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SUSCEPTIBILITY

In Vitro Susceptibility of *Plasmodium falciparum* Isolates from the China-Myanmar Border Area to Piperaquine and Association with Candidate Markers

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ABSTRACT *Plasmodium falciparum* from the Greater Mekong subregion has evolved resistance to the artemisinin-based combination therapy dihydroartemisinin and the partner drug piperaquine. To monitor the potential westward spread or independent evolution of piperaquine resistance, we evaluated the *in vitro* susceptibility of 120 *P. falciparum* isolates collected at the China-Myanmar border during 2007 to 2016. The parasite isolates displayed a relatively wide range of piperaquine susceptibility estimates. While 56.7% of the parasites showed bimodal drug response curves, all but five generated area-under-the-curve (AUC) estimates consistent with a susceptible phenotype. Using the piperaquine survival assay (PSA), 5.6% parasites showed reduced susceptibility. Of note, parasites from 2014 to 2016 showed the highest AUC value and the highest proportion with a bimodal curve, suggesting decreasing effectiveness in these later years. Unsupervised K-mean analysis of the combined data assigned parasites into three clusters and identified significant correlations between 50% inhibitory concentration (IC_{50}), IC_{90}, and AUC values. No parasites carried the E415G mutation in a putative exonuclease, new mutations in PfCRT, or amplification of the *plasmepsin 2* and *3* genes, suggesting mechanisms of reduced piperaquine susceptibility that differ from those described in other countries of the region. The association of increased AUC, IC_{50}, and IC_{90} values with major PfK13 mutations (F446I and G533S) suggests that piperaquine resistance may evolve in these PfK13 genetic backgrounds. In addition, the Pfmdr1 F1226Y mutation was associated with significantly higher PSA values. Further elucidation of piperaquine resistance mechanisms and continuous surveillance are warranted.

KEYWORDS *Plasmodium falciparum*, drug resistance, piperaquine, *pfcrt*, *pfmdr1*, plasmepsin, gene amplification

The Greater Mekong Subregion (GMS) of Southeast Asia is one of the most threatening foci of malaria because of the emergence and spread of multidrug-resistant (MDR) *Plasmodium falciparum* parasites. Decades ago, parasites resistant to chloroquine (CQ) and the antifolate drug pyrimethamine arose in this location and spread to Africa, causing malaria resurgence and loss of millions of lives (1–4). Artemisinin combination therapies (ACTs) have been adopted worldwide as the frontline treatment of uncomplicated *P. falciparum* malaria (5) and have played an indispensable role in reducing global malaria-associated mortality and morbidity (6). However, clinical artemisinin resistance, first detected in western Cambodia (7–9), is now seen in all GMS countries due to spread and independent emergence (10–16). Since the efficacy of ACTs relies on both the fast-acting artemisinin derivatives and long-lasting partner drugs, artemisinin resistance would leave a larger parasite mass for the partner drug to

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clear and increase the risk of resistance development to the partner drug. This would translate into clinical failures of ACTs. Indeed, a decade or so after the deployment of ACTs, clinical resistance to artesunate-mefloquine (ATS-MFQ) and dihydroartemisinin-piperaquine (DHA-PPQ) emerged in Cambodia (17–21). More recent multisite clinical studies showed that DHA-PPQ failure rates reached high levels in several sites of the eastern GMS (22), which urged the adoption of other ACTs and consideration of triple ACTs (23). As the GMS moves toward malaria elimination, the intensity of drug selection increases, leading to rapidly adapting MDR parasites in the dwindling remnant parasite populations with different genetic backgrounds (24, 25). This situation offers a unique setting to investigate the evolution of drug resistance since important lessons could be learned for other countries moving toward elimination in the future.

Clinical artemisinin resistance in Cambodia is associated with mutations in the Kelch domain protein PfK13 (26). Molecular surveillance showed that PfK13 mutations associated with artemisinin resistance were restricted to certain areas of the GMS (16, 26, 27), but such mutations were rare in Africa (28). Within the GMS, there is also significant geographic heterogeneity in both the patterns of the PfK13 mutations and their prevalence (15, 25, 29–32), possibly reflecting regionally different drug histories and evolutionary origins of the parasites (33). Parasites in the eastern GMS harbor the predominant PfK13 C580Y mutation, whereas the most prevalent PfK13 mutation in the western GMS is F446I (26, 29, 30, 32). Consequently, PPQ resistance in Cambodia primarily arose on a PfK13 C580Y background (34–37). Using a genome-wide association (GWAS) approach, clinical PPQ resistance in western Cambodia was found to be associated with amplification of the two aspartic protease genes *plasmepsin 2* and *plasmepsin 3* (*plasmepsin 2/3*) on chromosome 14 (35, 36) and a point mutation E415G (*exo-E415G*) in a putative exonuclease gene (*PF3D7_1362500*) on chromosome 13 (35). Knockout experiments showed that disruption of *plasmepsin 2/3* in the 3D7 parasite specifically sensitized the parasites to PPQ but not to other antimalarials, adding support for the roles of *plasmepsin 2/3* amplification in PPQ resistance (38). However, a subsequent study showed that overexpression of these two enzymes in the 3D7 genetic background did not change the parasites' susceptibility to PPQ, CQ, or artesunate (39), suggesting that further validation of *plasmepsin 2/3* amplification in PPQ resistance is needed.

Given the potential mode of action of PPQ in blocking heme detoxification in the food vacuole (FV) (40), two FV membrane-resident transporters *pfmdr1* and *pfcrt* have received much attention. Clinical efficacy studies revealed the reduced prevalence of multicopy *pfmdr1* after switching from ATS-MFQ to DHA-PPQ (34–37), suggesting that this is due to DHA-PPQ selection of single-copy *pfmdr1* or decreased use of MFQ, since *pfmdr1* amplification is associated with a fitness cost (41). Interestingly, accompanying the emergence of PPQ resistance in Cambodia were several new PfCRT mutations (H97Y, F145I, M343L, and G353V) that have evolved in a Dd2 PfCRT background (74I, 75E, 76T, 220S, 271E, 326S, 356T, and 371I) (42), which were all validated by genetic studies as conferring resistance to PPQ (43). Moreover, the PfCRT F145I mutation was also associated with *in vivo* DHA-PPQ treatment failure and decreased *ex vivo* PPQ susceptibility by GWAS (44), providing further evidence that these new PfCRT mutations can serve as molecular markers for PPQ resistance.

Because the PPQ dose-response curves from the traditional *in vitro* or *ex vivo* assays to determine 50% inhibitory concentrations (IC_{50}s) sometimes do not fit the sigmoid curve, a PPQ survival assay (PSA) was designed (42), which measures the parasite survival rates after exposure to a pharmacologically relevant dose of 200 nM PPQ for 48 h. The PSA estimate was found to be well correlated with the DHA-PPQ clinical failure phenotype. Subsequently, an *in vitro* study using a wide range of PPQ concentrations described a bimodal dose-response curve for some clinical PPQ-resistant parasites, showing increased parasite survival at PPQ concentrations of 100 nM to 10 μM (45). This second peak in the PPQ dose-response curve was proposed to be an alternative readout for PPQ resistance. Whereas these drug assays were developed based on

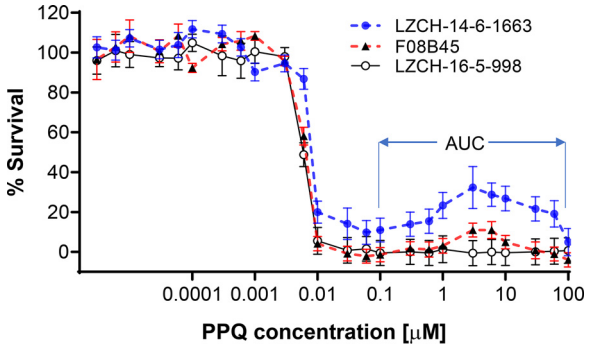

FIG 1 Piperaquine drug response curves of representative parasite isolates from the China-Myanmar border using the modified SYBR green I assay with 24 drug concentrations. One parasite isolate (LZCH-16-5-998) showed a typical sigmoid curve, while the other two isolates (LZCH-14-6-1663 and F08B45) showed a second peak in PPQ concentrations of 1 to 10 μM. The concentration range used to calculate the AUC is indicated.

clinical phenotypes observed in the GMS, their values in predicting resistance in other malaria regions remain to be evaluated.

Within the GMS, PPQ has the most prolonged use history in China, where it was first used in the early 1970 to supersede CQ as the first-line treatment for CQ-resistant *P. falciparum* (46). The emergence of PPQ resistance in the mid-1980s led to its diminished use in subsequent years (47–49). In a study conducted in the early 1990, the cure rates of a total dosage of 25 mg/kg PPQ dropped to ~33% (50). DHA-PPQ was then adopted as the first-line treatment for falciparum malaria in 2005 in China. In contrast to the rapidly declining clinical efficacy of DHA-PPQ in Cambodia, recent studies along the China-Myanmar border reported that DHA-PPQ remained highly efficacious for *P. falciparum* malaria (51, 52). In this study, we describe the features of *in vitro* susceptibility to PPQ, and the associated genetic adaptions, on the China-Myanmar border. Using culture-adapted clinical parasite isolates, we compared different readouts of *in vitro* PPQ susceptibility assays and genotyped multiple molecular markers associated with PPQ resistance in Cambodia.

RESULTS

***In vitro* susceptibility of parasite isolates to PPQ.** We tested *in vitro* susceptibilities of 120 archived *P. falciparum* isolates to PPQ using recently developed *in vitro* assays. These parasites are monoclonal, culture-adapted clinical isolates collected from the China-Myanmar border area during 2007 to 2016, which represent parasite populations after the adoption of DHA-PPQ as the frontline treatment for uncomplicated *P. falciparum* malaria in 2005. We first performed a modified 72-h standard drug assay using 24 dilutions of PPQ with the highest concentration being 100 μM to ensure complete parasite killing. Consistent with an earlier study (45), the drug response curves in 56.7% (68/120) of the parasite isolates exhibited a bimodal pattern with a second peak at PPQ concentrations of 100 nM to 100 μM (Fig. 1). We used the whole range of the drug concentrations to estimate the IC_{50} and IC_{90} values after removing the outliers following the strategy adopted in a previous *ex vivo* study (53). Overall, we found that the parasites were susceptible to PPQ with a median IC_{50} value of 5.6 nM (interquartile range [IQR], 4.1 to 7.1 nM) and a median IC_{90} value of 10.1 nM (IQR, 7.1 to 12.6 nM) (Fig. 2A and B). Despite this, the most and the least sensitive parasites had >6-fold differences in IC_{50} (1.8 to 11.0 nM) and ~8-fold differences in IC_{90} (3.0 to 23.5 nM), indicating the presence of parasites with reduced susceptibility to the drug. Over the 9 years, annual median PPQ IC_{50} varied between 4.8 and 6.8 nM (Table 1), but a significant difference was only identified between the 2014-2016 and 2007 parasites (Fig. 2A). Similarly, the annual median IC_{90} value fluctuated between 8.0 and 13.1 nM (Table 1). The IC_{90} in parasites from the most recent years (2014 to 2016) was significantly higher than the

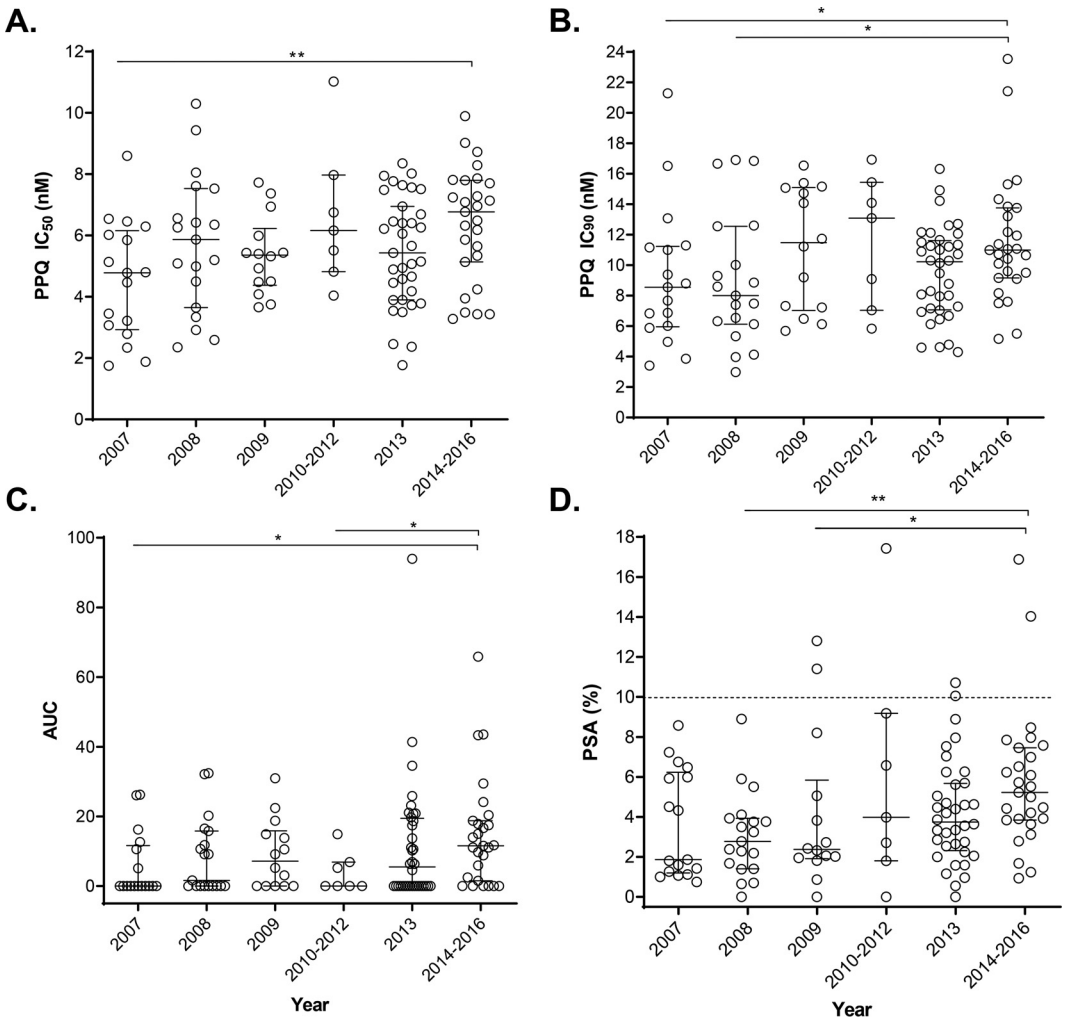

FIG 2 Dot plots of *in vitro* susceptibilities to piperaquine of longitudinally collected parasites from the China-Myanmar border area during 2007 to 2016. (A) IC_{50} values. (B) IC_{90} values. (C) AUC values. (D) PSA values. The dotted line represents the 10% survival rate cutoff that distinguishes piperaquine-resistant (≥10%) from piperaquine-sensitive (<10%) parasites in PSA. For samples from each year, the medians and interquartile ranges are shown. Asterisks indicate significant differences in drug sensitivity between 2 years. * and **, $P < 0.05$ and < 0.01, respectively (Mann-Whitney U test).

IC_{90}s in the earliest samples from years 2007 and 2008, suggesting declining PPQ susceptibility over the years (Fig. 2B). With regard to the fluctuation in the annual IC_{50} and IC_{90} data, the Mann-Kendall trend test did not detect a clear trend of increase in IC_{50} or IC_{90} ($P > 0.05$).

We then measured the AUC at PPQ concentrations 0.1 to 100 μM in the drug response curves. In different years, 35.3 to 77.8% of parasites displayed bimodal drug response curves (Table 1), and parasites collected in 2014 to 2016 had the highest proportion (77.8%). Similar to the IC_{50} and IC_{90} values, parasites from 2014 to 2016 had significantly higher AUC values than parasites from 2007 and from 2010 to 2012 (Fig. 2C). However, compared to the AUC range defined earlier (45), only five parasite isolates had the intermediate AUC values (35 to 100), whereas no parasites had an AUC of >100, a cutoff value found to correspond to PSA value of >10% (Fig. 2C).

We used PSA to assess the parasite's resistance to PPQ, which measures the parasite survival rate after exposure to 200 nM PPQ for 48 h (42). The overall parasite survival rate was low (3.8%), whereas parasites from 2014 to 2016 had significantly higher PSA values than parasites from 2008 and 2009 (Fig. 2D). There seemed to be a trend of gradual increase over the years (from 1.9% in 2007 to 5.2% in 2014 to 2016), albeit the

TABLE 1 *In vitro* susceptibilities of *Plasmodium falciparum* strains from the China-Myanmar border area to piperaquine[a]

		Median (IQR)			No. (%)		
Period (yr)	No.	IC_{50} (nM)	IC_{90} (nM)	PSA (%)	PSA ≥ 10%[b]	Bimodal[c]	Median AUC (IQR)[d]
2007	17	4.8 (2.9–6.2)	8.6 (6.0–11.2)	1.9 (1.2–6.2)	0	6 (35.3)	0.0 (0–11.6)
2008	19	5.9 (3.7–7.5)	8.0 (6.1–12.6)	2.8 (1.4–3.9)	0	10 (52.6)	1.7 (0–15.8)
2009	14	5.4 (4.4–6.2)	11.5 (7.0–15.1)	2.4 (1.9–5.8)	2 (14.3)	9 (64.3)	7.2 (0–15.9)
2010–2012	7	6.2 (4.8–8.0)	13.1 (7.0–15.5)	4.0 (1.8–9.2)	1 (14.3)	3 (42.9)	0.0 (0–6.9)
2013	36	5.4 (3.9–7.0)	10.2 (7.1–11.6)	3.7 (2.3–5.7)	2 (5.6)	19 (52.8)	5.5 (0–19.5)
2014–2016	27	6.8 (5.1–7.8)	11.0 (9.2–13.8)	5.2 (3.8–7.5)	2 (7.4)	21 (77.8)	11.2 (1.5–18.8)
Total[e]	120	5.6 (4.1–7.1)*	10.1 (7.1–12.6)**	3.8 (2.0–6.1)†	7 (5.8)	68 (56.7)	5.6 (0–16.5)‡

[a]AUC, area under the curve; IQR, interquartile range; PSA, piperaquine survival assay score.
[b]Number and percentage of parasites with a PSA of ≥10%.
[c]Number and percentage of parasites with bimodal PPQ response curves (67.4%).
[d]AUC for the drug response curve at PPQ concentrations of 10^2 to 10^5 nM.
[e]A Kruskal-Wallis test was used to compare results between years (*, $P = 0.0557$; **, $P = 0.1103$; †, $P = 0.0450$; ‡, $P = 0.1246$). A Mann-Kendall trend test was used to evaluate the presence of annual trends for IC_{50}, IC_{90}, PSA, and AUC values (all of which were nonsignificant).

trend was not significant (Table 1, $P > 0.05$, Mann-Kendall trend test). In total, there were only 7 (5.6%) parasite isolates displaying PSA values of >10% (Table 1 and Fig. 2D), the proposed cutoff value for PPQ resistance (42), and the highest PSA value was 17.4%. For the two earlier years, 2007 and 2008, all 36 parasite isolates tested had PSA values lower than 10%.

Comparison among different *in vitro* PPQ sensitivity assays. The PSA was developed to better differentiate recrudescent from nonrecrudescent *P. falciparum* cases after treatment with DHA-PPQ in Cambodia (42). Using the cutoff value of 10%, we compared the IC_{50}, IC_{90}, and AUC values between the 113 parasite isolates with PSA values <10% and the seven isolates with PSA values of ≥10%. The results showed no significant differences between the two groups in IC_{50}, IC_{90}, or AUC values (see Fig. S1 in the supplemental material), indicating that PSA and other parameters measured different aspects of PPQ susceptibilities.

To discover the underlying patterns of the *in vitro* assay data for PPQ and properly group the parasites based on their IC_{50}, IC_{90}, AUC, and PSA values, we subjected the *in vitro* assay results to analysis using the unsupervised K-means clustering algorithm. This clustering analysis assigned the parasites in three groups of 44, 46, and 26 parasites, respectively (Fig. 3). While this grouping identified significantly different clusters in AUC, IC_{50}, and IC_{90} values, PSA results remained indistinguishable among the three clusters (Table 2 and Fig. 3). Group 1 (44 parasites) contained mostly parasites having typical sigmoid *in vitro* drug response curves (median AUC = 0), with low IC_{50} (median, 3.9 nM) and IC_{90} (median, 6.6 nM) values. Group 2 (46 parasites) had intermediate AUC value (median, 5.8), but relatively high IC_{50} (median, 6.3 nM) and IC_{90} (median, 11.6 nM) values. Group 3 (26 parasites) had the highest AUC value (median, 22.1) and highest IC_{50} (median, 7.7 nM) and IC_{90} (median, 12.3 nM) values (Fig. 3). The results showed that IC_{50} and IC_{90} values were highly correlated ($r = 0.688$, $P < 0.05$, Pearson correlation), while they also exhibited significant correlations with the AUC results (Fig. 3, upper diagonal). However, results from the 72-h assays showed no correlation with the PSA results.

Association with known resistance markers. PPQ resistance has been associated with the E415G mutation in a putative exo-nuclease (35), and new mutations (H97Y, F145I, M343L, and G353V) in PfCRT (43, 44), and *plasmepsin 2/3* amplification (35, 36). Genotyping the 120 isolates from the China-Myanmar border by PCR and sequencing found no E415G mutation (data not shown). Also, real-time PCR analysis did not identify any parasites carrying more than one copy of *plasmepsin 2/3* (see Fig. S2). In order to determine mutations in the *pfcrt* gene, we used RT-PCR to circumvent problems associated with multiple AT-rich introns. Sequencing of the full-length *pfcrt* genes showed that the overwhelming majority (115/120) of the parasites carried the Dd2 haplotype (M74I, N75E, K76T, A220S, Q271E, N326S, I356T, and R371I). Compared with the Dd2 haplotype, one isolate carried an M74T mutation, one was wild-type (WT) at

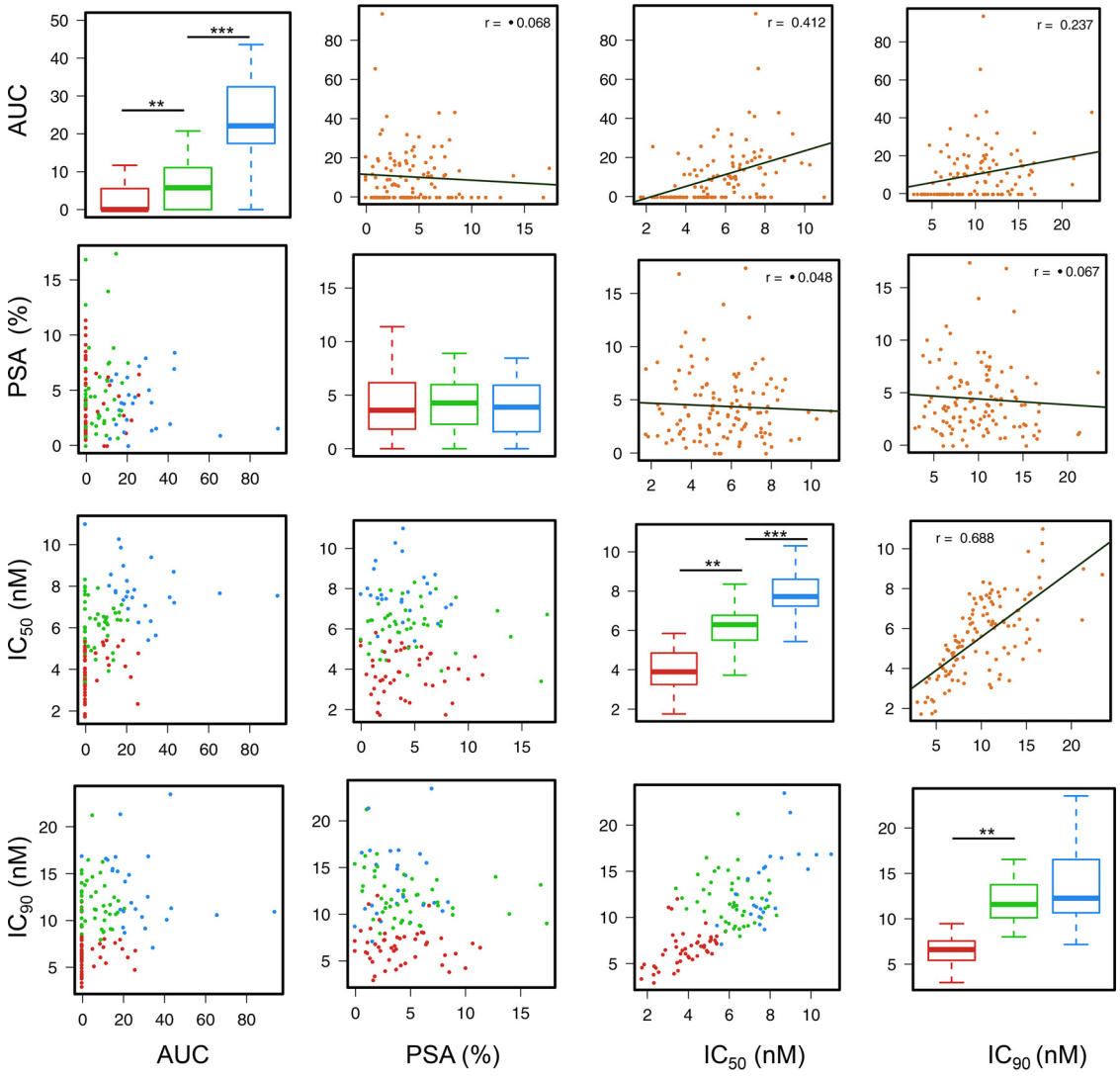

FIG 3 Clustering of parasite populations into three groups based on unsupervised K-means clustering analysis of piperaquine drug assay data. The diagonal panels show comparisons in AUC, PSA, IC_{50}, and IC_{90} values among the three groups. Groups 1, 2, and 3 are indicated in red, green, and blue, respectively. Panels below the diagonal are scatterplots showing the distribution of values of samples, which are color-coded based on the three K-mean groups. Panels above the diagonal are scatterplots showing the correlation between each pair of experimental data. Pearson correlation coefficient values are shown.

N326, one carried two additional mutations at L243S and K284E, and one had an additional mutation at F78S (Table 3). For the four new mutations described in Cambodia, only one parasite carried an H97L mutation in the Dd2 haplotype background.

PPQ-resistant parasites have evolved mainly in the background of PfK13 mutations and loss of amplification of the *pfmdr1* gene (34, 35, 42, 54). For PfK13, the predominant mutation in the China-Myanmar border area is the F446I, but in 2014 to 2016, the new mutation G533S appeared and reached 44% prevalence (55). Parasites with the NN insertion at amino acids 136 to 137 of PfK13 continuously increased in frequency and reached 100% in 2014 to 2016. For *pfmdr1*, gene amplification was rare in the parasite population from the China-Myanmar border (56). Only Y184F was consistently present throughout most of the years and slightly increased in the 2013-2016 samples. Whereas N1042D was only found in the 2009 samples, the N86Y, D90H, and E130K mutations appeared in samples collected in later years (2013 to 2016) at very low frequencies. It is noteworthy that the F1226Y mutation appeared from 2013 onward, but its frequency reached 44.4% in the 2014-2016 samples.

TABLE 2 PPQ susceptibilities categorized based on IC_{50}, IC_{90}, and the presence of bimodal curves and AUC[a]

Parasite group or *P* value	Median (IQR)			PSA ≥ 10%, no. (%)	Median PSA (IQR)
	AUC	IC_{50} (nM)	IC_{90} (nM)		
Groups					
1 (red)	0.0 (0.0–5.7)	3.9 (3.2–4.9)	6.6 (5.4–7.6)	3 (6.3)	3.6 (1.8–6.2)
2 (green)	5.8 (0.0–11.1)	6.3 (5.5–6.8)	11.6 (10.1–13.8)	4 (8.7)	4.3 (2.2–6.1)
3 (blue)	22.1 (17.2–33.0)	7.7 (7.2–8.6)	12.3 (10.6–16.6)	0 (0.0)	3.9 (1.6–6.0)
P values[b]					
*	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	ND	$P = 0.6450$
**	$P = 0.0034$ (1 vs 3) $P < 0.0001$ (2 vs 3) $P = 0.4108$ (1 versus 2)	$P < 0.0001$ (1 vs 2, 1 vs 3, 2 vs 3)	$P < 0.0001$ (1 vs 2, 1 vs 3) $P = 0.1230$ (2 versus 3)	ND	ND

[a]AUC, area under curve; IQR, interquartile range; PSA, piperaquine survival assay score; ND, not done.
[b]*, Comparison among three groups (Kruskal-Wallis test); **, comparison between two groups (Mann-Whitney U test).

To determine whether any of these mutations were associated with altered PPQ susceptibility, we performed Lasso regression analysis, assessing all mutations regardless of their prevalence. The PfK13 G533S and two rare mutations PfK13 C580Y and Pfmdr1 D90H were significantly associated with PPQ IC_{50} values (see Fig. S3A). For the mutations associated with increased PSA values, only the Pfmdr1 Y184F and PfCRT I356T mutations were relatively prevalent. In contrast, the PfK13 mutations and Pfmdr1 N86Y and E130K were very rare in the study parasite population (see Fig. S3B). We then used the Mann-Whitney U test to determine whether the relatively abundant (≥5%) mutations or haplotypes were associated with altered susceptibilities to PPQ. When parasites with PfK13 mutations in the propeller domain (>440 amino acids) were compared to the PfK13 WT parasites, we found that those with PfK13 mutations had significantly elevated AUC values (Fig. 4A, $P < 0.01$). Although these parasites with the PfK13 mutations also had higher IC_{50} and IC_{90} values than the WT parasites, the differences were only marginally significant (Fig. 4B and C, $P = 0.0709$). When comparing the two most predominant mutations F446I and G533S, we found that parasites carrying either of these two mutations had significantly higher AUC values than the WT parasites (Fig. 4E). In addition, parasites with the G533S mutation also had significantly higher PPQ IC_{50} values than the WT parasites ($P < 0.0001$) and parasites with the F446I mutation (Fig. 4F, $P < 0.01$). Compared to the WT parasites, the parasites with PfK13 mutations in the propeller domain (amino acids >440) or the predominant mutations showed no significant differences in their PSA values (Fig. 4D and H).

For the Pfmdr1 Y184F and F1226Y mutations, 1226Y was associated with significantly increased PPQ IC_{50} values, while 184F was associated with significantly higher PSA values (see Fig. S4). For the haplotypes, Y184/1226Y had significantly higher IC_{50} than the WT (Y184/F1226) and the 184F/F1226 haplotype ($P < 0.05$, Mann-Whitney U test). The 184F/F1226 haplotype had a significantly higher PSA value than the WT parasites (see Fig. S4, $P < 0.05$).

DISCUSSION

Surveillance for antimalarial drug resistance and elucidation of resistance mechanisms are essential for guiding effective treatment of malaria cases. This is especially true for eliminating malaria in the GMS, where parasites have developed resistance to all commonly used antimalarial drugs, especially the frontline ACT drugs ATS-MFQ and DHA-PPQ. Since the deployment of DHA-PPQ in Cambodia in 2008, failure rates to this ACT rapidly increased, which is associated with the increased prevalence of parasites with PfK13 C580Y mutation and *plasmepsin 2/3* gene amplification (22). Population genomics studies identified the expansion of an MDR colineage KEL1/PLA1 carrying these mutations in eastern GMS, which diversified into multiple subgroups with the emergence of novel *pfcrt* mutations (57). To evaluate the evolution of drug resistance in the pre-elimination setting of the GMS, we assessed the *in vitro* PPQ susceptibility of parasites collected from the China-Myanmar border. Despite the historical detection of

TABLE 3 Haplotypes of the *pfcrt* gene in the studied parasite population and association with altered piperaquine susceptibility

Isolate	*pfcrt* position[a]												Frequency, no. (%)	AUC	*P*[b]	PSA[c] (%)	*P*	IC_{50} (nM)	*P*	IC_{90} (nM)	*P*
	74	75	76	78	97	220	243	271	284	326	356	371									
3D7	M	N	K	F	H	A	L	Q	K	N	I	R	0	0	<0.0001	1.19	<0.0001	5.47	<0.0001	7.05	<0.0001
Dd2 type	**I**	**E**	**T**	F	H	**S**	L	**E**	K	**S**	**T**	**I**	115 (95.8)	6.14		3.84		5.66		9.55	
F09N29	**I**	**E**	**T**	F	H	**S**	L	**E**	K	N	**T**	**I**	1 (0.97)	0	<0.0001	0.87	<0.0001	5.32	0.0985	15.08	<0.0001
F07-29	**I**	**E**	**T**	F	H	**S**	**S**	**E**	**E**	**S**	**T**	**I**	1 (0.97)	0	<0.0001	1.86	<0.0001	3.45	<0.0001	6.87	<0.0001
NB-14-6-3	**I**	**E**	**T**	F	**L**	**S**	L	**E**	K	**S**	I	**I**	1 (0.97)	15.02	<0.0001	6.52	<0.0001	7.81	<0.0001	15.58	<0.0001
F09N6	**I**	**E**	**T**	**S**	H	**S**	L	**E**	K	**S**	**T**	**I**	1 (0.97)	5.22	<0.0001	1.82	<0.0001	4.5	<0.0001	14.72	<0.0001
NB13-1827	**T**	**E**	**T**	F	H	**S**	L	**E**	K	**S**	**T**	**I**	1 (0.97)	4.59	<0.0001	4.47	0.1795	5.07	0.0037	12.62	<0.0001

[a]Mutations in the Dd2 parasite are highlighted in boldface; new mutations are underlined.

[b]All *P* values were determined using a Wilcoxon matched-pairs signed-rank test, compared to the Dd2 type.

[c]PSA, piperaquine survival assay score.

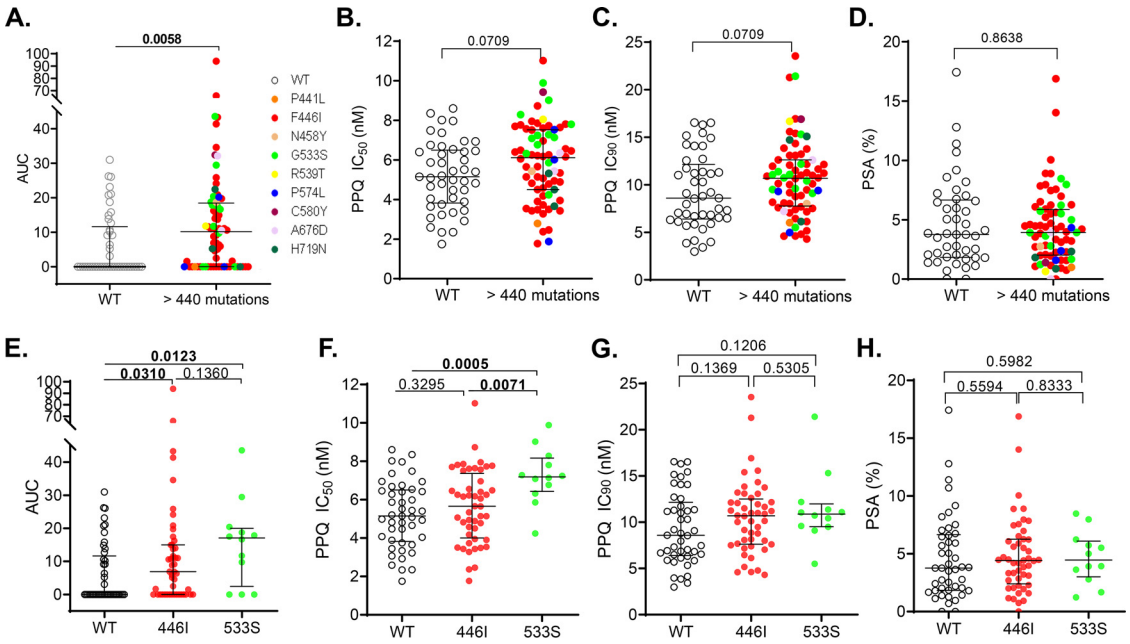

FIG 4 Comparisons of *in vitro* piperaquine susceptibility (AUC [A and E], IC_{50} [B and F], IC_{90} [C and G], and PSA [D and H]) between parasites with PfK13 mutations (colored dots) and wild-type parasites (WT, open circles). PfK13 mutations were indicated by different colors. AUC, area under curve. Horizontal lines indicate medians ± IQRs. *P* values are indicated, and significance is highlighted in boldface (Mann-Whitney U test).

clinical PPQ resistance in southern China when PPQ was used as monotherapy after the mid-1980s (47–50), our study suggests the lack of PPQ resistance in this region or that PPQ resistance in this parasite population may involve additional mechanisms. While the lack of clinical outcome data prevents us from connecting the *in vitro* assay data with the *in vivo* efficacy result, this assumption is nonetheless consistent with the excellent clinical efficacy of DHA-PPQ from recent studies in this region (51, 52).

The three assays, measuring the IC_{50}, IC_{90}, AUC, and PSA as the parasite's response to PPQ, present a complicated picture for identifying *in vitro* surrogates of clinical PPQ resistance. PSA measures the survivorship of parasites after exposure to 200 nM PPQ for 48 h (42). Using clinical DHA-PPQ efficacy data, a PSA value of ≥10% was found to be a relevant value to define PPQ resistance, and the PSA results were used to identify *plasmepsin 2/3* gene amplification (36). Using the PSA, we determined that 5.8% of the 120 parasites collected from the China-Myanmar border had PSA values of higher than 10%, conforming to the definition of PPQ resistance. However, the lack of parasites with *plasmepsin 2/3* amplification and new *pfcrt* mutations suggests that they may not represent the clinical PPQ resistance previously described. Thus, without clinical and molecular data as backups, the usefulness of PSA outside eastern GMS remains to be further evaluated. In evaluating the bimodal curve-based assay, Bopp et al. found that the PSA survival rates (0 to 30%) in a small set of nine representative isolates were significantly correlated with the AUC values, and an AUC of >100, corresponding to a PSA value of >10%, was considered to be PPQ resistant (45). In this study, comparing the results from these different assays did not identify any correlation between PSA and IC_{50}, IC_{90}, or AUC values. The relatively low IC_{50} and IC_{90} values and the finding that only five parasites had AUC values in the intermediate range of 35 to 100 are both consistent with the excellent DHA-PPQ efficacy of the China-Myanmar border parasite population (51, 52). This study identified correlations among the IC_{50}, IC_{90}, and AUC estimates, suggesting that the use of a wide range of PPQ concentrations with the high concentrations able to kill all parasites would allow a more realistic determination of these *in vitro* assay values (53). Furthermore, given that the use of the *ex vivo* IC_{50} or

IC_{90} estimates also allowed the identification of the *plasmepsin 2/3* amplification as associated with clinical PPQ resistance by GWAS (35, 44), we consider that the IC_{50} or IC_{90} values are useful parameters for predicting PPQ resistance.

Plasmepsin 2/3 amplification has previously been associated with clinical PPQ resistance and was a useful marker for predicting DHA-PPQ clinical failures in Cambodia and eastern GMS. However, validation of these two genes in the 3D7 background using genetic inactivation and overexpression produced conflicting results, suggesting that the effect of *plasmepsin 2/3* amplification on PPQ resistance may vary depending on the parasites' genetic backgrounds. This also undermines the use of *plasmepsin 2/3* amplification for monitoring PPQ resistance outside the eastern GMS. Novel *pfcrt* mutations were found to be associated with PPQ resistance, and there is genetic evidence showing that these novel mutations mediate PPQ resistance in the genetic background without *plasmepsin 2/3* amplification (43). Although we did not observe any of the new *pfcrt* mutations (H97Y, F145I, M343L, and G353V) described in the DHA-PPQ-resistant populations in Cambodia (42–44), one parasite collected in 2014 had an H97L mutation. This parasite had a significantly elevated CQ IC_{50} value (826.9 nM) compared to parasites carrying the Dd2 haplotype *pfcrt* (259.2 nM). It also had higher PPQ IC_{50}, IC_{90}, PSA, and AUC estimates than parasites carrying the Dd2 haplotype *pfcrt* (Table 3), but the limited data from a single parasite isolate carrying the H97L mutation warrant future studies. Of note, this mutation was identified in a single parasite from a set of 183 samples collected in ACT efficacy studies in Cambodia, but it was not associated with PPQ IC_{90} (44).

The PPQ resistance occurring in western Cambodia probably reflects a two-step selection process. The spread of artemisinin resistance has led to the limited genetic diversity of the parasite populations with most parasites carrying the PfK13 mutations (e.g., C580Y), and on top of these genetic backgrounds, PPQ resistance has emerged (34, 42). Within a short time period, parasite lineages harboring the PfK13 C580Y mutation and *plasmepsin 2/3* amplification rapidly spread in eastern GMS in the fashion of a hard selective sweep (37, 57). At the China-Myanmar border, *P. falciparum* had PfK13 alleles similar to those of parasites from northern Myanmar, with a predominance of the F446I mutation (29, 30). This mutation, when introduced into the 3D7 background, did not confer artemisinin resistance as measured by the *in vitro* ring-stage survival assay, but the parasites had a prolonged ring stage and showed no fitness cost (58). When we assessed the connection between the PfK13 mutations in the parasites studied here with PPQ *in vitro* assay results, we found that the total K13 mutations after the position 440 or the two predominant mutations F446I and G553S were all associated with increased IC_{50}, IC_{90}, and AUC values compared to the WT parasites. The F446I and G553S parasites developed significantly higher AUC values than WT, and the G553S parasites showed significantly higher IC_{50} values than WT and F446I parasites. Of note, both of these predominant PfK13 mutations were associated with significantly higher ring survival rates than the WT parasites (55). We speculate that they may not be causal for the elevated PPQ AUC and IC_{50} values, but, like the C580Y mutation in Cambodia, may represent the PfK13 background mutations on which PPQ resistance emerges. GWAS may reveal the genetic changes underlying the altered susceptibility to PPQ.

The ATS-MFQ combination has previously selected for increased copy number of *pfmdr1* in the eastern GMS, whereas DHA-PPQ used to replace ATS-MFQ appears to have selected for the opposite. Consistent with the extensive deployment of DHA-PPQ at the China-Myanmar border, parasites from this region mostly had a single copy *pfmdr1*. In these parasite isolates, we found that the Y184F mutation had an overall frequency of 36%, while the F1226Y mutations appeared after 2013 and reached a high frequency of 44% in the 2014-2016 samples. Whereas the Y184F mutation was not significantly associated with changes in PSA in the Cambodian parasites, we found that it was associated with an increased PSA value in the current work. Also, we found that the newly acquired F1226Y mutation in later years was associated with an increased PPQ IC_{50} value. It would be interesting to determine whether the F1226Y mutation was

selected by the extensive use of DHA-PPQ and whether it indeed mediates PPQ resistance.

We did not find clear evidence of PPQ resistance in the parasites from the China-Myanmar border area, nor did we observe temporally declining PPQ susceptibility. Alternatively, the fluctuating PPQ susceptibility over the years suggests that PPQ resistance emerged one or more times, but did not spread significantly. Despite this, we found that parasites collected in 2014 to 2016 had significantly higher IC_{50}, IC_{90}, AUC, and PSA values than those collected earlier (Fig. 2). These samples also appeared to be genetically distinct from those collected in earlier years for the *pfk13* gene. The F446I mutation showed a gradual increase in the frequency between 2007 and 2013, whereas the G533S mutation only emerged in the 2014-2016 parasite population and reached 44% (55). It would be interesting to test whether this parasite population was the result of intensive selection by DHA-PPQ, the most popular ACT deployed in this area, or the result of the spread of parasites from elsewhere in the GMS. Regardless of the origins, the drug resistance situation requires continued surveillance efforts using a combination of complementary tools to monitor clinical efficacy, *in vitro* drug susceptibility, and molecular epidemiology of resistance-associated markers. We did not identify clear evidence of PPQ resistance from *in vitro* assays or association with known molecular markers, consistent with the known sustained clinical efficacy of DHA-PPQ in the region.

MATERIALS AND METHODS

Parasite collection and culture adaption. Uncomplicated *P. falciparum* malaria was diagnosed by microscopy in patients attending local clinics located along the China-Myanmar border during 2007 to 2016. *P. falciparum*-infected blood samples were collected and cryopreserved in liquid nitrogen for laboratory culture adaption. Informed consent was obtained from all patients, and the study was approved by the institutional review boards of Kachin Health Bureau, Kunming Medical University, and Pennsylvania State University. A total of 120 parasite isolates (17 in 2007, 19 in 2008, 14 in 2009, 7 in 2010 to 2012, 36 in 2013, and 27 in 2014 to 2016), determined to be monoclonal infections by genotyping three polymorphic antigen markers—*msp1*, *msp2*, and *glutamate-rich protein*—were culture adapted and used for *in vitro* drug assay and genotyping (56). Parasites were cultured in type O^+ human red blood cells (RBCs) in a complete medium at 37°C in an incubator under 5% O_2, 5% CO_2, and 90% N_2.

Drug assays. PPQ was obtained from Chongqing Kangle Pharmaceutical Co., Ltd. (Chongqing, China). The stock solution of PPQ (10 mM) was prepared in a mixture of 90% methanol and 10% HCl. *In vitro* susceptibilities of the parasite isolates to PPQ were determined using the standard SYBR green I assay (66). Twenty-four dilutions of the drug were added to 96-well microplates, which cover both the high-range (100, 60, 30, 10, 6, 3, 1, 0.6, and 0.3 μM) and low-range concentrations (100, 60, 30, 10, 6, 3, 1, 0.6, 0.3, 0.1, 0.06, 0.03, 0.01, 0.006, and 0.003 nM). Parasite cultures were synchronized with 5% D-sorbitol treatment (59), and the drug assay was performed using 0.5% synchronized parasites at the ring stage and 2% hematocrit (60). A drug assay was carried out with three technical replicates and two biological replicates. For consistency and comparison, 3D7 was included throughout the study as an internal control.

PSA was performed as described earlier (42). Briefly, tightly synchronized 0- to 3-h ring-stage parasites in 2 ml of culture media, diluted to 2% hematocrit and 0.5% parasitemia, were exposed to 200 nM PPQ or distilled water as the control in 24-well plates for 48 h. The drug was then washed off with RPMI 1640, and parasites were cultured for an additional 24 h. Thin smears were stained with Giemsa, and the number of surviving parasites in 20,000 RBCs were independently enumerated by two investigators. A third independent assessment was conducted if the difference between the first two counts was >20%. Survival rates were calculated by determining the proportion of viable parasites (second-generation rings or trophozoites with normal morphology) at the 72-h time point in PPQ-treated versus control cultures. A survival rate of 10% was used as the cutoff value for PPQ resistance (42).

Single nucleotide polymorphisms and copy number variations. The E415G mutation in PF3D7_1362500 was determined by PCR and sequencing. For this, parasite genomic DNA was extracted from freshly cultured parasites using a genomic DNA extraction kit according to the manufacturer's instructions (Roche). The *exo-E415G* fragment was amplified in 25 μl of reaction mixture, which contained 12.5 μl of Premix *Taq* (TaKaRa *Taq* version 2.0), 1 μl of primers (0.1 μM) (5′-TTTCCTTCTGACCCCTTT-3′ and 5′-TCCCATTCGATATCTATACCTAT-3′), and 50 ng of genomic DNA. PCR was performed under the following conditions: 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 68°C for 1 min. For the year 2013 samples, the full-length *pfk13* gene and the two *pfmdr1* fragments covering codons 86, 184, 1042, and 1246 were amplified and sequenced as described previously (29, 61). For other samples, the *pfk13* and *pfmdr1* sequences were obtained from earlier studies (61, 62).

CNVs of the *plasmepsin 2/3* genes were evaluated by real-time PCR following an established procedure (35). Briefly, each 20-μl PCR contained 10 μl of Bestar SybrGreen qPCR master mix, 0.25 μM concentrations of each forward and reverse primer for the *plasmepsin 2/3* gene or the *β-tubulin* gene as an internal control, 0.4 μl of 5× ROX reference dye, and 50 ng of the parasite DNA. Real-time PCR was

performed with an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 56°C for 30 s, and 72°C for 30 s and then a final extension for 5 min at 72°C. A copy number ratio of ≥1.5 was considered an increased copy number.

The full-length coding sequence of the *pfcrt* gene consisting of 424 codons was obtained by RT-PCR. Total RNA was isolated from 1 ml of cultured parasites at 1% parasitemia using the TRIzol reagent (Life Technologies). cDNA was synthesized using a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, 1 μg of total RNA was mixed with 1 μl of Oligo(dT)$_{18}$ primer, 1 μl of RiboLock RNase Inhibitor, 2 μl of 10 mM dNTP mix, and 1 μl of RevertAid M-MuLV reverse transcriptase in a total volume of 20 μl, and the reaction mixture was incubated at 42°C for 60 min. The *pfcrt* gene was amplified using primers located in the 5′ untranslated region (5′ UTR; CCGTTAATAATAAATACACGCAG) and the 3′ UTR (ATTCCTTATAAAGTGTAATGCGA) of the *pfcrt* gene (63). PCR was performed in 25 μl, including 12.5 μl of PrimeSTAR Max Premix (2×) (TaKaRa PrimeSTAR Max DNA polymerase), 1 μl of each primer (0.1 μM), and 2 μl of cDNA. PCR conditions were initial denaturing at 95°C for 3 min, followed by 30 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 1 min. PCR products were sequenced in both directions using the dideoxy method, and sequences were aligned to the reference gene in 3D7 using ClustalW to identify the single nucleotide polymorphisms.

Statistical analysis. To calculate IC_{50} and IC_{90} values, drug response data in the PPQ concentration range of 0.03 to 100 nM were fit to a sigmoid curve after removing the outliers (64). Outliers were determined using Grubb's test as implemented in GraphPad Prism 6.0. If a parasite isolate had 3 or more of the 10 data values in the 0.1 to 100 μM PPQ range exceeding 3 × standard deviations of the baseline value (based on the value at 100 μM PPQ, approximating 100% growth inhibition), it was considered to have a bimodal PPQ response curve. The area under the curve (AUC) for the drug response curves at 100 nM to 100 μM was calculated using GraphPad Prism 6.0. Statistical analyses were performed using GraphPad Prism 6.0. Medians and IQRs were calculated, given that the data were not normally distributed. IC_{50}s and PSA survival rates between the groups were compared by the Kruskal-Wallis test. Correlations were determined using the Pearson's test. For the grouping of samples, we used an unsupervised machine-learning algorithm K-means to cluster the AUC, PSA, IC_{50}, and IC_{90} values of all the samples after the data were normalized by their z-scores. All possible pairs of AUC, PSA, IC_{50}, and IC_{90} values were plotted in scatterplots, and the regression lines were built using a least-squares approach. To identify the mutation markers associated with PPQ susceptibility data, the Lasso regression, a widely used method with proven better performance than linear regression and stepwise regression (65), was used first to analyze the 30 mutation markers in 120 samples. Each model was trained by 10-fold cross-validation, and the final parameters are listed in Table S1 in the supplemental material. The shrunk coefficient returned from Lasso regression usually indicates the association between a mutation marker and PPQ susceptibility. For further validation, a Wilcoxon test was performed. In addition, the predominant mutations in PfK13 or all propeller domain mutations combined, as well as the major haplotypes of *pfmdr1*, were analyzed by the Mann-Whitney U test.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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