A single point mutation in the *Plasmodium falciparum ftsh1* metalloprotease confers actinonin resistance.

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Abstract

The antibiotic actinonin kills malaria parasites (*Plasmodium falciparum*) by interfering with apicoplast function. Early evidence suggested that actinonin inhibited prokaryote-like post-translational modification in the apicoplast; mimicking its activity against bacteria. However, Amberg Johnson et al. (2017) identified the metalloprotease *Tg*FtsH1 as the target of actinonin in the related parasite *Toxoplasma gondii* and implicated actinonin in the inhibition of *P. falciparum* growth. The authors were not, however, able to recover actinonin resistant malaria parasites, leaving the specific target of actinonin uncertain. We generated actinonin resistant *P. falciparum* by *in vitro* selection and identified a specific sequence change in *PfftsH1* associated with resistance. Re-Introduction of this point mutation using CRISPr-Cas9 allelic replacement was sufficient to confer actinonin resistance in *P. falciparum*. Our data unequivocally identifies *Pf*FtsH1 as the target of actinonin and suggests that actinonin should not be included in the highly valuable collection of "irresistible" drugs for combatting malaria.

Introduction

Actinonin is an anti-bacterial and anti-parasitic antibiotic derived from streptomycete bacteria [1,2]. In bacteria, actinonin targets peptide deformylase (*Pfpdf* - PF3D7_0907900) [3], an enzyme involved in prokaryotic post-translational modification and also present in the relict plastid (apicoplast) of apicomplexan parasites. Actinonin causes defects in malaria parasite apicoplast development [4] and inhibits recombinantly expressed *Plasmodium falciparum* PDF *in vitro* [5] at concentrations consistent with its anti-parasitic activity, all of which led to the general conclusion that actinonin targets the apicoplast-localized *Pf*PDF in malaria parasites. However, the characteristics of actinonin — particularly the rapid mode of action and the unusual kinetics of apicoplast genome loss —are at odds with how all other drugs targeting apicoplast translation impact the parasite [6,7]. In a search for the target of actinonin, Amberg-Johnson et al. [6] used the related apicomplexan *Toxoplasma gondii* and identified a point mutation in the putative metalloprotease *TgftsH1* that confers a 3.5-fold resistance to actinonin. They also showed that actinonin inhibits recombinantly expressed human malaria parasite FtsH1 (*Pf*FtsH1) *in vitro* at levels comparable to its antimalarial activity [6]. Moreover,

parasites with reduced *Pf*FtsH1 expression were more sensitive to actinonin, all of which prompted the interim conclusion that *Pf*FtsH1, rather than *Pf*PDF, might be the target of actinonin and that *Pf*FtsH1 is a potential new antimalarial target [6].

Despite repeated attempts, Amberg-Johnson et al. [6] were not able to generate actinonin resistant *P. falciparum* parasites. Interestingly, the residue mutated from asparagine to serine (N805S) in *Tg*FtsH1 identified as conferring actinonin resistance by Amberg-Johnson et al. [6] is already a serine in *Pf*FtsH1 (Table 1), which begs the question of whether *Pf*FtsH1 is already 'resistant' to actinonin. This might mean that actinonin kills malaria parasites through a mechanism not involving *Pf*FtsH1, perhaps even inhibition of *Pf*PDF. Compounding this uncertainty is a report that *Pf*FtsH1 is localized in the mitochondrion [8], which would not be consistent with the demonstrated impact of actinonin on the malaria parasite apicoplast [4 7].

To determine if *Pf*FtsH1 is the target of actinonin, we generated *P. falciparum* parasites with robust resistance to actinonin, identified a point mutation conferring resistance, and recapitulated the resistance phenotype by introducing a single amino acid change using CRISPrCas9 genome editing.

Results and Discussion

P. falciparum strain D10 parasites were selected for resistance using stepwise increases in actinonin concentration. Ten million parasites were treated with twice the IC_{50} of actinonin, which resulted in no parasites being detectable in the culture by microscopy. Fresh, drug-containing media was regularly provided until parasites were again detectable by microscopy, and normal growth rate had resumed. Drug concentration was then increased two-fold and the process repeated until parasites were growing vigorously in media containing 20 µM actinonin. Both the parasite strain and selection methodology used differ from previous attempts to generate resistance [6], which may explain why we obtained resistance where others did not.

Table 1. The impact of mutations in *ftsh1* on parasite resistance to actinonin.

Parasite	FTSH1 Peptidase Motif (partial amino acid sequence)	Actinonin IC_{50} (µM)
***Tg* FTSH1 WT (TGGT_259260)**	804 FGRDALS**N**GASSDI 811	14[a]
***Tg* FTSH1 Act^R**	804 FGRDALS**S**GASSDI 811	44[a]
***Pf* 3D7 FTSH1 (PF3D7_1239700)**	481 FGKSETSS**G**ASSDI 494	1.99 (n=1)[b]
***Pf* D10 FTSH1 WT**	481 FGKSETSS**G**ASSDI 494	2.0 ± 0.2 (n=4)
***Pf* D10 FTSH1 Act^R**	481 FGKSETSS**C**ASSDI 494	73.3 ± 2.7 (n=4)
***Pf* D10 (apicoplast minus)**	481 FGKSETSS**G**ASSDI 494	43.1 ± 4.1 (n=2)[c]

[a]calculated from data provided in [6], [b] and [c] are both consistent with previously published data [4,6].

Several clones were generated from our actinonin resistant parasite line, and each showed consistent actinonin resistance, with IC_{50} values 18 to 35-fold higher than the parental line (Table 1, Supp Table 1). The clone with the highest level of resistance showed an IC_{50} of 73.3 µM actinonin (Table 1). We genotyped four actinonin resistant clones, and all retained wild type sequences of *Pf pdf*, *Pf formyl-methionyl transferase* (*Pffmt* - PF3D7_1313200), and *Pf methionyl amino peptidase* (*Pfmap* - PF3D7_0804400) suggesting that neither *Pf*PDF nor the related apicoplast post-translational protein modifying enzymes are the primary target of actinonin. Similarly, all four actinonin resistant clones retained wild type sequence for *PfRING* (PF3D7_1405700), another actinonin target candidate[6]. However, each of the clones harbors a single nucleotide polymorphism in *Pfftsh1* that changed amino acid 489 (adjacent *Tg*FtsH1 N805S) from glycine to cysteine (Table 1, Supplementary Table 1), strongly implying that *Pf*FtsH1 is the primary target of actinonin.

To unequivocally confirm that *Pf*FtsH1 is the primary target of actinonin, and that the G489C mutation is sufficient to confer resistance, we used CRISPr Cas9 mutagenesis to introduce the mutation (with minimal collateral genome disruption) into the native *Pfftsh1* gene (Figure 1A). Accordingly, a parasite clone carrying synonymous "shield" mutations in the *Pfftsh1* coding sequence designed to prevent ongoing Cas9 cleavage but retaining glycine 489 (rFtsH1^{G489G}) remained sensitive to actinonin (Figure 1B), whereas two independent clones (rFtsH1$^{G489Ca/b}$) modified to have the

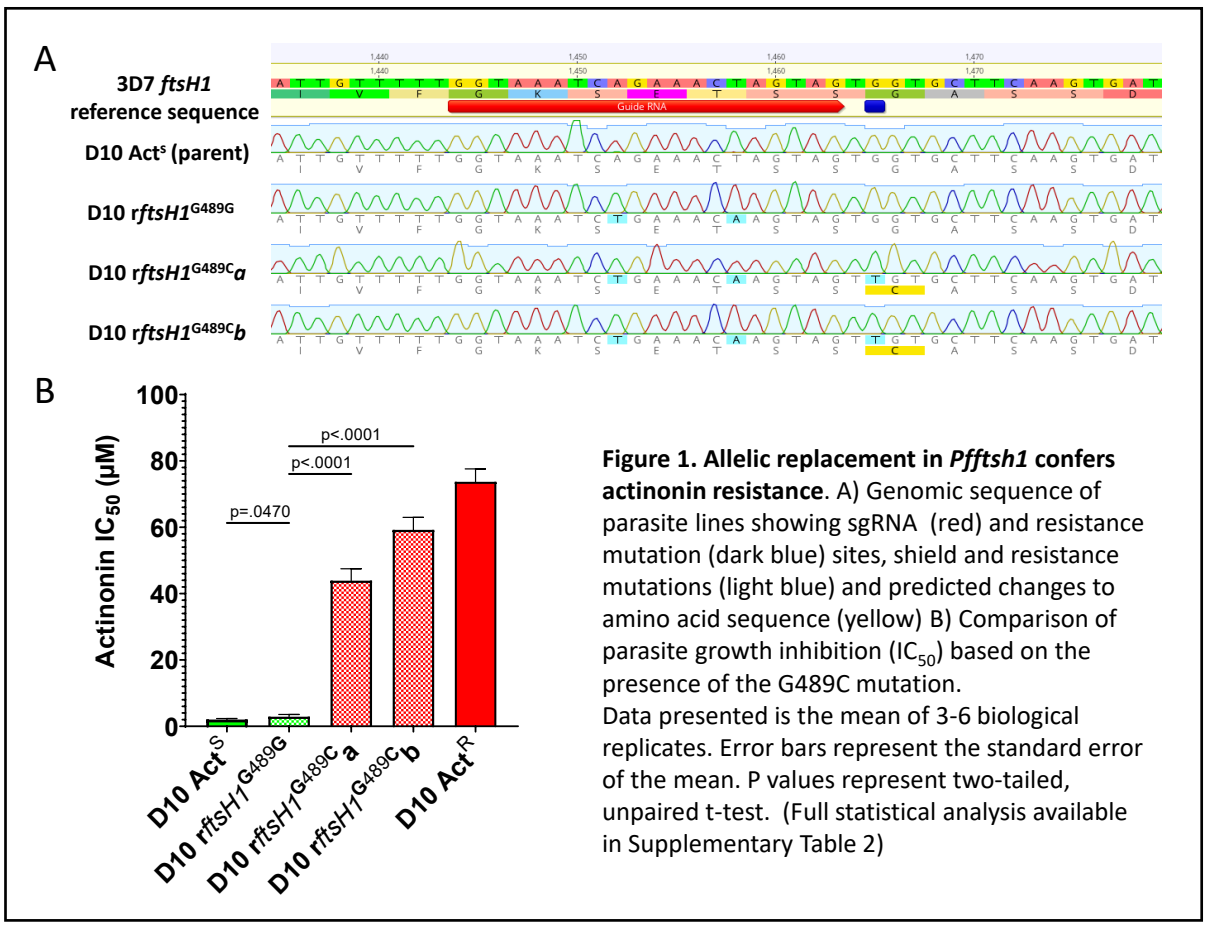

Figure 1. Allelic replacement in *Pfftsh1* confers actinonin resistance. A) Genomic sequence of parasite lines showing sgRNA (red) and resistance mutation (dark blue) sites, shield and resistance mutations (light blue) and predicted changes to amino acid sequence (yellow) B) Comparison of parasite growth inhibition (IC_{50}) based on the presence of the G489C mutation.
Data presented is the mean of 3-6 biological replicates. Error bars represent the standard error of the mean. P values represent two-tailed, unpaired t-test. (Full statistical analysis available in Supplementary Table 2)

G489C mutation (in addition to the "shield" mutations) showed actinonin resistance levels comparable to the actinonin-selected line (wtACT^{R}) (Figure 1B).

Robust actinonin resistance in *P. falciparum* resulting from the G489C mutation confirms that *Pf*FtsH1 is indeed the primary target of actinonin. That the resistance levels in *Pf*FtsH1 G489C parasites are of the same order of magnitude as that seen in lines that lack an apicoplast (Table 1), strengthens the conclusion that *Pf*FtsH1 has a role in apicoplast biogenesis, the anomalous localization [8] notwithstanding. The greater levels of resistance achievable through selection, while modest, suggests that these lines may have acquired other mutations that compensate for reduced *Pf*FtsH1 function and/or alter secondary actinonin targets, such as the other metalloproteases present in the genome[6]. Our ability to generate resistance to actinonin in a relatively small starting population of *P. falciparum* parasites means actinonin is not an "irresistible" drug[9], which tempers enthusiasm for development of *Pf*FtsH1 as an antimalarial target.

Material and Methods

P. falciparum D10 were cultured according to standard protocols[7,10]. Apicoplast-minus parasites were generated according to previously described methods[7,11]. To generate actinonin resistant parasites, 10^7 D10 parasites were treated with 2x the measured IC_{50} actinonin (Sigma-Aldrich) concentration and cultured until parasites began growing robustly. The actinonin concentration was then increased 2-fold and the culturing repeated until parasites grew normally at 20 µM actinonin. Resistant parasites were cloned by limiting dilution and retested to confirm the resistance phenotypes. Drug effects were assayed after 72 hours of drug exposure using the SYBR Green (ThermoFisher) method [7,12].

Genomic DNA was isolated using 200 µL of parasite culture (*Isolate II Genomic DNA kit*, Bioline). Candidate actinonin resistance genes were amplified using the primers listed in Supplementary Table 3. CRISPr edited FtsH1 clones were amplified using primers in Supplementary Table 4. Products were purified (*Isolate II PCR and Gel kit*, Bioline) and Sanger sequenced (Australian Genome Research Facility, Parkville). Alignment and analysis of sequenced genes was done using Sequencher (Gene Codes Corporation, Ann Arbor, MI USA) and Geneious Prime (www.geneious.com).

CRISPr-Cas9 mediated gene-editing utilized pAIO[13] expressing Cas9 and the *Pfftsh1* specific sgRNA 5'-GTAAATCAGAAACTAGTAG-3' inserted according to standard protocols [14] . Two allelic replacement vectors—pFtsH1^{G489G} carrying two synonymous "shield" mutations and pFtsH1^{G489C} carrying a further G to T mutation at base 1465 (Figure 1A)—were created by cloning a PCR amplified segment of *Pfftsh1* into pGEM-T Easy (Promega). Quickchange XL (Clontech) was used to make sequential modifications for allelic replacement constructs. The shield mutations were introduced first and then the plasmid carrying the confirmed shield mutations was modified to also include the putative

resistance mutation (G1465T). All constructs were confirmed by sequencing.

Each allelic replacement vector was linearized by digestion with *Eco*RI and co-transfected with pAIO-*Pfftsh1*[15]. Transfected parasites were selected by including 10 DSM-1 (Sigma-Aldrich) in the culture media for 14 days (rFtsH1^{G489G} and rFtsH1^{G489Ca}) or 7 days (rFtsH1^{G489Cb}) followed by 10-14 days of culture without drug until parasites grew normally in culture. Parasites were cloned by limiting dilution and three to five clones of each line were screened for actinonin sensitivity and successful modification of the *Pfftsh1* allele. All clones from rFTSH1^{G489G} and rFtsH1^{G489Ca} had the expected gene modifications while only one of five clones from rFtsH1^{G489Cb} did. Actinonin sensitivity was correlated to the presence of the G489C mutation in all clones tested. One clone from each recombinant line was selected for complete characterization of actinonin sensitivity.

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Competing Interests

The authors declare no financial or non-financial competing interests

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Supplementary Data

Supplementary Table 1. Screen of D10 clones for *Pfftsh1* sequence and actinonin resistance.

Act^R clone	**Nucleotide sequence of PF3D7_1239700 (*Pf*FtsH1)**	**Actinonin IC$_{50}$ (µM)**
Pf D10 WT	1451 CAGAAACTAGTAGT**G**GTGCTTCAAGTGATA 1480	2.0 ± 0.2 (n=4)
Pf D10 Act^R Clone 1	1451 CAGAAACTAGTAGT**T**GTGCTTCAAGTGATA 1480	73.3 ± 2.7 (n=3)
Pf D10 ACT^R Clone2	1451 CAGAAACTAGTAGT**T**GTGCTTCAAGTGATA 1480	61.6 ± 14.4 (n=2)
Pf D10 ACT^R Clone3	1451 CAGAAACTAGTAGT**T**GTGCTTCAAGTGATA 1480	51.4 (n=1)
Pf D10 ACT^R Clone4	1451 CAGAAACTAGTAGT**T**GTGCTTCAAGTGATA 1480	36.2 (n=1)

Supplementary Table 2: Descriptive statistics for growth inhibition trials in Figure 1.

	D10 Act^S	D10 r*ftsH1*^{G489G}	D10 r*ftsH1*^{G489C}a	D10 r*ftsH1*^{G489C}b	D10 Act^R
n	4	6	3	5	3
Mean	2.024	2.894	43.88	59.19	73.29
Median	2.117	2.887	43.60	58.71	71.69
SD	0.32	0.68	3.61	3.78	4.64
SEM	0.16	0.28	2.08	1.70	2.68
95% CI	1.52-2.53	2.18-3.61	34.92-52.84	54.49-63.88	61.76-84.82

Supplementary Table 3: Oligos for PCR amplification of potential actinonin targets.

GeneID	Forward primer	Reverse primer
PF3D7_0907900 *Pfpdf*	TCACTCAGGAAAACCACAACT	CCCCCAGAAACAAAAGAACA
PF3D7_1313200 *Pfftsh1*	TCCAATCTAAGAAATATTCGACCCCT	AATGGAGAGAATTCTATGCCTCTT
PF3D7_0804400 *Pfmap*	GCTTCCTGTTGGGGTGTTT	AAAATTGTCATTTTGTTTAACACTT
PF3D7_1239700 *Pffmt*	GAGCAATTGAAAGGATGGAA	TTTTCCAAAACAACAAATAAAACA
PF3D7_1405700 *PfRING*	AAAATCCTCTTCGCACATTTTT	TTGATTATCACAAATGCTCATTCA

Supplementary Table 4: Oligos for generation and sequencing of allelic replacement constructs.

Pfftsh1 primer	Forward primer	Reverse primer
sgRNA	TAAGTATATAATATTTTCTGTTTTATTGCATGATGGTTTTAGAGCTAGAA	TTCTAGCTCTAAAACCATCATGCAATAAAACAGAAAATATTATATACTTA
Genome segment for allelic replacement	CCTAGGAAATGGGTGCTAGAATGCC	GCATGCAGCGACTAGTAAGAATATTTAT
sgRNA sequencing	AAAAAATTCTTGCTTGTTCAGA	CGGCCGCTATTTCATCTATTT
Shield mutations	CTTGAAGCACCACTACTTGTTTCAGATTTACCAAAAACAATTTCTTCAGC	GCTGAAGAAATTGTTTTTGGTAAATCTGAAACAAGTAGTGGTGCTTCAAG
G489C mutation	ATATATCACTTGAAGCACAACTACTTGTTTCAGATTTACCAAAAACAATTTCTTCAG	CTGAAGAAATTGTTTTTGGTAAATCTGAAACAAGTAGTTGTGCTTCAAGTGATATAT
Mutation sequencing	TGAAGCTGGTCATGCTATCG	TGATCTCTTCTCCGGATAAGG