Title: Exploring the impact of DNA replication and repair genes on drug resistance in *Plasmodium falciparum*

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Abstract

Malaria remains a major global health problem, with *Plasmodium falciparum* resistance to frontline drugs highlighting the need for new therapies. *In vitro* resistance generation helps reveal mechanisms of action and resistance, while measuring the minimum inoculum for resistance (MIR) assesses resistance risk. However, these approaches require large parasite numbers, and frequently inability to generate resistance for some compounds. To address this challenge, our lab generated a parasite line (Dd2-Polδ) carrying mutations in the DNA polymerase delta gene. These mutations impair proofreading activity, leading to a mildly elevated mutation rate. The Dd2-Polδ line developed resistance to compounds that were previously difficult to select, demonstrating its enhanced capacity for resistance generation. In this study, we validated the Dd2-Polδ line for quantitative *in vitro* resistance generation using multiple antimalarial drugs. Comparative MIR assays revealed that the Dd2-Polδ line developed resistance at lower inoculum than the parental Dd2 wild-type strain for next generation compounds such as KAE609 and GNF179 in line with the increased mutation rate of the mutator parasite. In contrast, no difference in MIR values was observed for DSM265 and atovaquone. Atovaquone targets a mitochondrial-encoded protein and is not expected to be impacted by the disrupted polymerase proofing, which replicates and repairs the nuclear genome. Next, to further enhance the mutator phenotype, we investigated additional DNA replication and repair genes. *P. falciparum* exonuclease 1 (*Pfexo1*) was selected based on previous reports indicating it confers a mild mutator phenotype. *PfExo1* (predicted to be non-essential) removes mismatched bases during mismatch repair (MMR) and participates in DNA end resection during homologous repair (HR). Using targeted genome editing, a stop codon and frameshift mutation were introduced at the start of *Pfexo1* in both Dd2 and Dd2-Polδ parasite backgrounds. The gatekeeper (24-well) MIR assays revealed that the Dd2-Polδ+Exo1 line generated resistance to GNF179 and KDU691 with a lower inoculum compared to Dd2, Dd2-Polδ and Dd2+Exo1 lines, indicating an enhanced mutator phenotype in this double mutant parasite line. Furthermore, the Dd2-Polδ+Exo1 line returned recrudescent parasites fastest in the gatekeeper MIR assay. Collectively, these findings support developing parasite lines for miniaturized, higher-throughput resistance generation, facilitating identification of resistance mechanisms and potential new drug targets for compounds where resistance is difficult to evolve.