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Malaria Drug Resistance is Associated with Defective DNA Mismatch Repair

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Abstract

Malarial parasites exhibit striking genetic plasticity, a hallmark of which is an ever-increasing rate of resistance to new drugs, especially in Southeast Asia where multi-drug resistance (MDR) threatens the last line of antimalarial drugs, the artesunate compounds. Previous studies quantified the accelerated resistance to multiple drugs (ARMD) phenomenon, but the underpinning mechanism(s) remains unknown. We utilize a forward genetic assay to investigate a new hypothesis that defective DNA mismatch repair (MMR) contributes to the development of MDR by *P. falciparum* parasites. We report that two ARMD parasites, W2 and Dd2, have defective MMR, as do the chloroquine-resistant parasites T9-94, 7C12, and 7G8. By contrast, the chloroquine-sensitive parasites HB3, D6 and 3D7 were MMR proficient. Interestingly, W2 was unable to repair substrates with a strand break located 3' to the mismatch, which is attributable to a large observed decrease in PfMutLα content. These data imply that antimalarial drug resistance can result from defective MMR.

The inability of cells to maintain genomic integrity leads to the rapid accumulation of DNA mutations, a scenario that underlies many diseases. In particular, the loss of DNA mismatch repair activity is linked to several forms of cancer, microsatellite instability, and chemotherapeutic drug resistance [1]. Given *P. falciparum*'s rapid development of drug resistance, a characteristic of a mutator phenotype, it is reasonable to hypothesize that drug resistant parasites have inefficient post-replication repair efficiency. The consequences of defective mismatch repair (MMR) are mutagenic parasites that generate increased genome heterozygosity in the form of new mutations that include alterations in key drug resistance genes. This increased genetic plasticity contributes to the development of MDR, particularly in parasites from Southeast Asia, which have been described as hypermutable [2]. Malaria parasites with the accelerated resistance to multiple drugs (ARMD) phenotype develop resistance to antimalarial drugs at an increased rate compared to other resistant strains [2].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

These parasites likely acquired a mutator phenotype that provides them with a greatly enhanced genetic diversity compared to drug sensitive parasites. Mutator phenotypes that have elevated mutation rates due to defective MMR are common in both natural and pathogenic isolates of *E. coli*, *P. aeruginosa* and *Salmonella* and have been implicated in the development of drug resistance mechanisms [3-5]. While the ARMD phenotype was identified more than 10 years ago, the molecular mechanism(s) that govern it remain enigmatic. We propose that 1) drug resistant *P. falciparum* parasites have defective MMR; 2) parasites with defective MMR are selected for under drug pressure and 3) defective MMR is the underlying mechanism in the development of antimalarial drug resistance (Figure 1A).

Previous work has sought a link between defective DNA repair and drug resistance in *Plasmodium* parasites. Bethke *et al.* [6] successfully deleted the MMR gene MSH2-2 in *P. berghei*, but found no correlation between the acquisition of drug resistance to 5-fluoroorotate and loss of MSH2-2 in blood stages. Another study looked at the repair of UV light-induced DNA damage in *P. falciparum* lysates [7]. In that study, the antimalarial drugs mefloquine, halofantrine, quinine and chloroquine (CQ) all inhibited repair reactions, but that investigation only addressed the repair of UV light induced lesions, which are processed through the nucleotide excision repair (NER) pathway. Therefore, the mechanism we propose is novel for *P. falciparum* and has not been substantiated in other malaria parasites.

We chose to examine MMR activity in the chloroquine-sensitive (CQS) parasite, HB3 and the chloroquine-resistant (CQR), ARMD parasite, Dd2, since the analysis of a genetic cross between these parasites provided the identity of the CQR determinant, *pfprt* [8]. We also chose to investigate the MMR phenotype of the MDR parasite, W2; Dd2 is a clone derived from W2 after long term exposure to mefloquine [9-10]. To do so, we adapted an assay to measure MMR activity in *P. falciparum* lysates prepared from HB3, Dd2 or W2 parasites. Initial measurements used DNA substrates which contained a nick in one DNA strand that was located 3' to an A:A or G:G mismatch or a single nucleotide (T) insertion/deletion loop (IDL) [11, see Supplementary data for methods]. HB3 possessed MMR activity and repaired the three lesions with similar efficiency (Fig. 1B). This activity was robust, reproducible (triplicate measurements with independent lysates), and strand-specific, *i.e.* directed towards repair of the mismatched base in the strand containing the nick. In contrast to HB3 parasites, the MMR activities of the Dd2 and W2 parasites were much lower, *i.e.* at levels representing the background noise of the assay, which we defined as the frequency of repair in the absence of parasite lysate (Fig. 1B).

Since the repair of mispaired substrates occurs bi-directionally *in vivo*, we further investigated repair of DNA substrates containing a nick located 5' to the mismatches. Again, HB3 repaired the different DNA mismatches with similar efficiency (Fig. 1C). While MMR activity was modestly higher than that measured for the 3' nicked substrates (compare Figs. 1B and 1C), Dd2 repair efficiency was still at or below background levels. However, in contrast to results for the 3' nicked substrates, W2 repaired all the mismatched substrates with a 5' nick to a similar or greater extent than HB3. Thus, unlike Dd2, the MMR defect of W2 parasites became evident only when the nick was located 3' to the mismatch.

MMR activity on mispaired DNA substrates utilizes a distinct repertoire of proteins depending on the location of the nick, with respect to the mismatch. Repair of a 5' nicked substrate requires the following six components: MutS α (MSH2-MSH6 heterodimer), exonuclease I, replication protein A, replication factor C, proliferating cell nuclear antigen, and DNA polymerase δ [Supplementary Table 1]. The repair of a 3' nicked substrate requires the previous listed proteins plus an additional complex, MutL α (MLH1-PMS2 heterodimer) [12]. The orientation-dependent defect in strand-specific MMR, similar to what we observed in the W2 parasites, has been previously reported for extracts from drug

resistant ovarian tumor cell lines [13]. Drug resistance was associated with the virtual absence of MLH1 and greatly reduced levels of PMS2. MMR activity was restored upon the addition of purified MutL α heterodimer to the extract. We investigated whether a similar scenario may explain the deficient 3' repair activity for W2.

To measure mRNA expression levels quantitative RT-PCR was performed on all of the five core PfMMR genes (PfMSH2-1, PfMSH2-2, PfMSH6, PfMLH1, and PfPMS1) with only PfMLH1 mRNA levels showing a significant decrease (55%) in W2 parasites, when compared to HB3. A modest decrease (13%) was observed for Dd2 parasites (Fig. 2A). The other MMR gene mRNA levels were similar to what was observed for HB3 parasites (data not shown). In order to discern protein expression levels, we obtained a commercially available antibody raised against amino acids 60-75 of human MLH1, which had a 70% sequence identity with *P. falciparum* MLH1 (PF11_0184) and recognized a protein in parasite lysates near the predicted molecular weight of PfMLH1 (~118 kDa) (Fig. 2B). Densitometry of Western blots showed a 94% decrease in W2 parasites and 38% in Dd2 for PfMLH1, when compared to HB3 protein levels (Fig. 2B). Moreover, the exogenous addition of purified human MutL α to repair reactions was able to restore 3' nick directed MMR for W2 (Fig. 2C). However, the addition of human MutL α to Dd2 lysate failed to restore the defective 3' nicked directed repair activity (Fig. 2C). This data shows that 3' repair activity for W2 parasites is MLH1 dependent. Furthermore, it would suggest that the defect in Dd2 parasites involves proteins other than or in addition to MutL α , possibly the MSH2/MSH6 heterodimer since it is essential for both 5' and 3' repair activity [14]. Preliminary analysis of the MSH2 and MSH6 genes has shown that there are no SNP's contained within the ORFs between HB3 and Dd2 parasites. Initial attempts to sequence the 5' and 3' UTRs for these genes was unsuccessful, so further analysis is required to determine if there is a mutation within these regions or if some epigenetic factor regulating expression is effecting this heterodimer.

The differences in MMR activity between the W2 and Dd2 parasites were unexpected since clone W2 is the progenitor of clone Dd2. Upon further examination, we discovered that Dd2 was a clone of W2-mef, which resulted from exposure of W2 to 96 weeks of continuous culture under high concentrations of mefloquine [9,10, see Supplementary Table 2]. Consequently, it is possible that the adaptation to mefloquine reflected a predisposition of W2 to accumulate mutations due to defective MMR on which subsequent drug pressure acted to select for stable resistance. Furthermore, additional divergence between W2 and Dd2 MMR may have coincided with the emergence of mefloquine resistance.

The five core PfMMR genes have been sequenced for HB3 and Dd2. Comparisons of the MLH1 gene revealed a single nucleotide polymorphism leading to a non-synonymous mutation between the HB3 (Ile371) and Dd2 (Thr371) sequences. However, this amino acid change does not map to any functionally homologous region for MLH1. We sequenced the 5' and 3' UTRs as well as the predicted open reading frame (ORF) for PfMLH1 in HB3, W2 and Dd2 parasites. We were able to detect some microsatellite (MS) regions within the 5' and 3' UTRs that were different when compared to HB3, however W2 and Dd2 parasite sequences in these regions were identical (data not shown). Furthermore, we found that the ORF for W2 and Dd2 were also identical. The sequencing data would suggest that the reduction in PfMLH1 protein in W2 and Dd2 could be due to any of several post-transcriptional or post-translational regulatory mechanisms, such as post-transcriptional regulation via RNA binding proteins, or other possible epigenetic mechanisms.

We have begun to expand our characterization of MMR activity to other laboratory strains with varying degrees of drug resistance. We observed that the CQS parasites D6 and 3D7 had repair efficiencies similar to HB3 parasites (Fig. 3). The CQR parasites 7C12 and 7G8

as well as the moderately CQR (CQRM) parasite, T9-94, all had repair efficiencies at or below the 10% background level measured for Dd2 (Fig. 1 B, C). We have not completed comprehensive analysis with all of the substrates used for HB3, W2 and Dd2, so further work will be necessary to determine if D6, 3D7, 7C12, 7G8 or T9-94 have a substrate bias similar to what was observed for W2. However, this data lends further support to our hypothesis that there is a causal relationship between defective MMR and the acquisition of drug tolerance by *P. falciparum* parasites.

A phenotype database has been compiled for the progeny of the HB3 × Dd2 cross for a series of 30 drug sensitivity and growth profiles [15,16] as well as transcript levels for 5200 genes. This provides a framework to mine these data for genetic determinants and gene interactions that connect traits. In future experiments, we will seek links of the MMR phenotypes to specific genetic loci and mechanisms using QTL mapping and genome wide mutation detection. Moreover, with the accumulation of data from progeny of the HB3 × Dd2 cross, we can identify relationships between MMR defects and resistant phenotypes. In Fig. 3, we showed that the CQR HB3 × Dd2 progeny, 7C12 [17], inherited the Dd2 form of deficient MMR. In this case, the MMR deficiency is co-inherited with the drug resistant phenotype, however if the drug resistance mechanism is not linked to MMR, these phenotypes will segregate independently in the progeny. Conversely, if MMR provides a key mechanism for parasites to evolve resistance in the first place, we would expect MMR and CQR to exist in the same genome of naturally occurring isolates and parent clones (e.g. Dd2) of the genetic crosses.

In considering defective MMR as the source of antimalarial drug resistance, one possible scenario is that parasites in endemic regions with defective MMR acquired beneficial point mutations in genes, e.g. *pfprt*, *pfdhfr*, that led to resistance to CQ and other drugs. It is conceivable that the mutations in the MMR pathway that led to repair deficiency could have disappeared from the population over time. This would be an anticipated scenario if the mutations were to bear a negative fitness cost. Seemingly, this is not the case for the multi-drug resistant parasite clones we analyzed in this investigation, since Dd2, W2, 7C12 and 7G8 are CQR and MMR defective. Supporting evidence from *P. falciparum* field isolates lends further weight to our hypothesis, since CQR parasites flourish in Cambodia even though CQ treatment was abandoned more than 20 years ago [18]. Furthermore, bacterial strains possessing high mutations rates have been found in natural populations, suggesting that there is little detrimental cost to these mutator phenotypes [19]. Future investigations characterizing MMR activity in a panel of geographically diverse natural isolates with differing drug sensitivities are needed to gain further insight into the mechanisms regulating the development of antimalarial drug resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

(A.) Model proposing the role of defective MMR in *P. falciparum* multi-drug resistance. We surmise that *P. falciparum* drug sensitive parasites have efficient MMR activity (left branch), which allows them to maintain genomic fidelity. The lack of mutations in critical antimalarial drug target genes enables the compounds to elicit their deleterious effects upon the parasites. By contrast, drug-resistant parasites have defective MMR (right branch). This can cause increased mutational frequency, which could ultimately lead to the alteration of critical genes that are either antimalarial drug targets or reside within a biochemical pathway affected by the drug. Addition of antimalarial drugs enriches the parasite population for mutator phenotypes that are MMR deficient and as a result, drug resistant. (B.)

Characterization of MMR activity in *P. falciparum* lysates from different parasite strains on 3' nicked mispaired DNA substrates. Whole cell lysate was generated from sorbitol synchronized parasites as described previously [7]. MMR repair assays were performed as described previously, with the exception that parasite lysate was substituted [11]. Briefly, 5 ng of heteroduplex DNA containing either a single base mismatch (G:G or A:A) or a 1 base IDL within the LacZ gene of the bacteriophage vector was incubated with the parasite lysate, recovered after the completion of the reaction, and electroporated into MMR deficient *E. coli* for blue/white plaque counting. Percent repair was calculated based on blue/white and mixed burst screening using the following equation: % repair = $1 - (\% \text{ mixed burst experimental} / \% \text{ mixed burst mock (no lysate)}) \times 100$ as described previously [11]. (C.) Characterization of MMR activity in *P. falciparum* lysates from different parasite strains on 5' nicked mispaired DNA substrates. Three independent parasite lysates prepared from three different synchronized cultures were assayed, and each lysate was analyzed in triplicate for all substrates. Error bars indicate SEM. * $P < 0.0001$.

Fig. 2.

Molecular basis for the 3' nicked substrate MMR deficiency in W2 parasites. (A.) qRT-PCR analysis of PfMLH1 mRNA. PfMLH1 mRNA was normalized to Pfhsp70-1 and mRNA expression levels of PfMLH1 in the W2 and Dd2 clones were calibrated to the HB3 clone. (B.) Western blot measuring PfMLH1 content in HB3, W2 and Dd2 parasites. Approximately 50 μ g of whole parasite lysate protein from all three strains was probed with a human anti-MLH1 antibody (Abcam, MA). Lysates were also probed with an anti-Pfhsp70-1 antibody (kind gift from Nirbhay Kumar) which was used as a loading control. Results from drug resistant strains were normalized for PfMLH1 concentration to the HB3 strain. Three independent lysates were analyzed for each parasite clone and blots were analyzed using ImageJ software. (C.) The effect of the addition of exogenous human MutL α (MLH1-PMS1 heterodimer) to parasite lysates from W2 and Dd2 on 3' nick directed repair. 320ng of hMutL α was added (+) to W2 and Dd2 lysates and MMR reactions performed as previously described and compared to reactions without the addition of exogenous hMutL α (-). Reactions were done with both G:G (shown) and A:A substrates (not shown) with the results being substrate independent. Error bars represent standard deviation of the normalized values. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.0001$.

Fig.3.

Repair reactions were performed on multiple parasite strains with varying sensitivities to CQ. For 3D7, T9-94 and 7G8, results from repair assays using a 3' G:G substrate are shown. A 5' G:G substrate was utilized for D6 and 7C12. Previous results obtained from repair reactions for HB3 and Dd2 using a 5' G:G substrate are shown for reference. Error bars represent the standard deviation of at least 3 separate experiments. D6 and 3D7 have CQ sensitive phenotypes, T9-94 is moderately resistant (CQR_m) and 7C12 and 7G8 are CQR.