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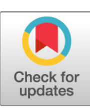
MECHANISMS OF RESISTANCE



Associations between Varied Susceptibilities to PfATP4 Inhibitors and Genotypes in Ugandan *Plasmodium falciparum* Isolates

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ABSTRACT Among novel compounds under recent investigation as potential new antimalarial drugs are three independently developed inhibitors of the *Plasmodium falciparum* P-type ATPase (PfATP4): KAE609 (cipargamin), PA92, and SJ733. We assessed *ex vivo* susceptibilities to these compounds of 374 fresh *P. falciparum* isolates collected in Tororo and Busia districts, Uganda, from 2016 to 2019. Median IC_{50}s were 65 nM for SJ733, 9.1 nM for PA92, and 0.5 nM for KAE609. Sequencing of *pfatp4* for 218 of these isolates demonstrated many nonsynonymous single nucleotide polymorphisms; the most frequent mutations were G1128R (69% of isolates mixed or mutant), Q1081K/R (68%), G223S (25%), N1045K (16%), and D1116G/N/Y (16%). The G223S mutation was associated with decreased susceptibility to SJ733, PA92, and KAE609. The D1116G/N/Y mutations were associated with decreased susceptibility to SJ733, and the presence of mutations at both codons 223 and 1116 was associated with decreased susceptibility to PA92 and SJ733. In all of these cases, absolute differences in susceptibilities of wild-type (WT) and mutant parasites were modest. Analysis of clones separated from mixed field isolates consistently identified mutant clones as less susceptible than WT. Analysis of isolates from other sites demonstrated the presence of the G223S and D1116G/N/Y mutations across Uganda. Our results indicate that malaria parasites circulating in Uganda have a number of polymorphisms in PfATP4 and that modestly decreased susceptibility to PfATP4 inhibitors is associated with some mutations now present in Ugandan parasites.

KEYWORDS KAE609, PA92, PfATP4, *Plasmodium falciparum*, SJ733, Uganda, cipargamin, drug resistance, malaria

Resistance to antimalarial drugs has confounded the treatment and control of malaria for decades. Currently, artemisinin-based combination therapy (ACT) is the recommended treatment for falciparum malaria in nearly all countries, but ACTs are challenged by the emergence of resistance to artemisinins and to some partner drugs in Southeast Asia (1). Worrisome reports suggest the spread of ACT resistance to other regions (2). New treatments, ideally directed against novel targets, are needed (3, 4).

Novel candidate antimalarials have been identified through screens of chemical libraries (5–7). A number of these compounds have moved to advanced preclinical and clinical development (3). In parallel, the targets of multiple compounds have been

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determined by characterizing genetic alterations associated with resistance selected *in vitro*. Interestingly, three independently developed compounds, KAE609 (cipargamin), PA92, and SJ733, were found to act against the same target, the *Plasmodium falciparum* P-type ATPase 4 (PfATP4) (PFL0590c; PF3D7_1211900) (4, 7–9). PfATP4 is a Na^+ efflux pump that serves to maintain a low cytosolic Na^+ concentration via exchange with H^+ (10, 11). Inhibition of Na^+ efflux results in an increased cytosolic pH, osmotic fragility, swelling, disruption of lipid homeostasis, premature schizogony-like events, and, ultimately, death of intraerythrocytic parasites (2, 9, 10, 12–16).

The PfATP4 inhibitor candidate antimalarials KAE609, PA92, and SJ733 and their backups are currently in different stages of development. KAE609 is a spiroindolone that exhibited activity against asexual (8, 17) and sexual stages of *P. falciparum* (18). Major advantages of KAE609 were very fast action, marked potency (50% inhibitory concentration [IC_{50}] of $<$10 nM against cultured parasites), a good safety profile, and excellent efficacy against uncomplicated malaria in a phase II clinical trial (8, 17, 19, 20). KAE609 is now under development for single dose treatment of uncomplicated or severe falciparum malaria (17, 20, 21) (https://clinicaltrials.gov/ct2/show/NCT04675931). PA92, a pyrazoleamide, was highly active against erythrocytic *P. falciparum* (IC_{50} of 5 to 13 nM against laboratory strains and mean IC_{50} of 18 nM against Indonesian isolates) and prevented formation of male (IC_{50}, 39 nM) and female (IC_{50}, 74 nM) gametes (9). PA92 led to rapid parasite clearance when administered orally to *P. falciparum*-infected immunodeficient mice. SJ733, a dihydroisoquinolone, had potent activity against erythrocytic *P. falciparum* (IC_{50} 10 to 60 nM) and yielded rapid parasite clearance when administered to *P. falciparum*-infected immunodeficient mice (22). It showed promising results as a multiple dose antimalarial in preclinical studies and phase I clinical trials, with favorable pharmacokinetic and safety profiles as well as rapid but not sustained parasite clearance (4, 7, 23). For all three PfATP4 inhibitors, resistance was selected *in vitro* by culture with defined concentrations of the drugs and resistant parasites were evaluated. Decreased susceptibility to all three PfATP4 inhibitors was associated with mutations in the predicted transmembrane domain of PfATP4, near the inhibitor-binding pocket (8, 9, 11, 12, 22, 24); mediation of resistance by PfATP4 mutations was further confirmed with CRISPR-Cas9 editing (25). Thus, the PfATP4 target is capable of alteration under drug selection, and some mutations are associated with altered drug susceptibility (8, 9, 11, 22, 25, 26). However, data on the diversity of PfATP4 in parasites now circulating in endemic countries and the consequences of this diversity are not available.

To gain insights into PfATP4 diversity and drug susceptibility, we assessed PfATP4 inhibitor susceptibility among *P. falciparum* field isolates from eastern Uganda and characterized associations between inhibitor susceptibility and *pfatp4* genotypes.

RESULTS

Susceptibilities of Ugandan isolates to novel PfATP4 inhibitors. *Ex vivo* susceptibilities to three lead PfATP4 inhibitors were determined for 374 fresh Ugandan *P. falciparum* isolates collected from patients presenting with uncomplicated malaria between 2016 and 2019 in Busia and Tororo districts. We observed a rank order of potency of KAE609$>$PA92$>$SJ733, with all compounds active at low to mid nM concentrations (median IC_{50}s: 65 nM for SJ733, 9.1 nM for PA92, and 0.5 nM for KAE609) (Fig. 1). Small numbers of isolates had IC_{50} values 2-fold or greater above the median IC_{50} values for the tested isolates. Pairwise comparisons of susceptibilities to PfATP4 inhibitors showed strong positive correlations between the three lead PfATP4 inhibitors, with strongest association (Spearman's correlation coefficients $>$ 0.5) between PA92 and both KAE609 and SJ733 (Fig. 2). Overall, PfATP4 inhibitors were highly active against Ugandan *P. falciparum* isolates but variations in susceptibilities were observed.

PfATP4 was highly polymorphic in Ugandan isolates. *Pfatp4* sequences were determined for 218 Ugandan isolates representing a range of susceptibilities for all three inhibitors. *Pfatp4* was highly polymorphic, with a large number of synonymous (44% of isolates) and nonsynonymous (89% of isolates) single nucleotide polymorphisms (SNPs) compared to the reference 3D7 strain sequence (Fig. 3A). The most common mutations led to the amino acid substitutions Q1081K/R (69%: 62% mixed, 38%

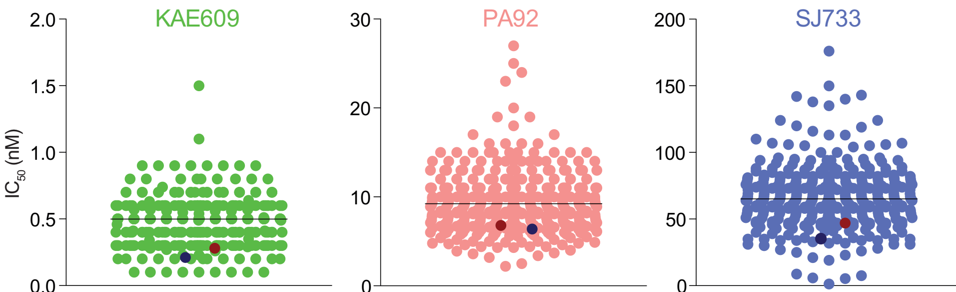

FIG 1 *Ex vivo* susceptibilities of Ugandan isolates to three PfATP4 inhibitors. Each circle represents a different isolate. Horizontal bars show medians. Dark blue and red circles depict mean 3D7 and Dd2 IC_{50}s across all experiments, respectively.

pure mutant), G1128R (68%: 61% mixed, 39% pure mutant), G223S (25%: 72% mixed, 28% pure mutant), N1045K (16%: 86% mixed, 14% pure mutant), and D1116G/N/Y (16%: 70% mixed, 30% pure mutant) (Fig. 3B). This level of polymorphism for a housekeeping protein was unexpected, prompting us to examine the potential structural significance of the identified mutations. PfATP4 belongs to a superfamily of P-type transporters that use phosphorylation-dephosphorylation cycles and ATP hydrolysis to transport ions across membranes. PfATP4 is classified as a type P2D transporter, for which a molecular structure is not available. However, all P-type pumps share certain structural domains, even where sequence homology is limited (27). In the modeled structure for PfATP4 (Fig. 3C), mutations identified in Ugandan isolates are predicted to localize to the nucleotide binding, actuator, extracellular loop, transmembrane, and cytoplasmic domains of the protein; none of the mutations were in the predicted phosphorylation domain (27). The majority of high-frequency mutations were predicted to localize to the extracellular loop. Previous studies identified over 30 PfATP4 mutations in *P. falciparum* selected *in vitro* for resistance to PfATP4 inhibitors (8, 9, 12, 14, 22, 26). Except for G223S, none of the mutations seen in Ugandan isolates were at positions previously selected *in vitro*. Interestingly, a mutation at that same codon, G223R, was previously selected by incubation of a laboratory strain of *P. falciparum* with NITD678, an analog of KAE609, and this mutation mediated low-level resistance to KAE609 (8). In the Ugandan isolates, the G223S mutation, encoded by the g667a substitution, was common, and identified in pure mutant (6.4%) and mixed (18.8%) isolates. Also, 44% of the mixed isolates harboring the G223S mutation had a synonymous c669a SNP in the

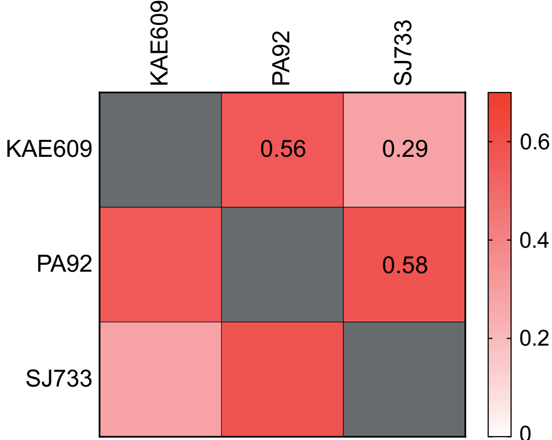

FIG 2 Heat map demonstrating correlations between results of individual isolates for different PfATP4 inhibitors (collected 2016 to 2020). Spearman's correlation coefficients are indicated numerically and by the color scale. All associations were statistically significant, $P < 0.001$.

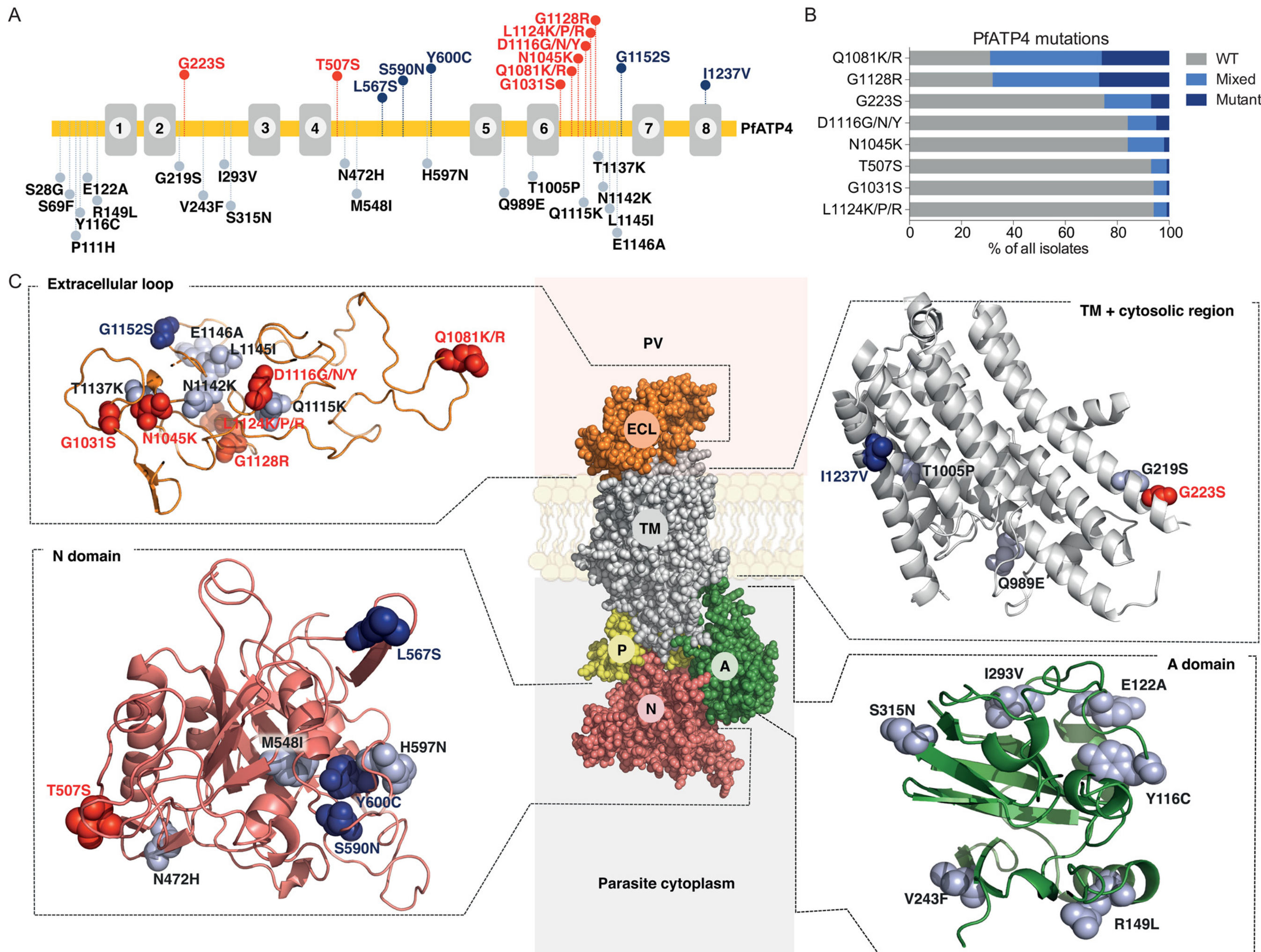

FIG 3 PfATP4 mutations in Ugandan isolates. (A) PfATP4 protein sequence depicted as a linear model with the transmembrane domains numbered and colored in gray. Mutations observed in Ugandan isolates are marked; the colors indicate the prevalence: red (>5%), dark blue (0.5 to 5%), and gray (<0.5%). (B) Mutations with >5% prevalence among Ugandan isolates, including percentages of observed WT, mixed, or mutant sequences. (C) Domain architecture of PfATP4 is depicted as a space-fill model (center), with actuator (A), nucleotide (N), phosphorylation (P), and transmembrane (TM) domains and the extracellular loop (ECL) shown as ribbon diagrams. Domains in the model are based on extensive structural information available for P-type ATPases (27). PV, parasitophorous vacuole. Mutations are annotated as spheres in representations of individual domains, with the same color scheme indicating prevalence as noted for panel A.

same codon. The combination of the two SNPs in that codon would encode G223R, the mutation previously selected *in vitro*. However, it cannot be determined if mixed isolates included this sequence or only one parasite strain with the common G223S mutation plus another strain with a synonymous mutation. In any event, PfATP4 was highly polymorphic in the studied Ugandan isolates.

Association between genotypes and *ex vivo* susceptibility to PfATP4 inhibitors. Previous studies have shown mutations in PfATP4 to influence inhibitor activity. We assessed whether the mutations present in Ugandan *P. falciparum* isolates were associated with variations in susceptibility. The G223S mutation was associated with decreased susceptibility to all three PfATP4 inhibitors (G223 versus 223S KAE609: $P = 0.037$, PA92: $P < 0.001$, SJ733: $P = 0.034$) (Fig. 4A, Table S2 in the supplemental material). Mutations at another codon, D1116G/N/Y, were associated with decreased susceptibility to SJ733 ($P = 0.016$) (Fig. 4B, Table S2). A haplotype including both the G223S and D1116G/N/Y mutations was associated with decreased susceptibilities to PA92 ($P = 0.004$) and SJ733 ($P < 0.001$) (Fig. 4C, Table S2). In all of these cases, absolute differences in susceptibilities of WT and mutant parasites were modest. Associations were not seen between other identified PfATP4 mutations and susceptibilities to the

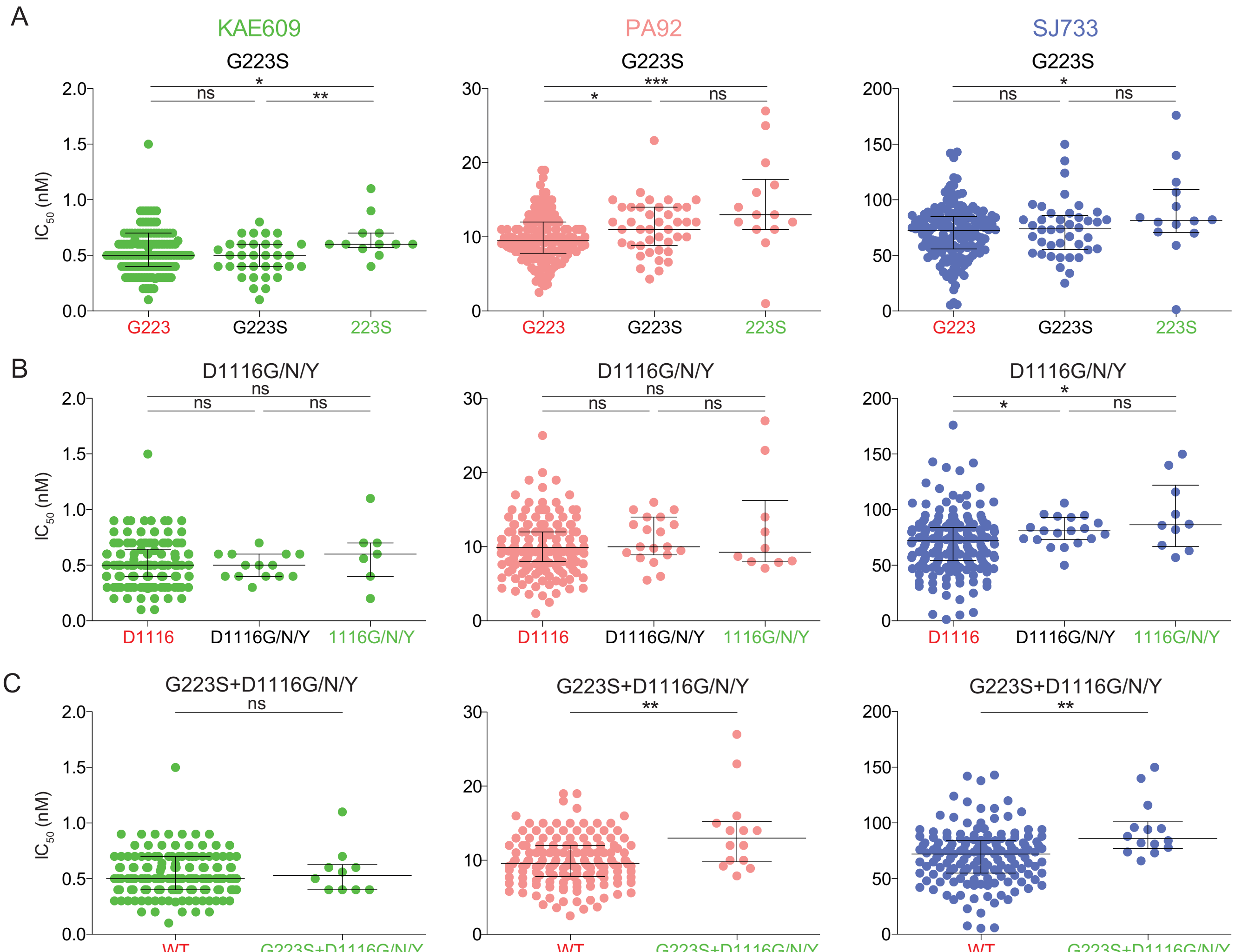

FIG 4 Associations between PfATP4 mutations and susceptibility to PfATP4 inhibitors. Susceptibilities are shown for WT (G223, red) versus mixed (G223S, black) and mutant (223S, green) sequences at codon 223 (A), WT (D1116, red) versus mixed (D1116G/N/Y, black) and mutant (1116G/N/Y, green) sequences at codon 1116 (B), and WT versus mixed and mutant (G223S+ D1116G/N/Y, green) for the combined G223S and D1116G/N/Y codons (C). Horizontal bars show medians and vertical bars the interquartile ranges (IQRs). Significance values are based on the Mann-Whitney U test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

tested inhibitors (not shown). Taken together, our findings suggest associations between the G223S and D1116G/N/Y mutations present in circulating Ugandan parasites and susceptibilities to PfATP4 inhibitors.

PfATP4 inhibitor susceptibility of clones selected from mixed Ugandan isolates. Our analyses of *ex vivo* susceptibilities were complicated by the presence of polyclonal isolates. To examine the contributions of WT and mutant strains in mixed isolates, individual strains were cloned by limiting dilution and then characterized. Sequencing confirmed isolation of both WT and mutant clones from three different isolates (Fig. 5E to G). Mutant clones were consistently less susceptible than WT clones to PfATP4 inhibitors (Fig. 5A to D, Table S3). As assessed by the Friedman test, pairwise comparison of individual runs showed a rank order of potency between WT clones, mixed isolates, and mutant clones (Table S3). These results support the consideration of susceptibilities for mixed isolates as representative of susceptibilities of *P. falciparum* with various genotypes circulating in Uganda.

PfATP4 G223S mutation prevalence across Uganda. We assessed the genotypes of isolates collected from 13 different Ugandan sites as part of ongoing surveillance. The G223S and D1116G/N/Y mutations were seen in isolates from nearly all of the sites studied, mainly with mixed WT/mutant genotypes (Fig. 6). Our findings indicate that

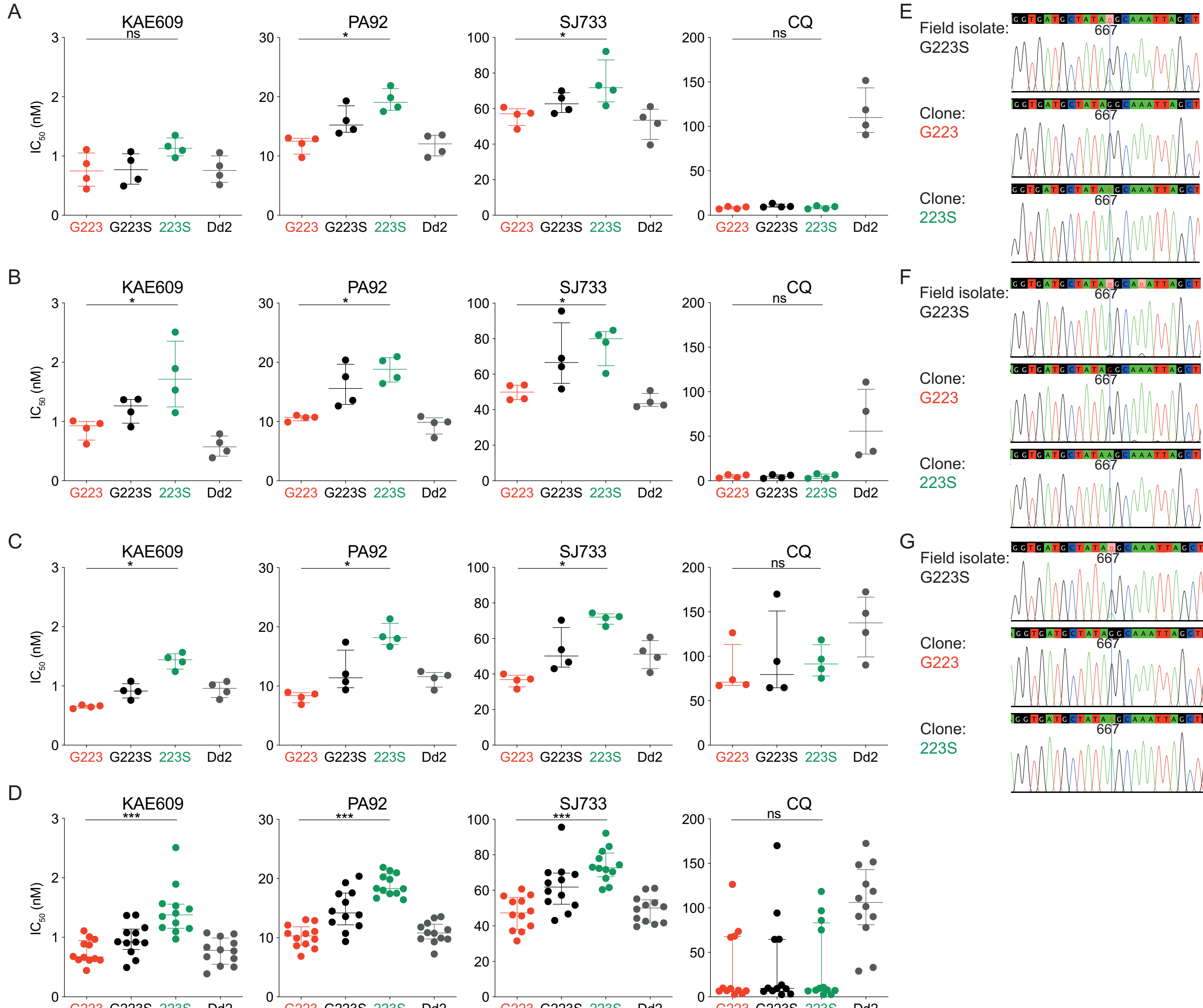

FIG 5 Reduced susceptibility of mutant 223S clones to PfATP4 inhibitors. Susceptibilities to the three studied PfATP4 inhibitors for parasites with different genotypes are shown. Original mixed isolates (G223S, black) were cloned to carry either the G223 (WT) or 223S (mutant) sequence and susceptibilities were compared to those of the laboratory strain Dd2 and with chloroquine (CQ). Results for three independent isolates (A to C) and composite results (D) represent four independent biological replicates with two technical replicates per assay. Error bars show medians with IQRs. (E to G) Electropherograms show *pfatp4* sequences for bases 656 to 675 to demonstrate base 667 as the WT (g) or mutant (a) genotype. Significance values are based on the Mann-Whitney U test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

diversity of PfATP4, and in particular diversity at the 223 and 1116 codons, was high among isolates from sites across Uganda.

DISCUSSION

Compounds that inhibit PfATP4 are among the most promising new antimalarials under development. A potential liability of antimalarials that target an essential enzyme is the selection of resistance mediated by mutations in the target enzyme. Resistance to each of the three PfATP4 inhibitors was previously selected for *in vitro*, and resistance was linked to a number of mutations in PfATP4. However, *in vitro* studies of laboratory strains offer limited information on the potential for altered drug susceptibility after clinical use of a drug. To gain insights into baseline susceptibilities of *P. falciparum* circulating in Uganda, we determined *ex vivo* susceptibilities of Ugandan isolates to the three lead PfATP4 inhibitors. Results for the three compounds showed low nanomolar activities, and activities were correlated for the isolates studied,

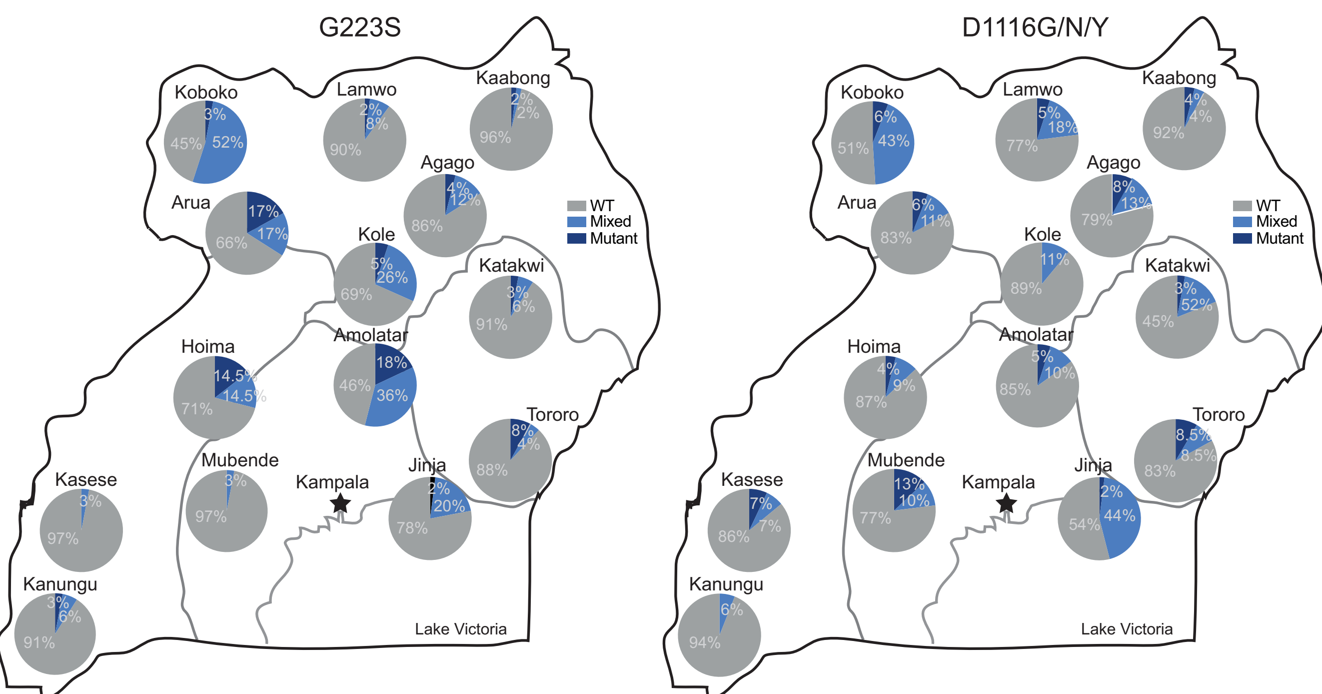

FIG 6 Prevalence of the G223S and D1116G/N/Y mutations in *P. falciparum* isolates collected from 13 different sites around Uganda in 2018 and 2019. The results shown are for 10 to 50 isolates per site.

suggesting similar mechanisms mediating varied susceptibility. The *pfatp4* gene was highly polymorphic in Ugandan parasites, and two common mutations were associated with modest alterations in PfATP4 inhibitor susceptibilities. Our results offer insights into inhibitor susceptibilities and PfATP4 sequences of parasites now circulating in Africa. Importantly, although some mutations were associated with altered drug susceptibility, changes in susceptibility were modest, suggesting good activities of these compounds against African parasites.

Ex vivo susceptibilities to all three PfATP4 inhibitors varied among Ugandan *P. falciparum* isolates, with IC_{50}s up to three times the median values for a small number of isolates. In considering the *ex vivo* IC_{50} results, it is important to appreciate that these assays necessarily cannot be repeated and are subject to some variation due to the complex nature of the assay. Nonetheless, the observed susceptibility ranges for the three inhibitors were similar to previous results for laboratory strains (8, 9, 22). Additionally, small field studies of isolates from Indonesia and the Thai-Burmese border reported similar susceptibility ranges for PA92 and KAE609, respectively (8, 9). Our results indicate that Ugandan isolates were generally highly susceptible to PfATP4 inhibitors, albeit with modest variations potentially explained in part by observed PfATP4 mutations.

The *pfatp4* sequences were highly polymorphic in Ugandan *P. falciparum* isolates. The most common mutations were Q1081K/R and G1128R, consistent with available sequences from eastern Africa, which showed 48% and 50% prevalence of these variants, respectively (www.malariagen.net). However, these and most other observed mutations were not associated with decreased susceptibility of parasites to PfATP4 inhibitors. In contrast, the G223S and D1116G/N/Y mutations were associated with decreased susceptibility to the tested PfATP4 inhibitors. Interestingly, a mutation at codon 223 was previously selected in the Dd2 strain pressured independently with NITD678, an analog of KAE609 (8), and with SJ733 (22). Dd2 harbors a synonymous *pfatp4* SNP (c669a), compared to the 3D7 reference strain, in the same codon as the nonsynonymous g667a SNP. Together, g667a/c669a encodes the G223R mutation, whereas the single g667a SNP encodes the G223S mutation. Ugandan isolates

harbored both SNPs; 25% had the g667a nonsynonymous mutation and 24% had the c669a synonymous mutation; 34% of the isolates with the c669a mutation were mixed, with WT and mutant sequence at bases 667 and 669. It is difficult to assign haplotypes for mixed infections, and pure mutants were only observed for the 223S mutation. Isolates carrying the G223S mutation were significantly less susceptible to all three tested PfATP4 inhibitors, although changes in susceptibility were modest. Surveillance studies across Uganda indicated varied prevalence of the G223S mutation, consistent with sequences of parasites collected across Africa (www.malariagen.net). In these, data frequencies of 3 to 18% for the G223S mutation and 12 to 62% for the synonymous c669a SNP, which can be selected to the G223R mutation, suggest a potential for the emergence of parasites resistant to PfATP4 inhibitors. Further studies using cloning techniques to search for the uncommon 223R mutation and *in vitro* characterization of Ugandan parasites after introduction of mutations of interest could help to elucidate the relevance of observed mutations in Ugandan isolates. However, the modest changes in inhibitor susceptibility associated with mutations already circulating in Uganda will probably not impact importantly on the clinical efficacies of the tested inhibitors.

PfATP4 enables *P. falciparum* to maintain ion homeostasis by efflux of sodium from the cytoplasm in exchange for protons (10, 12). Parasites selected *in vitro* for resistance to PfATP4 inhibitors harbored mutations in and around the modeled protein transmembrane domain (8, 9, 22) (Fig. 3). Changes in or near the transmembrane domain are predicted to block binding of inhibitors and perturb the ion-transporting channel, respectively (22). Among mutations identified in Ugandan isolates, only the G223S mutation was common and located near the transmembrane domain in our modeled protein structure. The G223R mutation was previously shown to impact inhibitor susceptibility at a modest level (22). This is consistent with our findings that the 223S mutation decreased PfATP4 inhibitor susceptibility by less than 2-fold. The high level of polymorphism in PfATP4 leads to questions as to selective pressures that may underlie its evolution. The highest frequency mutations are predicted to occupy the extracellular loop of the protein. This region, likely to be exposed in the bloodstream during the merozoite stage, may be under immune pressure, leading to mutations to evade immune responses. Previous studies have suggested that some resistance-associated mutations in PfATP4 are accompanied by fitness costs (22). At present, we do not know whether the naturally occurring variations in PfATP4 have any effect on parasite fitness.

Our study captures the genetic complexity and impact of this complexity on susceptibility to PfATP4 inhibitors in Ugandan parasites. Further, it offers insights into the background of circulating African parasites upon which additional polymorphisms, potentially leading to true clinical resistance, may be selected with widespread use of PfATP4 inhibitors to treat malaria. Varied susceptibilities to PfATP4 inhibitors were observed in Ugandan *P. falciparum* isolates. Even though PfATP4 was highly polymorphic, only two mutations were associated with modest decreases in susceptibility in circulating strains. The clinical relevance of the identified polymorphisms is unknown, but it is reassuring that no mutations identified in circulating Ugandan parasites mediated high-level resistance to candidate PfATP4 inhibitor antimalarials.

MATERIALS AND METHODS

Study location, patient inclusion, and sample collection. Subjects 6 months of age or older presenting between July 2016 and June 2020 at the outpatient clinics of the Tororo District Hospital, Tororo District or the Masafu General Hospital, Busia District, Uganda with clinical suspicion for malaria and a positive Giemsa-stained blood film for *P. falciparum* and without signs of severe disease were enrolled after informed consent. Blood was collected in a heparinized tube before initiation of treatment with artemether-lumefantrine, following national guidelines. For surveillance across Uganda, blood from up to 50 patients age 6 months or older diagnosed with *P. falciparum* malaria was collected as spots dried on filter paper at 16 sites in both 2018 and 2019 (28). The studies were approved by the Makerere University Research and Ethics Committee, the Uganda National Council for Science and Technology, and the University of California, San Francisco Committee on Human Research.

***Ex vivo* drug susceptibility assay.** Samples with parasitemias of ≥0.3% were placed into culture and susceptibilities to PfATP4 inhibitors and the control chloroquine (CQ) were determined as previously described (29). *Ex vivo* assays were performed on fresh blood specimens and thus necessarily could not be replicated. KAE609, PA92, and SJ733, provided by Medicine for Malaria Venture, were prepared as 10 mM stock solutions in dimethyl sulfoxide. Chloroquine was diluted in distilled water or 70% ethanol to a 10 mM stock. Stock solutions were stored at −20°C, and working solutions were freshly prepared within 24 h of susceptibility tests and stored at 4°C. SYBR green-based 72 h drug susceptibility assays were performed as previously described (30). Drugs were serially diluted 3-fold in 96-well assay plates in complete medium (RPMI 1640 medium supplemented with 25 mM HEPES, 0.2% NaHCO3, 0.1 mM hypoxanthine, 100 μg/ml gentamicin, and 0.5% AlbuMAX II [Invitrogen]) before adding parasite culture to reach a parasitemia of 0.2% and hematocrit of 2%. Drug-free and parasite-free controls were included. Plates were incubated for 72 h in a humidified modular incubator under blood gas mixture (91% N_2, 6% CO2, 3% O_2) at 37°C, and growth of control and treated cultures was then assessed by measuring SYBR green fluorescence, as previously described (29, 30). Laboratory control strains Dd2 and 3D7 were included in the assays on a monthly basis. Half-maximal inhibitory concentrations (IC_{50}s) were determined by plotting fluorescence intensity against log drug concentration in Prism 8.4.3 (GraphPad Software). Data were curve fit with a variability slope function and well-to-well and signal to noise ratios were calculated using the test statistic: $Z = 1\text{-}([3SD_{infected} + 3SD_{uninfected}]/[signal_{infected}\text{-}signal_{uninfected}])$ (29). Results with poor curve fit (typically $Z < 0.5$) or obvious poor fit on visual inspection were discarded.

Sequencing of Ugandan parasites. *P. falciparum* DNA was extracted from blood spots using Chelex-100. For dideoxy sequencing of 110 isolates, *pfatp4* was amplified from sample DNA by PCR with Phusion High-Fidelity DNA polymerase using primers PfATP4_1F (ATGAGTTCTCAAAATAATAAT AAACAGGGTGAC) and PfATP4_1R (GGTACTTCTATCAAGTAA TCTATCAGGTG) for the upstream region, and PfATP4_2F (CAC CTGATAGATTACTTGATAGA AGTACC) and PfATP4_2R (TTAATTCTTAATAGTCATATATTTTCTTCTATATATAACC TTTGG) for the downstream region. PCR products were purified with Ampure beads (Beckman Coulter) and sequencing was performed. Sequences were analyzed with CodonCodeAligner (CodonCode Corporation) against the reference strain 3D7 *pfatp4* sequence.

Pfatp4 from 108 additional isolates and samples collected from across Uganda were analyzed as part of a study to determine sequences of known and potential drug targets in Ugandan *P. falciparum* field isolates using molecular inversion probe (MIP) capture and deep sequencing, with library preparation and sequencing as previously reported (28). The *pfatp4*-specific probes (see Table S1 in the supplemental material) were designed using MIPTools software (v.0.19.12.13; https://github.com/bailey-lab/MIPTools). MIPTools was used to analyze raw sequencing data and call variants (28).

Culturing and cloning of field isolates. Ugandan isolates were cultured for 2 to 3 weeks and, when robust growth was established, cryopreserved in 50% glycerolyte (Fenwal) solution and stored in liquid nitrogen. Subsequently, parasites were thawed using a stepwise process in 12% NaCl solution, 1.6% NaCl, and then 0.2% glucose/0.9% NaCl, and cultured in complete RPMI medium (25 mM HEPES pH 7.4 [GIBCO], 0.5% Albumax, 0.2% NaHCO3, 2 mM L-glutamine, 100 μM hypoxanthine and gentamicin) at 37°C under 3% O_2, 6% CO_2, and 91% N_2, at 2% hematocrit. Parasites from isolates with mixed G223S genotypes, confirmed by dideoxy sequencing of cultured isolates, were cloned by limiting dilution at 0.5 parasites/well in 200-μl cultures to obtain pure wild-type (WT) and mutant parasites. Cloned cultures were expanded, DNA was extracted by Saponin/Chelex-100 DNA extraction, and *pfatp4* sequences were determined by PCR and dideoxy sequencing, as described above. The drug susceptibility of parental isolates and cloned parasites was determined based on SYBR green fluorescence, as described above (29), after synchronization with 10% sorbitol (Fisher Scientific). Fluorescence was determined using a Varioskan (Thermo Fisher Scientific) reader at excitation 485 nm, emission 520 nm. Fluorescence data were curve fit with a variable slope function to estimate IC_{50} values using Prism version 8.4.3.

Modeled structure of PfATP4. A homology model of PfATP4, based on the 3D7 strain sequence, was generated using the SWISS MODEL database (31). The database retrieved 7 models, of which SERCA 2b (PDB model number: 6LN7; a type P2A pump) was chosen because of maximum sequence coverage (>91%) with PfATP4. Mutations relative to 3D7 were annotated using the PyMOL molecular graphics system (Version 1.2r3pre, Schrödinger, LLC). Transmembrane helices of PfATP4 were predicted using constrained consensus TOPology prediction server (CCTOP), a software that uses an algorithm based on the framework of the hidden Markov model. The linear model of PfATP4 (Fig. 3A) was generated based on transmembrane predictions from CCTOP.

Statistical analysis. Associations between IC_{50} values and *pfatp4* genotypes were determined with the Mann-Whitney U test and between IC_{50} values for different compounds with the Spearman's rank correlation coefficient using Prism version 8.4.3. The direction and magnitude of associations between IC_{50} values for different compounds were quantified with the Spearman's rank correlation coefficient. The Friedman test was used to compare rank order of potency between WT and mutant clones of individual runs. All statistical tests were two-tailed, and considered statistically significant at $P < 0.05$.

Data availability. Sequencing reads are available in the NCBI Sequence Read Archive (accession numbers PRJNA655702, PRJNA660547, and MW881877 to MW881988).

MIP probes and PCR primers used in this study are listed in Table S1 in the supplemental material. MIPWrangler (https://github.com/bailey-lab/MIPWrangler) and MIPTools (https://github.com/bailey-lab/MIPTools) software is available on GitHub. All additional data is available on request from the authors (Philip.rosenthal@ucsf.edu).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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O.K., S.A.R., P.K.T., T.K., M.O., O.B., and S.O. assisted in study design, performed *ex vivo* IC_{50} assays, and archived data. S.L.N., M.D., and J.L. provided project administrative and logistical support. O.K., S.A.R., M.D.C., V.A., O.A., and J.B. performed and analyzed genotyping studies. A.A.R. and A.B.V. assisted in generating the protein model. O.K., B.R.B., and R.A.C. verified and analyzed data and performed statistical analysis. All authors contributed to the writing of the manuscript.

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