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## Supplementary appendix

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## Supplementary appendix

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Lancet Infect Dis 2023; published online.

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## Supplementary Methods

### Generation of isogenic parasite clones harboring single or multicopy *pm2/3* from the S170 field isolate

The S170 field isolate from French Guiana was collected in 2015 by Dr. Lise Musset and colleagues. The parasite line was culture-adapted to *in vitro* conditions shortly before individual clones were generated by limiting dilution. All parasite cultures were maintained at 3% hematocrit in RPMI 1640 medium supplemented with 25 mM HEPES, 2.1 g/L sodium bicarbonate, 10 µg/mL gentamicin, 50 µM hypoxanthine, 0.5% (wt/vol) AlbuMAX II (Thermo Fisher Scientific), and 7.5% (v/v) human serum (Interstate Blood Bank). Parasites were maintained at 37°C under 5% $O_2$ and 5% $CO_2$ conditions.

### Piperaquine survival assays

*In vitro* susceptibility to piperaquine (PPQ) was examined using PPQ survival assays (PSAs), carried out as previously described,[1] with the following modifications. Sorbitol-synchronized ring-stage parasites were seeded at 1% parasitemia and 1% hematocrit and exposed to a 14-point, 2-fold dilution series of PPQ concentrations ranging from 1.6 nM to 12,800 nM in technical duplicates. The master stock of 5 mM PPQ was dissolved in 0.5% lactic acid. Parasites were incubated with PPQ alongside drug-free controls for 48h at 37°C. PPQ was then removed by washing the plates three times with complete media and contents were transferred to fresh plates using the Freedom EVO MCA96 liquid-handler (Tecan). Assay plates were further incubated for 24h in drug-free media. In parallel, we also exposed parasites to PPQ continuously for 72h without drug removal. Parasitemia of the drug-treated and drug-free wells from the 48h and 72h exposure assays were measured at 72h by flow cytometry, as previously described.[2] Briefly, parasites were incubated with 1× SYBR Green and 100 nM MitoTracker Deep Red for 30 min at 37°C, followed by quenching with 1× PBS. An average of 10,000 cells per sample were analyzed using the iQue Screener Plus cytometer (Sartorius). Viable parasites were defined as the percentage of MitoTracker-positive and SYBR Green-positive labelled cells.

### *In vitro* drug susceptibility assays with cultured parasites

Parasite susceptibility to antimalarial drugs was measured by exposing parasites starting at 1% parasitemia and 1% hematocrit to a 10-point, 2-fold dilution range of drug concentrations for 72h in technical duplicates, alongside drug-free control wells. Drug susceptibility assays were performed with PPQ, monodesethyl-amodiaquine (the *in vivo* metabolite of amodiaquine), monodesethyl-chloroquine (the *in vivo* metabolite of chloroquine), pyronaridine, quinine, lumefantrine, mefloquine and dihydroartemisinin (DHA). Master stocks of these drugs were dissolved in DMSO, except chloroquine that was dissolved in water. For all drug assays, the S170 and NF54 parasites were included as reference lines. Parasitemia of the drug-treated and drug-free wells from these dose-response assays were measured by flow cytometry as described above.

### *Calculation of PSA, AUC, $IC_{50}$ and $IC_{90}$ values*

For all drug assays conducted herein**,** we included kill controls in which 1 µM DHA-treated parasites were used as a background control to achieve complete parasite killing. We subtracted this percent parasitemia from the total parasitemia measured for each well. Parasite survival for PSA was calculated by dividing the background-subtracted parasitemias in the PPQ-treated samples by the parasitemia of the no-drug control. The Area Under the Curve (AUC) values for PPQ was determined based on total parasite survival across the range of 6.2 nM to 12,800 nM using Prism 8.3.1 (GraphPad). $IC_{50}$ and $IC_{90}$ values were calculated by applying a non-linear regression model (sigmoidal dose-response) on the normalized % survival across the log-transformed drug concentrations using Prism v8.3.1 (GraphPad). Statistical significance was determined by two-tailed unpaired Student's t test tests using Prism 8.3.1 (GraphPad).

### Determination of *pm2/3* copy from parasite lines

Genomic DNA was extracted from the S170 isogenic clones using the QIAamp DNA Blood Kit (Qiagen). Copy number of *pm2* (PF3D7_1408000) was measured by quantitative PCR (qPCR) on the QuantStudio 3 instrument (Thermo Fisher Scientific), as previously described.[1] Reactions were multiplexed for *pm2* together with the single copy *β-tubulin* (PF3D7_1008700) reference gene. Each clonal parasite line was tested in triplicates alongside standards containing predefined ratios of *pm2* and *β-tubulin* gene fragments, as well as NF54 and S170 lines. The *pm2* copy number was calculated after normalizing to *β-tubulin* and standardizing to NF54 single-copy values. Whole-genome sequencing was further performed on these clones to confirm the *pm2/3* copy number status. Libraries were prepared from genomic DNA using Illumina DNA Nextera Flex Kit according to manufacturer's instructions and sequenced using 150 bp paired-end reads on the Illumina NextSeq platform. Sequence data were aligned to the *P. falciparum* 3D7 genome (PlasmoDB version 36.0) using Burrow-Wheeler Alignment. Reads that did not map to the reference genome and optical duplicates were removed using SAMtools and Picard. The reads were realigned around indels and base quality scores were recalibrated using Genome Analyses Tool Kit RealignerTargetCreator and BaseRecalibrator, respectively. BIC-Seq was used to identify copy number variations using

the Bayesian statistical model. Copy number differences between the clones was only observed in the chr14 segment harboring the tandem *pm2/3* genes.

## Acknowledgements

We sincerely thank Dr. Lise Musset (Laboratoire de parasitologie, World Health Organization Collaborating Center for Surveillance of Antimalarial Drug Resistance, Centre Nationale de Référence du Paludisme, Institut Pasteur de la Guyane) for kindly providing the S170 clinical isolate. We are grateful to Mr. Felix D. Rozenberg and Dr. Anne-Catrin Uhlemann for performing the whole-genome sequencing and to Mr. Tomas Yeo for analyzing the genome data of the parasite clones.

## Supplementary References

1. Ross LS, Dhingra SK, Mok S, et al. Emerging Southeast Asian PfCRT mutations confer *Plasmodium falciparum* resistance to the first-line antimalarial piperaquine. *Nat Commun* 2018; **9**: 3314.

2. Dhingra SK, Small-Saunders JL, Menard D, Fidock DA. *Plasmodium falciparum* resistance to piperaquine driven by PfCRT. *Lancet Infect Dis* 2019; **19**: 1168-9.

3. Duru V, Khim N, Leang R, et al. *Plasmodium falciparum* dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine *in vitro* assays: retrospective and prospective investigations. *BMC Med* 2015; **13**: 305.

**Figure S1. plasmepsins 2 and 3 copy number in the parasite lines and S170 isogenic clones. (A)** Bars represent the copy number of the *pm2* gene in the S170 clones (orange and blue), the S170 bulk (yellow) and NF54 (dark grey) parasite lines, as determined by qPCR. Standards containing predefined ratios of *pm2* to *β–tubulin* gene fragments (grey bars) were assayed in parallel. Shown are mean ± SEM data across 3 technical replicates. Numbers listed above the bars indicate the *pm2* copy number for each parasite line. **(B)** Whole-genome sequence read counts showing the amplification of *pm2* and *pm3* on chr14 in 3 out of 6 isogenic S170 clones as well as the S170 bulk line. Results show that the breakpoints surrounding the *pm2/3* amplified locus in the S170 French Guiana isolate are similar to that in the RF7 Cambodian isolate (also known as PH1008C and earlier reported as having three copies of *pm2/3*[1]). The amplicon segment spans from the 5' upstream region of *pm2* gene to the 3' end of the *pm3* coding sequence (orange box). Blue boxes indicate the full length transcripts including the 5' and 3' untranslated region (UTR) sequences whereas the dark grey boxes indicate the coding sequences. Gold boxes above indicate the positions of the *pm2* and *pm3* genes. *pm4*, plasmepsin 4; *pm1*, plasmepsin 1; *pm2*, plasmepsin 2; *pm3*, plasmepsin 3, *ap2-g2*, AP2 domain transcription factor AP2-G2.

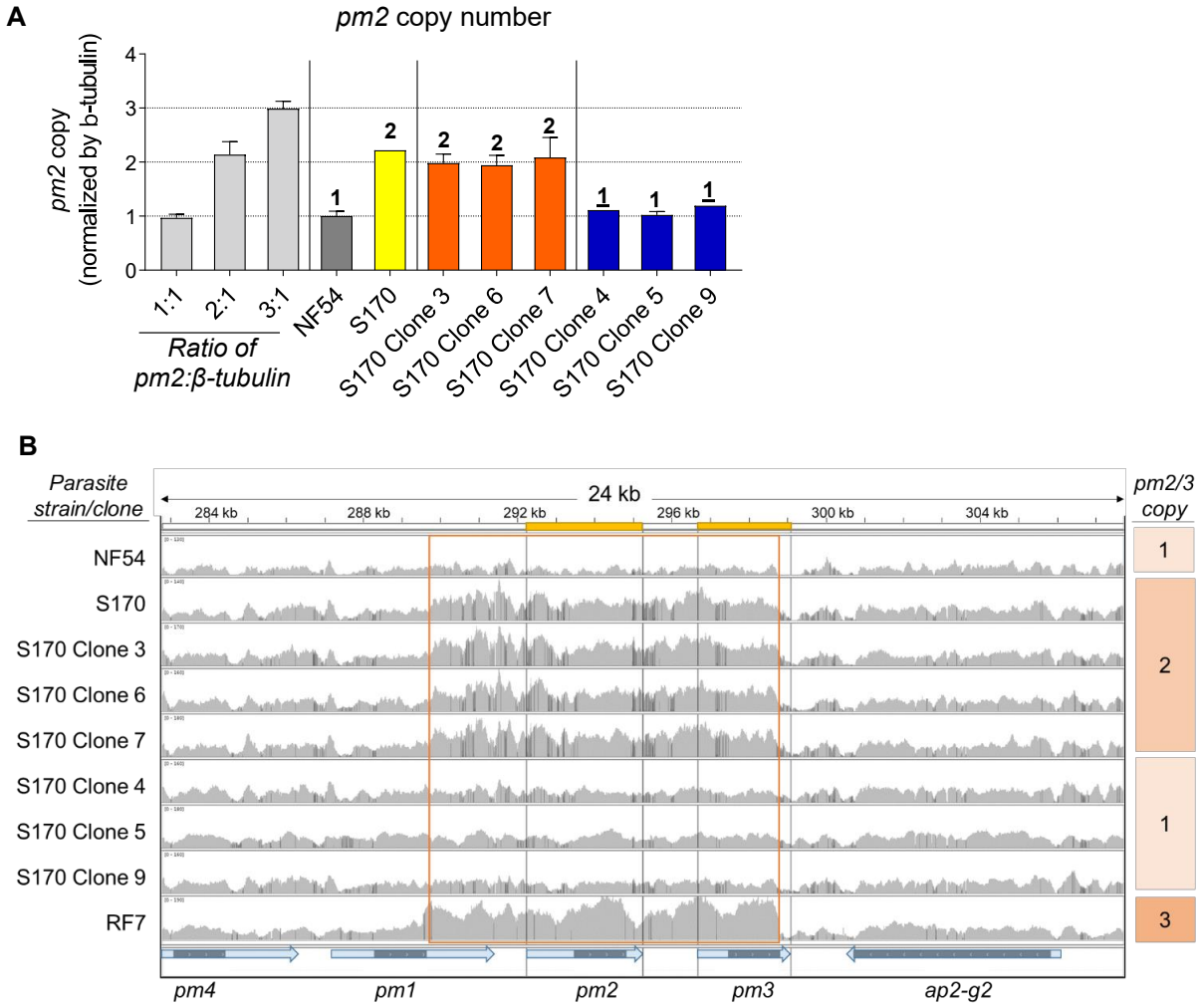

**Figure S2. Dose-response curves for isogenic PfCRT C350R mutant clones exposed to piperaquine for 48h or 72h**. **(A** and **B)** Survival of individual S170 isogenic clones exposed to a range of piperaquine concentrations for **(A)** 48h or **(B)** 72h. Shown are mean ± SEM data across 2-4 independent experiments performed for each clone. The 10% cutoff represents the threshold for resistance at 200 nM piperaquine for 48h.[3] 2x *pm2/3*, 2 copies of plasmepsins 2/3; 1x *pm2/3*, 1 copy of plasmepsins 2/3. Note that the 72h assay shows a greater differential PPQ response between the single- and double-copy *pm2/3* clones.

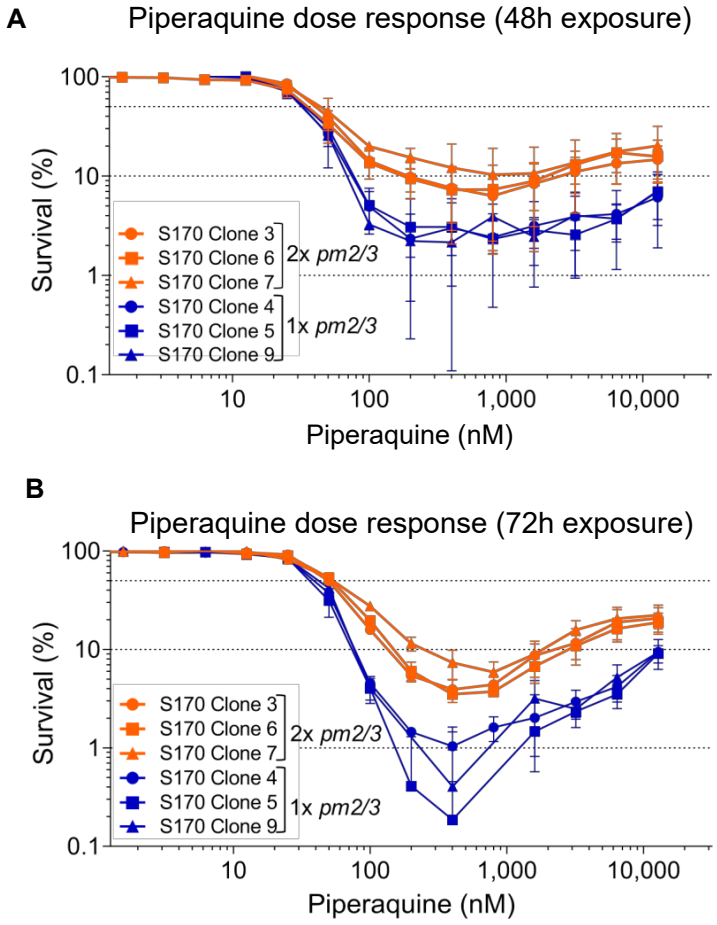


**Figure S3. $IC_{50}$ and $IC_{90}$ values of individual isogenic PfCRT C350R mutant clones tested against a panel of first-line antimalarials. (A** and **B)** Bars represent the mean ± SEM $IC_{50}$ values **(A)** or $IC_{90}$ values **(B)** for each S170 isogenic parasite clone profiled in 3 to 5 independent assays in duplicate. The NF54 and S170 bulk lines were assayed alongside. S170 and its derivative clones all express the PfCRT 7G8 (SVMNT) + C350R haplotype, whereas NF54 expresses wild-type (CVMNK) PfCRT.

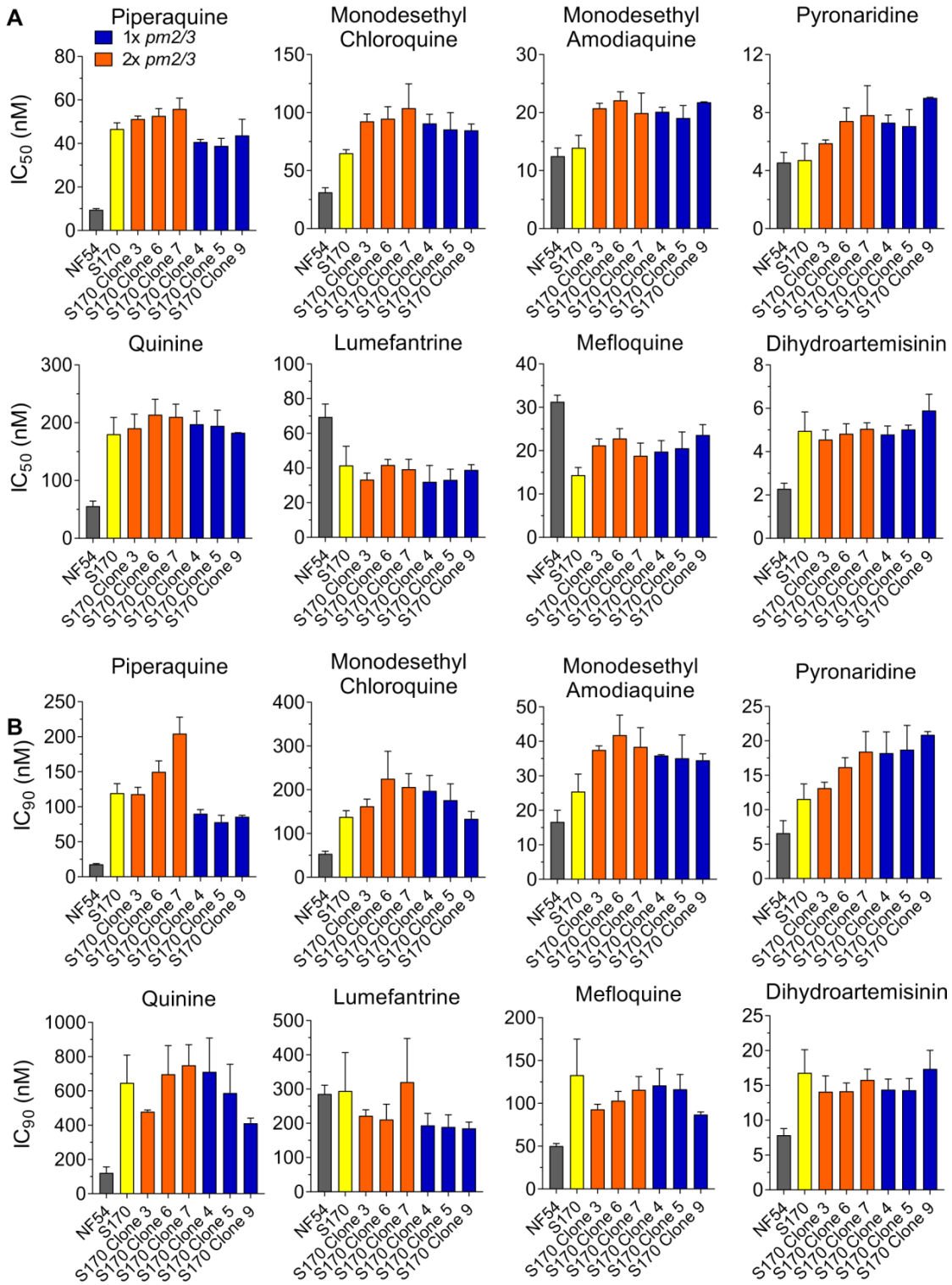


**Figure S4. Aggregate $IC_{50}$ and $IC_{90}$ values of isogenic PfCRT C350R mutant clones grouped by plasmepsins 2/3 (*pm2/3*) copy number and tested against a panel of first-line antimalarials.** Data are mean ± SEM for the S170 isogenic clones in each group. Significance between groups was tested using two-tailed unpaired Student's t-test. ns, non-significant. *$P$<0.05;

**$P$<0.01. Fold-shift in mean IC values are listed above the points. 2x *pm2/3*, 2 copies of plasmepsins 2/3; 1x *pm2/3*, 1 copy of plasmepsins 2/3.