolone Enrofloxacin), and MP Biomedicals' Mycoplasma Removal Agent (a proprietary fluoroquinolone).

Unfortunately, nearly all these products have been found to also inhibit the growth of *Plasmodium falciparum* [6-8] or other apicomplexan parasites [9,10] at similar or lower concentrations than those required to effectively clear mycoplasma [11-14]. This poor selectivity is due the effect of these antibiotics on the parasite apicoplast, an essential chloroplast-derived organelle that retains translation and DNA-replication machinery of cyanobacterial origin. The lone validated exception is Mycoplasma Removal Agent (MRA), which contains an undisclosed fluoroquinolone and has successfully been used to clear mycoplasma from cultures of apicomplexan parasites, including *P. falciparum* [6], *Toxoplasma gondii,* and *Neospora caninum* [15]. At the recommended concentration of 0.5 µg/mL clearance of mycoplasma with MRA can take 1-3 weeks, is relatively expensive at $270 per liter of culture medium, and some mycoplasma strains have shown resistance to clearance with MRA [12,13].

Here we report that the fluoroquinolone Sparfloxacin is a safe, effective, and low-cost alternative to MRA for treatment of mycoplasma contamination. Sparfloxacin cleared both MRA-sensitive and MRA-resistant mycoplasma species from *P. falciparu*m cultures at 1 and 4 µg/mL, respectively. Furthermore, we show that cultures of three different apicomplexan parasites can be maintained without deleterious effects on parasite growth at concentrations of Sparfloxacin required to clear mycoplasma.

RESULTS & DISCUSSION

Dose-response of Apicomplexan parasites to Sparfloxacin

To establish the maximal concentrations of Sparfloxacin that apicomplexan parasites would tolerate, we measured the effect of Sparfloxacin on the growth of three geographically diverse strains of *Plasmodium falciparum* (Figure 1a), as well as, two other commonly cultured apicomplexan parasites, *Toxoplasma gondii* and *Babesia divergens (*Figure 1b). EC50 values of the tested isolates all fell within a narrow range of 3.1-8.6 µg/mL. The effect of drugs inhibiting apicoplast replication doesn't manifest until the subsequent cell cycle, a phenomenon known as delayed death [16].

The growth assays used for *Toxoplasma* and *Babesia* parasites covered multiple lytic cycles and therefore capture any delayed death effects of Sparfloxacin on apicoplast replication. However, since *P. falciparum* divides by schizogony and apicoplast replication only occurs every 48 hours, our 72 hour growth assays may not have fully captured potential delayed death effects by Sparfloxacin. To evaluate *whether P. falciparum* cultures exhibited any delayed death, we measured the effect of Sparfloxacin over 4 replication cycles (Figure 1c). Growth rates were constant over 8 days at all concentrations Sparfloxacin, excluding the possibility of substantial delayed death effects, which would have resulted in a decrease of the growth rate after the first replication cycle. Compared to untreated cultures, there was a 10-12% decrease in the growth rate when were maintained in the presence of 1 and 2 µg/mL of Sparfloxacin or MRA (0.5 µg/mL). At 4 µg/mL growth of *P. falciparum* strain NF54 cultures slowed by 31% but cultures could be maintained continuously for at least 3 weeks at this concentration. While we did not measure the effect continuous growth, all the other *P. falciparum* strains, as well as *Toxoplasma* and *Babesia*, could be maintained at 4 µg/mL for prolonged periods.

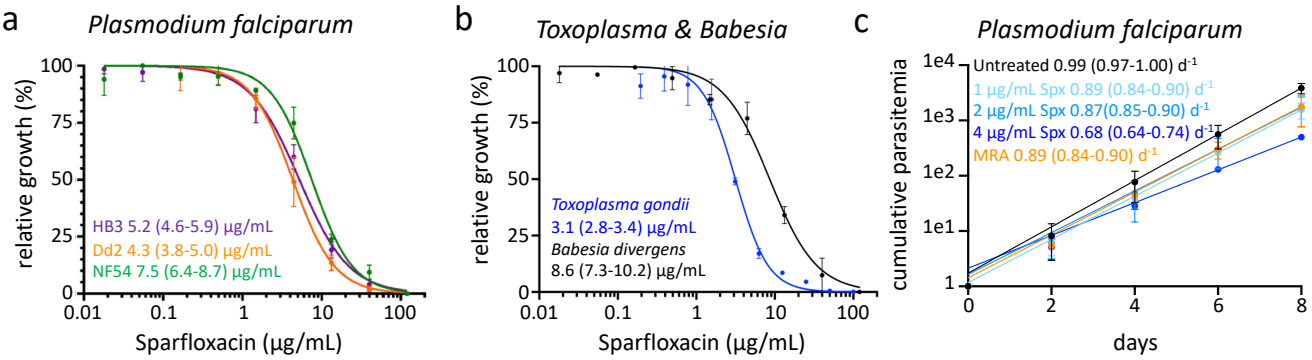

Figure 1. Dose-response of apicomplexan parasites to Sparfloxacin.
a) Sparfloxacin dose-response of *Plasmodium falciparum* isolates of African (NF54, green), Southeast Asian (Dd2, orange) and South American (HB3, blue) origin based on 72-hour SYBR Green growth assays. Estimated EC50 with 95% confidence are indicated based on n=2-3. **b)** Sparfloxacin dose-responses of *B. divergens* isolate Rouen 1987 and *T. gondii* were measured using on 72-hour SYBR Green or 96-hour luciferase growth assays, respectively. Estimated EC50s with 95% confidence are indicated based on n=2-3. **c)** Representative growth curves of *P. falciparum* NF54 maintained at 1/2/4 µg/mL Sparfloxacin, MRA (0.5 µg/mL), or no drug. Growth rates with 95% confidence interval are shown in days^{-1} based on n=2.

A low-cost, in-house PCR assay with internal controls is effective at detecting mycoplasma contamination.

We compared the ability to detect mycoplasma in DNA isolated from cultures of *P. falciparum* for an in-house PCR assay, based on universal mycoplasma primers targeting 425 bp of the locus encoding the 16S ribosomal RNA [17], to a the commercially available e-Myco Mycoplasma detection kit. To provide an internal PCR control for negative mycoplasmas results, we included small amount of pUC18 plasmid and primers to amplify 316 bp of the plasmid backbone. The commercial PCR product was able to detect mycoplasma in DNA extracted from mycoplasma-positive cultures even at 1:1000 dilution at a per-sample cost of $6.15. The in-house PCR assay was also effective

at detecting mycoplasma at a per-sample cost of $0.20 but the limit of detection was slightly lower at between 1:100 and 1:1000 (Figure 2). Carryover if amplicons from earlier PCRs can easily result in false positive results. To avoid this, we included thermolabile Uracil DNA glycosylase (UDG) and substituted dUTP for dTTP and in the PCR master mix. UDG generates breaks in dUTP-containing double-stranded DNA [18] during an initial incubation at 37°C, thus preventing previous amplicons from serving as templates [19], before being inactivated in the subsequent 94°C initial denaturation step.

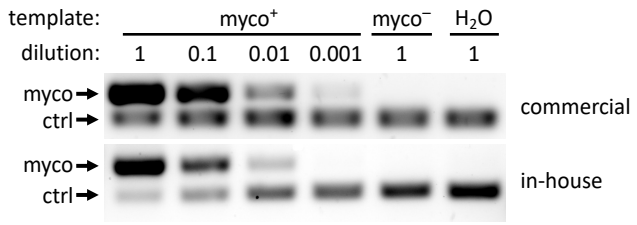

Figure 2. Detection of mycoplasma in *P. falciparum* cultures with commercial or in-house PCR assays.
A commercial PCR assay was able detect mycoplasma in DNA of mycoplasma-positive (myco$^+$) cultures even at 1:1000 dilution while the limit of detection for the in-house assay was between 1:100 and 1:1000. Results from undiluted mycoplasma-negative (myco$^-$) or no template (H_2O) are shown as controls. Images are representative of n=2.

Sparfloxacin is effective at clearing Mycoplasma, even when MRA resistant.
To evaluate the ability of Sparfloxacin to clear mycoplasma, mycoplasma-positive cultures were maintained either without treatment, with the manufacturer recommended 0.5 µg/mL of MRA, or with 1, 2, or 4 µg/mL of Sparfloxacin. For most cultures, one week of treatment with MRA and all concentrations of Sparfloxacin was able to reduce mycoplasma below the level of detection of the in-house PCR assay (Figure 3a). However, mycoplasma contamination in one culture proved to be resistant to clearance with MRA even after three weeks of treatment but was successfully cleared after one week of treatment with 4 µg/mL Sparfloxacin (Figure 3b).

This demonstrates that Sparfloxacin can be used to treat even MRA-resistant mycoplasma strains at concentrations that are well tolerated by a variety of apicomplexan parasites at a cost of $0.06 per liter of culture medium, making it a safe, effective, and low-cost alternative to MRA with its cost of $696 per liter of culture medium.

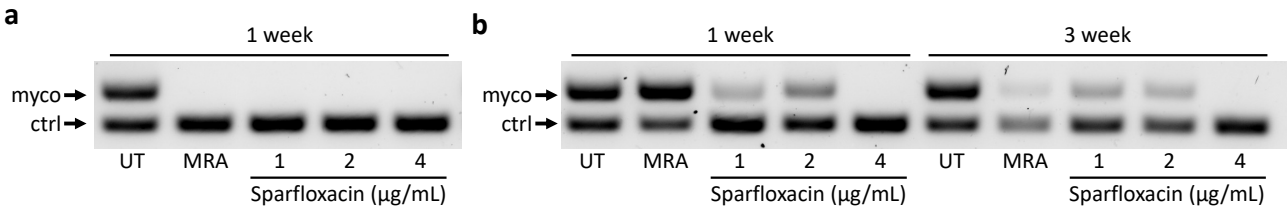

Figure 3. Effective clearance of mycoplasma with Sparfloxaxin.
a) Sparfloxacin was able to reduce MRA-sensitive mycoplasmas below the level of detection of the in-house PCR assay after one week of treatment at all concentrations tested. **b)** Sparfloxacin was able to clear MRA-resistant mycoplasma species from *P. falciparum* cultures after one week of treatment at 4 µg/mL when MRA at the recommended 0.5 µg/mL failed even after 3 weeks of treatment. Mycoplasma was detected using the in-house PCR assay and images are representative of n=2.

MATERIALS & METHODS

Parasite strains and maintenance

Plasmodium falciparum strains NF54, Dd2 and HB3 and *Babesia divergens* strain Rouen 1987 [20] were maintained using standard malaria culturing techniques [21]. *Toxoplasma gondii* strain RHΔku80::NLuc were maintained in Human Foreskin Fibroblasts (ATCC SCRC-1041) using standard culturing techniques [22]. Briefly, tissue cultures were maintained at 37°C with 5% CO_2 in D10 medium (Dulbecco's Modified Eagle Medium, 4.5 g/L glucose, VWR) supplemented with 10% Cosmic Calf serum (HycloneTM, GE Healthcare Life Sciences SH30087.03), 10 mM HEPES, additional 2 mM L-glutamine, and 10 mM Pen/Strep.

Mycoplasma detection

Mycoplasma contamination was detected using the e-Myco Mycoplasma detection kit (LiliF Diagnostics) according to manufacturer instructions. Alternatively, mycoplasma was detected using a custom PCR using Taq polymerase manufacturer instructions and 0.4 µM degenerate universal mycoplasma detection primers (GTGGGGAGCAAAYAGGATTAGA/GGCATGATGATTTGACGTCRT) that target the 16S locus [17] (see Figure 4). As an internal control, each reaction also contained 100 fg of pUC18 plasmid and 0.1 µM of primers (CCTGACGAGCATCACAAAAA / AGTCGTGTCTTACCGGGTTG) that amplify a 316 bp section of the plasmid backbone. PCR master mix reagents: *Taq* polymerase and 10X Reaction Buffer (NEB M0273S), nucleotide mix containing 10 mM dATP, dGTP, dCTP (NEB N0446S) and dUTP (NEB N0459S) each, thermolabile Uracil DNA glycosylase (Invitrogen, 239828) and pUC18 (or any other plasmid with pBlueScript II backbone). 20 µL aliquots of master mix were stored at -20 °C until use.

Number of Reactions	1	64
Nuclease-free Water	14	896
1 U/µL Uracil DNA glycosylase	0.2	12.8
10X Reaction Buffer	2.5	160
5 U/µLTaq Polymerase	0.2	12.8
nucleotide mix with dUPT	0.5	32
10 µM BKO_2364	1	64
10 µM BKO_2365	1	64
10 µM BKO_2377	0.2	12.8
10 µM BKO_2378	0.2	12.8
10 pg/µL pUC18	0.2	12.8
Master Mix Total	20	1280

	Temp. (°C)	Time (min:sec)	number of cycles
prior amplicon degradation	37	10:00	1
initial denaturation	94	10:00	1
denaturation	94	0:30	
annealing	58	1:00	45
extension	68	1:00	
final extension	68	∞	1

Figure 4: Mycoplasma Detection PCR master mix composition and cycling conditions. Note that the PCR is maintained at a final temperature of 68 °C instead of 4°C as suggested when including Uracil DNA glycosylase.

Dose-response assays

Sparfloxacin (ThermoScientific J66358-09) was dissolved in DMSO to a stock concentration of 2 mg/mL. For *P. falciparum* and *B. divergens* asexual blood-stages the dose response to Sparfloxacin was determined using the 72-hour SYBR Green Growth Assay [23] as described in detail in [24]. For *T. gondii,* 1,500 RHΔ*ku80*::*NLuc* tachyzoites were inoculated per-well in a 96-well plate format and the dose response was determined by a 96-hour luciferase-based growth assay as described in detail in [22]. In brief, the NanoLuc luciferase-expressing *Toxoplasma* parasites were allowed to grow in human foreskin fibroblasts for 4 hrs. Non-invaded parasites were washed off and the media were replaced with replaced with fresh D10 supplemented with Sparfloxacin. Ten concentrations generated by 2-fold serial dilution were tested starting at 100 µg/mL.

After 96-hour incubation with Sparfloxacin, the media were gently aspirated and the infected host cells were treated in the lysis buffer (100 mM 4-Morpholineethanesulfonic acid (MES) pH 6.0, 1 mM trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), 0.5% (v/v) Tergitol, 0.05% (v/v) Mazu DF 204, 150 mM KCl, 1 mM DTT, and 35 mM thiourea) containing 12.5 µM Coelenterazine H and incubated at room temperature for 10 min prior to luminescence quantification using a BioTek Synergy H1 Hybrid plate reader. The luminescence signals from sparfloxacin-treated cells were normalized against that from infected cells grown in plain D10 medium. The IC50 concentration for each species was estimated using non-linear fit using Prism (GraphPad).

Effect of Sparfloxacin on *P. falciparum* growth rate

Growth of P. falciparum cultures were followed by measuring culture parasitemia every other day for 8 days by flow cytometry after staining live cultures with 16 µM Hoechst 33342 and 200nM Thiazole Orange for 30 min at 37°C. Cultures above 2% were diluted to 0.5% and the dilution factor was noted. Cumulative parasitemia was calculated by multiplying the percentage of infected cells by the overall dilution factor and the growth rate was determined by exponential fit using Prism (GraphPad).

ACKNOWLEDGMENTS

Funding: This work was supported by funds from Weill Cornell Medicine (BFCK), NIH 1R01 AI141965 (BFCK), 1R21AI166436 (BFCK), NIH 1R01 AI138499 (BFCK), the Frueauff Family Foundation (BFCK), Clemson Startup fund (to Z.D.), Knights Templar Eye Foundation Pediatric Ophthalmology Career-Starter Research Grant (to Z.D.), a pilot grant of the NIH COBRE grant P20GM109094 (to Z.D.), and NIH R01AI143707 (to Z.D.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions: Conceptualization: BFCK, KK; Methodology: BK, KK, ZD; Investigation: DRMR; Writing – Original Draft: BFCK Writing – Review & Editing: BFCK, KK; Visualization: KK, BFCK; Supervision: BFCK, KK, ZD; Project Administration: BFCK; Funding Acquisition: BFCK, ZD.

Competing interests: The authors declare no competing interests.

Data and materials availability: No new materials were generated in this study.

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