Whole genome sequencing to evaluate the resistance landscape following antimalarial treatment failure with fosmidomycin-clindamycin

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Abstract

Novel antimalarial therapies are needed in the face of emerging resistance to artemisinin combination therapies. A previous study found a high cure rate in Mozambican children with uncomplicated *Plasmodium falciparum* malaria 7 days post treatment with a fosmidomycin-clindamycin combination. However, 28-day cure rates were low (45.9%), due to parasite recrudescence. We sought to identify any genetic changes underlying parasite recrudescence. To this end, we utilized a selective whole genome amplification method to amplify parasite genomes from blood spot DNA samples. Parasite genomes from pre-treatment and post-recrudescence samples were subjected to whole genome sequencing to identify nucleotide variants. We find that our data do not support the existence of a genetic change responsible for recrudescence following fosmidomycin-clindamycin treatment. Additionally, we find that previously described resistance alleles for these drugs do not represent biomarkers of recrudescence. Future studies should continue to optimize fosmidomycin combinations for use as antimalarial therapies.
Background

Malaria remains a serious global health concern, with ~214 million cases and 483,000 deaths due to malaria in 2015 [1]. A majority of severe malaria cases occur in pregnant women and children under five years of age and result from infection with the parasite Plasmodium falciparum [1]. Artemisinin combination therapies (ACTs) represent the current first-line treatment in endemic areas. Historically, the development of drug resistance has hindered malaria control. As delayed parasite clearance has emerged for ACTs in Southeast Asia and has continued to spread [1,2], new antimalarials are urgently needed.

One such potential therapeutic is the phosphonic acid antibiotic fosmidomycin (FSM). FSM is a well-characterized inhibitor of the first committed step of isoprenoid precursor synthesis via the methylerythritol phosphate (MEP) pathway [3–5]. Because humans synthesize isoprenoids via an enzymatically distinct metabolic route (mevalonate pathway), FSM is a highly specific inhibitor of malaria parasite growth [3,5] and is well-tolerated in humans [6,7].

FSM was originally developed as an antibacterial [8] and is currently under evaluation as a partner agent for combination therapy against uncomplicated *P. falciparum* malaria (clinicaltrials.gov identifier NCT02198807). Several studies have paired FSM with the antibiotic clindamycin (CLN), a protein translation inhibitor (clinical trials NCT02198807, NCT01361269, NCT01002183, NCT00214643, and NCT00217451) [6,9,10]. One such
Phase II clinical trial, performed in Mozambique in 2010, evaluated the efficacy of a FSM-CLN combination on uncomplicated malaria in children aged 6-35 months, the youngest cohort tested to date [11,12]. A high parasite cure rate was observed at day 7 post-treatment with FSM-CLN (94.6%). Unfortunately, the cure was not durable, and the post-treatment PCR-corrected day 28 cure rate was 45.9% (17/39), due to recrudescent infections. Parasite recrudescence is common in clinical studies of FSM efficacy, with an overall day 28 cure rate of ~70% in adults [12].

The safety and specificity of MEP pathway-targeting therapeutics, such as FSM, are highly desirable. However, the failure of FSM treatment to result in lasting cure has led to concern regarding the clinical utility of FSM or related antimalarials. We recently found that FSM resistance is readily achieved in culture due to mutations in the metabolic regulator PfHAD1 (PlasmoDB ID PF3D7_1033400) [13]. Additionally, CLN resistance has been reported in clinical isolates of P. falciparum, attributed to mutation in the apicoplast 23S rRNA [14].

In this study, we address whether observed recrudescence is produced by selection for mutations in previously identified candidate genes or novel resistance loci. Using selective whole genome amplification (SWGA) and sequencing, we characterize P. falciparum field isolates from blood spot samples to evaluate the genetic landscape of drug resistance before and after FSM-CLN treatment. Specifically, we evaluate for enrichment in genetic changes associated with decreased susceptibility to either FSM or CLN.
Methods

Study information
The study criteria have been previously described [11]. The study evaluated 37 children ages 6-35 months with uncomplicated malaria. Inclusion and exclusion criteria are outlined in the original study [11]. Patients were administered a three-day, twice-daily course of oral FSM-CLN. Blood spots were collected at days 0, 7, 14, and 28, and if applicable, upon recrudescence.

Clinical trial information
The original trial in Mozambique, sponsored by Jomaa Pharma, was conducted in 2010 according to the ICH Good Clinical Practice guidelines. The protocol was approved by the National Mozambican Ethics Review Committee and the Hospital Clinic of Barcelona Ethics Review Committee. The clinicaltrials.gov identifier is NCT01464138.

Selective whole genome amplification
Blood spotting and DNA isolation has been previously described [11]. SWGA of \textit{P. falciparum} genomes was performed as described previously [15], including primer sets (Supplementary Table 2) and reaction conditions. Reactions contained 10 ng template and 3.5 μM primer set 6A. Reactions were cleaned using Ampure beads (Beckmann Coulter) at a 1:1 DNA:bead ratio. Samples underwent a second round of SWGA using primer set 8A and 15-30 ng DNA.
**PCR of SWGA products**

Selective amplification of *P. falciparum* genomes was verified by PCR using *P. falciparum*- or human-specific primers (Supplementary Table 1). Human DNA and human-specific primers were kindly provided by Shiming Chen (Washington University). Reactions contained 2 μM each primer, 25-50 ng bead-cleaned SWGA product, and BIO-X-ACT Short PCR Mix (Bioline). Vendor-recommended cycling conditions were used, with modifications: (55 °C and 60 °C annealing for *P. falciparum*- and human-specific primers, respectively; 68 °C extension). Amplicons were visualized by agarose gel electrophoresis.

**Whole genome sequencing**

Library preparation, Illumina sequencing, read alignments, and variant calling were performed by the Washington University Genome Technology Access Center (GTAC). Bead-cleaned SWGA product DNA (0.5-1.2 μg) was sheared, end-repaired, and adapter-ligated. PCR-based libraries were sequenced on an Illumina HiSeq 2500 to generate 101 bp paired end reads. Reads were aligned to the 3D7 reference (PlasmoDB v24) using Novoalign (V2.08.02) [16]. Duplicate reads were removed. SNPs were called using samtools (mpileup) [17], filtered (quality ≥20, read depth ≥5), and annotated using snpEff (3.3c, build 2013-06-28) [18].

*P. falciparum* reads are available through the NCBI BioProject database (PRJNA315887) and Sequence Read Archive (SRP072442).
For some analyses (comparison of SNPs in resistance genes, GO analysis), only sample pairs with both exomes showing sufficient coverage (≥60% covered at ≥5X, indicated in Supplementary Table 3) were used. Given a per-base error rate of 0.1-0.5% for Illumina sequencing [19,20], 5X coverage equates to >99.9% accuracy. Studies have indicated reasonable sensitivity and accuracy for our variant caller at this cutoff [21].

**SNPhylo plot generation**

Multi-sample VCFs were converted to hapmap format. SNPhylo [22] was run with the default settings, with an LD cutoff of 0.8.

**Multiplicity of infection (MOI) determination**

MOI was determined using the WHO-recommended PCR-based genotyping procedures at the *MSP1* (PF3D7_0930300), *MSP2* (PF3D7_0206800), and *GLURP* (PF3D7_1035300) loci [23].

**Sequencing of the apicoplast 23S rRNA locus**

The apicoplast 23S rRNA (PlasmoDB ID PCF10_API0010:rRNA) locus was amplified by PCR. Reactions contained 2 μM of each primer 23S_1 and 23S_6 (Supplementary Table 1), 4-5 μL of blood spot DNA, and CloneAmp HiFi PCR Premix (Clontech). Vendor-recommended cycling conditions were used (55 °C primer annealing).
Amplicons were Sanger sequenced using primers 23S_1 – 23S_6 (Supplementary Table 1).

**GO analysis**

GO term enrichment of genes containing SNPs unique to post-treatment samples was determined using the PlasmoDB Gene Ontology Enrichment tool [24] (Bonferroni-corrected p-value <0.01).

**Results**

Selective whole genome amplification of blood spot DNA generates parasite templates for whole genome sequencing

We evaluated blood spot DNA (mixed human and *P. falciparum*) from 12 patient samples with microscopic recrudescent infection (12 pre- and post-treatment pairs, 24 samples total). Pilot unmodified, low-input library preparation methods resulted in <5% reads mapping to the *P. falciparum* genome. For this reason, we used SWGA to amplify *P. falciparum* DNA. This method uses the processive Φ29 DNA polymerase and genome-specific primers to selectively amplify a target genome from a mixed sample [25–27] and has been recently used to characterize chimpanzee *Plasmodium* genomes [15].

Enrichment of the *P. falciparum* genome was verified by PCR (Figure 1). We amplified *P. falciparum* genomes from a broad range of parasite densities (18-315,064
parasites/μL whole blood). Amplified samples were used to prepare libraries for Illumina sequencing.

The samples displayed varying degrees of sequencing success (Supplementary Table 3). An average (± S.E.M.) of 55.1% ± 2.9% of reads mapped to the *P. falciparum* genome, resulting in 48.1% ± 3.5% of the genome covered at ≥5X. This is comparable to previous studies using SWGA to sequence microbial genomes [27,28]. The genome coverage we obtain is consistent with very low proportions (<0.01%) of parasite DNA in the blood spot samples [15]. While more reads will increase genome coverage, including typically low-coverage intragenic regions, coverage is likely to be limited by incomplete genome representation in the sample, as very low proportions of parasite DNA are likely to result in incomplete or inefficient SWGA of some genome regions.

Given the low coverage of AT-rich intragenic regions, our analyses focused on protein-coding regions of the *P. falciparum* genome. We observe an average of 69.0% ± 4.4% of the exome covered ≥5X, with as much as 90% of the exome covered ≥5X (Supplementary Table 3).

We observe an average of 35,451 ± 1,220 total genome SNPs in our sequenced samples, consistent with recent studies of African field isolates [29,30]. Of these, 15,862 ± 534 are exome SNPs. We find an average of 10,755 ± 349 non-synonymous SNPs in our samples with sufficient exome coverage (≥60% covered at ≥5X).
SNP profiling confirms recrudescent infections

Maximum-likelihood phylogenetic tree construction from our exome SNP profiles from the 12 pre- and post-treatment sample pairs demonstrates clustering by patient-of-origin (Figure 2). These data support the conclusion that all infections analyzed indeed reflect failure to completely clear the original infection (recrudescence), as opposed to novel, independent infections. Pre-treatment and post-treatment samples share a majority (66.0 ± 3.4%) of non-synonymous SNPs, while independent (between-patient) pre-treatment infections are less related and share only 24.6 ± 0.4% of non-synonymous SNPs (strains with ≥60% of the exome at ≥5X, N=8). These data confirm the genotyping reported in the original study [11], which established recrudescence through WHO-recommended PCR-based genotyping at three *P. falciparum* loci: *MSP1*, *MSP2*, and *GLURP* [23].

Multiplicity-of-infection (MOI) pre- and post-treatment

In high transmission areas, patients may be simultaneously infected with multiple parasite strains. This genetic variation provides a potential reservoir for the development of resistance to antimalarials, both within a given patient and in the larger *P. falciparum* population. Multiplicity-of-infection (MOI) has been shown to decrease after recrudescence following chloroquine treatment [31], and, unsurprisingly, increased MOI is correlated with treatment failure [32]. However, during any individual infection, selective pressures during infection (such as immune evasion) and drug treatment are expected to decrease genetic diversity. We therefore assessed MOI before and after recrudescence.
All infections were polyclonal, with a mean pre-treatment MOI of 3.9 ± 0.3. This is slightly higher than MOIs reported in other pediatric studies in Mozambique [33,34]. MOI decreased modestly in two-thirds of the samples after treatment and recrudescence, with a mean post-treatment MOI of 3.2 ± 0.2 (p=0.0065, paired t-test). This reduction was not a result of lower parasitemias in recrudescent infections, as average post-treatment parasite density was approximately equal to average pre-treatment parasite density (76,225 ± 33,944 vs. 70,977± 20,498 parasites/μL). Additionally, MOI and parasite density were not correlated (Pearson r = -0.041, p = 0.849).

Of note, loss-of-function mutations in the FSM-resistance gene PfHAD1 have been infrequently reported in the genomes of field P. falciparum isolates (PlasmoDB, accessed April 2016), suggesting that such alleles are rare [24]. Therefore, if resistance emerges during the course of infection in a given patient, recrudescence due to outgrowth of a rare resistant clone should result in a dramatic decrease in MOI. This was not observed in our population, suggesting that FSM resistance alone is unlikely to account for recrudescence of patients treated with FSM-CLN.

**Genotyping of known resistance loci does not reveal a change in the resistance landscape between pre- and post-treatment samples**

To evaluate whether FSM-CLN recrudescent parasites have genetic changes associated with drug resistance, we investigated the genomes of paired pre- and post-treatment parasites for genetic markers of antimalarial resistance. We evaluated loci
previously implicated in resistance to FSM or CLN, as well as loci documented to contribute to resistance to other clinically available antimalarials.

FSM targets the isoprenoid synthesis enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (PfDXR, PF3D7_1467300). Following FSM-CLN treatment, recrudescent samples are not enriched in alternative PfDXR alleles (Figure 3, Supplementary Table 4). One sample (114) possesses the Q361R PfDXR allele, previously reported in African isolates [24]. The homologous residue (R276) in FSM-sensitive Escherichia coli DXR (NCBI reference NP_414715.1) is identical to the mutated residue (361R). Therefore, the DXR Q361R variant is unlikely to confer FSM resistance.

We have recently identified a metabolic regulator, PfHAD1, whose loss results in FSM resistance in cultured P. falciparum [13]. No PfHAD1 loss-of-function alleles were identified in our samples (Figure 3, Supplementary Table 4), consistent with a known lack of variation in this gene in field samples [24]. The N70S allele seen in a number of strains has been previously reported as a common wild-type variant [24], which does not confer FSM resistance [13]. Two pre-treatment samples (103 and 118) possessed additional polymorphisms in PfHAD1, S265L and E150K, respectively (Figure 3, Supplementary Table 4). These alleles are not predicted to affect PfHAD1 function [Polyphen-2 scores < 0.005 (benign)] [35] and are not enriched in the recrudescent samples. Altogether, our data indicate that known markers of FSM resistance in PfDXR and PfHAD1 do not emerge during the course of FSM treatment. Thus, known
mechanisms of FSM resistance observed in the laboratory are unlikely to represent the underlying mechanism behind FSM-CLN failure. Since recrudescent parasites were not culture-adapted, we cannot distinguish whether these parasites were resistant or just failed to be cleared. Therefore, it is possible that resistance-causing mutations in \textit{PfDXR}, \textit{PfHAD1}, or other loci might occur if parasites were subjected to longer drug exposures.

Resistance to the FSM partner agent, CLN, has also been reported in \textit{P. falciparum} [14]. In this previous study, mutation (A1875C) in the 23S rRNA locus (PFC10\_API0010) is associated with an approximately 100-fold increase in clindamycin \textit{IC}_{50} for clinical isolates [14]. Insufficient coverage of the plastid genome was obtained from whole genome sequencing; we therefore used targeted gene amplification and sequencing to interrogate for polymorphisms in PFC10\_API10010. No samples possessed the A1875C variant. All sequenced samples matched the FSM- and CLN-sensitive 3D7 control strain (Supplementary Data 1). Notably, from our sequencing of laboratory 3D7 and HB3 strains, we identify variations not reported in the genome reference (T451A, A454T, and 401insG) (Supplementary Data 1) [24]. These changes may have emerged over time during culturing of laboratory isolates.

Finally, we interrogated the prevalence of other drug resistance markers before and after FSM-CLN treatment (Figure 3, Supplementary Table 4). Specifically, we evaluated variation in the locus encoding the multi-drug transporter PfMDR1 (PF3D7\_0523000), which modulates parasite sensitivity to hydrophobic antimalarials, such as mefloquine.
and halofantrine [36]. Strains possessing PfMDR1 mutations are sensitive to FSM and PfMDR1 mutations are not predicted to impact FSM effectiveness [3,37]. Three PfMDR1 variants (wild-type, Y184F, and N86Y) were identified in our analyzed strains, but PfMDR1 haplotype frequencies were not significantly different in pre- and post-treatment populations (p>0.5).

ACTs, introduced in Mozambique in 2004 [38], may have selected for parasites able to withstand drug treatment such as FSM-CLN. Alleles of PfK13 (Kelch13, PF3D7_1343700) have been implicated in artemisinin resistance. We identify two PfK13 variants in our population (K189T and A578S), neither of which has been implicated in laboratory artemisinin resistance. The K189T variant is common in African isolates [39,40] and is not believed to cause increased clearance times following ACT. While the A578S variant has been associated with increased clearance times [41], this mutation was only observed in a small fraction of our strains. Additionally, strains possessing known resistance mutations in PfK13 are FSM sensitive (Edwards RL et al., submitted) suggesting that selection for artemisinin resistance does not result in FSM resistance.

In our small study population, selection with FSM-CLN did not appear to alter frequency of PfMDR1 alleles or alleles of additional known genetic loci associated with antimalarial resistance, including PfCRT (chloroquine), PfATP4 (multiple drug classes), PfDHFR (antifolates), and PfDHPS (antifolates) (Figure 3, Supplementary Table 4) [42–46].
Our approach permitted an unbiased search for any novel non-synonymous SNPs that are associated with recrudescence following FSM-CLN treatment. To better understand SNPs that were unique to or enriched in recrudescent samples, we also subtracted pre-treatment non-synonymous SNPs from post-treatment non-synonymous SNPs. The eight recrudescent strains analyzed had an average of 3,448 ± 604 unique non-synonymous SNPs (approximately 33% of their total non-synonymous SNPs).

Sixty-eight SNPs were shared in ≥50% of the samples (Supplementary Table 5). However, no non-synonymous SNPs were shared between all 8 recrudescent samples, demonstrating that, in this small population, a genetic marker of recrudescence was not present.

Selective pressures in vivo are likely to be distinct from those described in vitro. We hypothesized that certain biological processes may be enriched for genetic variation in our post-treatment samples. We therefore performed gene ontology (GO) analysis on the genes with SNPs shared in ≥50% of recrudescent samples (Supplementary Table 5) to understand the functions of genes containing SNPs enriched upon recrudescence. Our analysis reveals enrichment for immune evasion and parasitism-related functions (Table 1). This result has been observed in other studies of variation in *P. falciparum* populations [14,30]. Because these genes are among the most variable in a population, they are likely to display changes in allele frequency following a population bottleneck, such as recrudescence. Notably, GO analysis did not reveal enrichment in pathways associated with drug resistance or with the mechanism of action of FSM (isoprenoid...
synthesis) or CLN (protein translation). While a novel genetic route to FSM or CLN resistance is possible, we see no evidence for enrichment of new SNPs or pathways in our unbiased genome analysis.

Discussion

Fosmidomycin (FSM) is an antimalarial with a novel, parasite-specific mechanism-of-action, a well-characterized target, and exceptional clinical safety. Despite this promise, FSM combination treatment of uncomplicated *P. falciparum* infection in children was unsuccessful due to unacceptably high rates of parasite recrudescence [11]. Parasite FSM resistance arises readily in culture and has been attributed to loss-of-function mutations in *PfHAD1* [13]. These in vitro studies suggested that selection for FSM resistance alleles during clinical infection and/or FSM treatment may represent a mechanism to explain clinical failures following FSM treatment.

To address this concern, we have surveyed the genetic diversity in Mozambican pediatric *P. falciparum* malaria infections before and after treatment failure with FSM-CLN. Overall, our data indicate that drug resistance does not account for treatment failures following FSM-CLN therapy. Our results confirm that treatment and recrudescence represent a population bottleneck, as MOI is decreased in recrudescent samples. Since resistance alleles are thought to represent only a miniscule proportion of the pre-treatment population, the modest decrease in MOI that we observe is inconsistent with the selection of resistant strains from the population.
Importantly, we do not find evidence of SNPs enriched in parasites following FSM-CLN treatment. Specifically, we do not identify enrichment for alleles already experimentally implicated in FSM or CLN resistance. Our use of whole genome sequencing permits an unbiased screen for additional SNPs that may contribute to resistance, regardless of whether these alleles are directly responsible or otherwise associated with a recrudescent phenotype. While we are limited by the retrospective nature of our study and our inability to phenotype culture-adapted recrudescent parasites, both FSM and CLN have single, well-characterized targets and known SNPs underlying resistance [13,14]. We therefore conclude that neither FSM nor CLN resistance is responsible for clinical failure of FSM-CLN.

Our study was designed to evaluate the hypothesis that a simple coding mutation may underlie recrudescence in FSM-CLN-treated parasites. The results of our study cannot exclude other potential routes to resistance, such as non-coding mutations resulting in regulatory variation or non-genetic changes in gene expression or homeostatic responses. Further studies may address if and how these mechanisms contribute to resistance to FSM, CLN, and other antimalarials.

Our study highlights an important caution in applying the results of forward genetic screening in cultured parasites to clinical populations. As resistance alleles are identified in vitro, it is important to recognize that selective pressures during natural human infection (immune pressure, metabolic requirements) are likely to be distinct. Our data indicate that mutation in *PfHAD1* is not readily achieved in clinical populations.
Perhaps mutation of *PfHAD1* comes at a fitness cost in *P. falciparum*, similar to what has been found for other resistance loci, such as *PfCRT* and *PfATP4* [47,48]. However, *PfHAD1* mutation appears to reduce fitness during human infection and not during culture, as loss of PfHAD1 is easily achieved in laboratory selections.

Finally, our study illustrates the utility of SWGA for the analysis of *P. falciparum* genomes from blood spot samples. This method has applications for future field studies, as blood spots are easier to acquire than whole blood. Further optimization will facilitate the extraction of more data from these types of samples. Furthermore, our data provide additional validation of PCR-based strategies to determine MOI and recrudescence. We find that the high rate of recrudescence following FSM-CLN treatment in children was not overestimated due to use of the standard 3-locus PCR genotyping protocol [11]. Eventual use of whole genome sequencing for genotyping field populations will provide more information regarding variation within a geographic region and within patients.

This work supports the current hypothesis that the disappointing clinical efficacy of FSM combinations is likely due to challenges of partner drug selection and formulation [12]. The short serum half-life of both FSM and CLN (1-3 h) limits parasite exposure and presumably reduces the selective pressure for resistance [8,49]. However, this limited serum exposure almost certainly contributes to decreased clinical efficacy. Currently, FSM is being evaluated in combination with the bisquinolone piperaquine in a Phase II clinical trial in Gabon (NCT02198807). Piperaquine has a notably long half-life (>20 days) [50], which holds promise to limit recrudescence when paired with FSM. Our
findings support the continued development of antimalarials targeting PfDXR and the MEP pathway, as well as the development of alternative FSM combinations.

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Conflicts of Interest

The original clinical trial conducted in the year 2010 in Mozambique was sponsored by Jomaa Pharma. The company has played no role in the subsequent analyses presented in this study.
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References


Figure Legends

Figure 1. SWGA causes enrichment of *P. falciparum* DNA from mixed samples that can be detected by PCR. Shown is an agarose gel of SWGA amplicons obtained using *P. falciparum*-specific (top) and human-specific (bottom) primers. Markers indicate DNA size in base pairs.

Figure 2. Samples cluster by patient, indicating recrudescent infections. SNPhylo [22] was used to construct a maximum likelihood phylogenetic tree of SNP profiles from initial (pre-treatment) and recrudescent (post-treatment) infections. Bootstrap values were 100 for all pre- and post-treatment branch points. Samples are numbered by infection. Scale bar represents units of substitution.

Figure 3. Resistance landscape before and after recrudescence. Only infection pairs with sufficient exome coverage were analyzed (≥60% at ≥5X, N=8). Shown are the percentages of pre- and post-treatment samples with indicated non-reference (3D7) alleles at resistance loci. Any codons not shown match the 3D7 reference in all samples.
Table 1. Gene ontology (GO) analysis of genes containing SNPs unique to recrudescent samples.

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