scientific reports

Check for updates

OPEN

Gossypol is a natural product with good antimalarial activity against *Plasmodium falciparum* clinical isolates

Jersley D. Chirawurah[1,2], Felix Ansah[1,2], Samuel Blankson[1], Bridget Adikah[1,2], Silas Nkansah Yeboah[1,2], Lucas Amenga-Etego[1,2], Gordon A. Awandare[1,2]✉ & Yaw Aniweh[1,2]✉

Gossypol has demonstrated significant antimalarial activity against chloroquine-resistant and susceptible *Plasmodium falciparum* parasites. However, data on its potency in clinical isolates of *P. falciparum* remains limited. This study aimed to assess the potency of gossypol against six laboratory strains and twenty-one clinical isolates of *P. falciparum* using optimized growth inhibition assays. Additionally, parasites with reduced susceptibility to gossypol were selected using the *P. falciparum* Dd2 background (Dd2_3.5 µM) and tested for cross-resistance to chloroquine, dihydroartemisinin (DHA), and three Malaria box compounds (MMV006087, MMV085203, and MMV008956). On average, gossypol was found to be twice as potent against the laboratory strains compared to the clinical isolates, with IC_{50} values of 6.490 µM and 11.670 µM, respectively. Notably, Dd2_3.5 µM parasites displayed increased sensitivity after three months of exposure but developed decreased susceptibility after six months. Importantly, these gossypol-tolerant parasites showed no cross-resistance to chloroquine, DHA, or the three Malaria box compounds. These findings suggest that gossypol is effective against *P. falciparum* and holds potential as part of combination therapy with existing antimalarials. Furthermore, these results may support the identification of new antimalarial agents that are effective against drug-resistant malaria parasites.

The global malaria incidence declined from 216 million cases in 2009 to 198 million cases in 2013[1,2]. However, since 2014, malaria cases have risen, reaching an estimated 249 million cases in 2022[3]. This represents an increase of about 35 million cases compared to those reported in 2015[4,5]. This trend corresponded to growing reports of resistance to artemisinin and its combination therapy, initially reported in Southeast Asia and Western Cambodia[4,6,7]. More recently, there are growing reports indicating delayed clearance or resistance to artemisinin-based treatments in sub-Saharan Africa[4,8], where over 90% of global malaria cases occur. Studies in Eritrea[9], Rwanda[10], Tanzania and Uganda[11] have reported partial artemisinin resistance linked to clonal expansion of *Pfkelch13* mutations. The emergence of partial resistance to artemisinin and its partner drugs in sub-Saharan Africa presents a significant health risk to reducing the burden of malaria globally. Considering the limited efficacy of available malaria vaccines[12], chemotherapeutics remain a formidable force in our quest to eliminate malaria globally[13].

To accelerate the development of new chemotherapeutic agents for the treatment of drug-resistant malaria parasites, institutions such as Medicines for Malaria Venture (MMV) are collaborating with GlaxoSmithKline and St. Jude Children's Research Hospital to promote research and the rapid development of new antimalarial agents (malaria or pathogen box)[14]. Also, several other studies have explored new classes of antimalarial compounds that target crucial parasite proteins expressed at different stages of the parasite life cycle[15–17]. With all these efforts to increase the repertoire of antimalarial agents in the drug development pipeline, there is also the need to explore antimalarial agents which have not made it to clinical studies. By using new ideas and current research tools, it is possible to discover novel therapeutic targets from these antimalarial agents that are crucial for developing new antimalarial drugs. Towards this goal, this study further explores gossypol, a natural product from cottonseed. This compound has been tested and validated for good antimalarial activities

[1]West African Centre for Cell Biology of Infectious Pathogens, University of Ghana, Accra, Ghana. [2]Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Accra, Ghana. ✉email: gawandare@ug.edu.gh; YAniweh@ug.edu.gh

against chloroquine-resistant and susceptible *P. falciparum* parasites with IC_{50}values ranging between 5 and 13 μM[18]. Despite the historic interest in gossypol's antimalarial potential, its toxicity led to a disinterest in further researching this compound[19]. However, other studies have shown that less toxic derivatives of gossypol can retain potency similar to that of thbases with amino groups of proteinse parent compound[19], suggesting renewed potential for gossypol as an antimalarial. The high toxicity associated with gossypol is attributed to its two functional aldehyde groups forming Schiff's bases with amino groups of proteins[20]. However, studies in rats indicate that gossypol's toxicity is related to the dose and duration of administration[21], with recovery observed after withdrawal. In light of these studies, we believe that exploring the antimalarial potential of gossypol could offer new targets for drug development and combination therapies.

This study evaluated the antimalarial activity of gossypol by screening it against laboratory and clinical *P. falciparum* isolates. The clinical isolates were included to provide robust testing, an approach validated in our previous studies[22,23]. We first confirmed gossypol's efficacy against laboratory strains and clinical isolates. We then selected *P. falciparum* parasites with reduced susceptibility to gossypol (Dd2_3.5 μM) and tested them for cross-resistance with chloroquine, dihydroartemisinin (DHA), and three Malaria box compounds. Finally, we investigated the impact of multiclonality and polymorphisms in the *pfcrt* and *pfmdr1* genes on *P. falciparum* sensitivity to gossypol. This study represents one of the largest validations of gossypol's potency across diverse *P. falciparum* parasites.

Results

Evaluating the potency of gossypol against laboratory strains and clinical isolates of *P. falciparum*

Since protocols for setting assays may vary from one laboratory to another, it was important to independently validate the potency of gossypol using our optimized protocol for setting growth inhibitory assays[23]. To achieve this objective, we first screened six (6) laboratory strains of *P. falciparum* (Table 1) against gossypol at final-well concentrations from 1.56 μM to 100 μM. The IC_{50} values observed ranged from 3.829 μM to 10.190 μM (Supplementary Fig. S2) with an average IC_{50} of 6.11 μM (Fig. 1). Gossypol was most efficacious against W2mef (IC_{50} of 3.829 μM) and least efficacious against NF54 ($IC_{50}$of 10.190 μM). As we previously established the significance of including clinical isolates during antimalarial compound screen activities[23], we further validated the antimalarial properties of gossypol using twenty-one (21) clinical isolates at final-well concentrations from 1.56 μM to 100 μM (Table 1). The observed IC_{50} values varied widely between 1.058 μM and 24.79 μM (Supplementary Fig. S3), with an average IC_{50} of 11.67 μM (Fig. 1). The overall average IC_{50} value for gossypol against both the laboratory and clinical isolates of *P. falciparum* was 10. 46 μM. Thus, the IC_{50} values observed in our study were within the range of IC_{50} values previously reported for gossypol. Furthermore, an independent t-test was conducted to compare the mean IC_{50} values between the laboratory strains ($n = 6$) and the clinical isolates ($n = 21$). The susceptibility of the laboratory strains to gossypol (Mean = 6.11, SD = 2.25) was significantly higher than the clinical isolates (Mean = 11.67, SD = 7.44), t (24.73) = 2.98, $p < 0.006$). The F test confirmed

Laboratory strain	IC_{50} (μM) (95%CI)	Clinical Isolate	IC_{50} (μM) (95%CI)	Clinical Isolate	IC_{50} (μM) (95%CI)
W2mef	3.829	A006	1.058	A059	10.54
	(3.266–4.488)		(0.194–1.511)		(7.915–15.130)
7G8	4.893	A156	2.034	173	11.06
	(3.847–6.224)		(1.175–2.647)		(9.930–12.330)
Dd2	5.049	A031	3.819	14414	11.45
	(4.291–5.883)		(2.790–5.411)		(18.410–21.840)
3D7	5.801	A005	3.985	A376	12.64
	(5.163–6.367)		(1.100–5.841)		(11.000–14.890)
GB4	6.919	A010	6.883	A377	13.07
	(5.630–9.591)		(5.299–8.037)		(8.949–25.180)
NF54	10.19	A011	4.056	A378	15.55
	(8.371–12.460)		(3.214–5.125)		(11.660–21.300)
		14400	6.655	K239	20.07
			(5.877–7.562)		(17.170–24.990)
		A058	8.399	A362	22.2
			(6.201–13.260)		(18.040–30.330)
		179	9.704	A354	22.99
			(8.974–10.470)		(16.470–43.200)
		A375	9.939	A007	24.19
			(8.239–11.770)		(19.630–55.260)
				A018	24.79
					(18.040–45.870)

Table 1. Summary of the IC_{50} values in the clinical isolates and laboratory strains of *P. falciparum* parasites. The 95% confidence intervals (95%CI) are shown in the brackets.

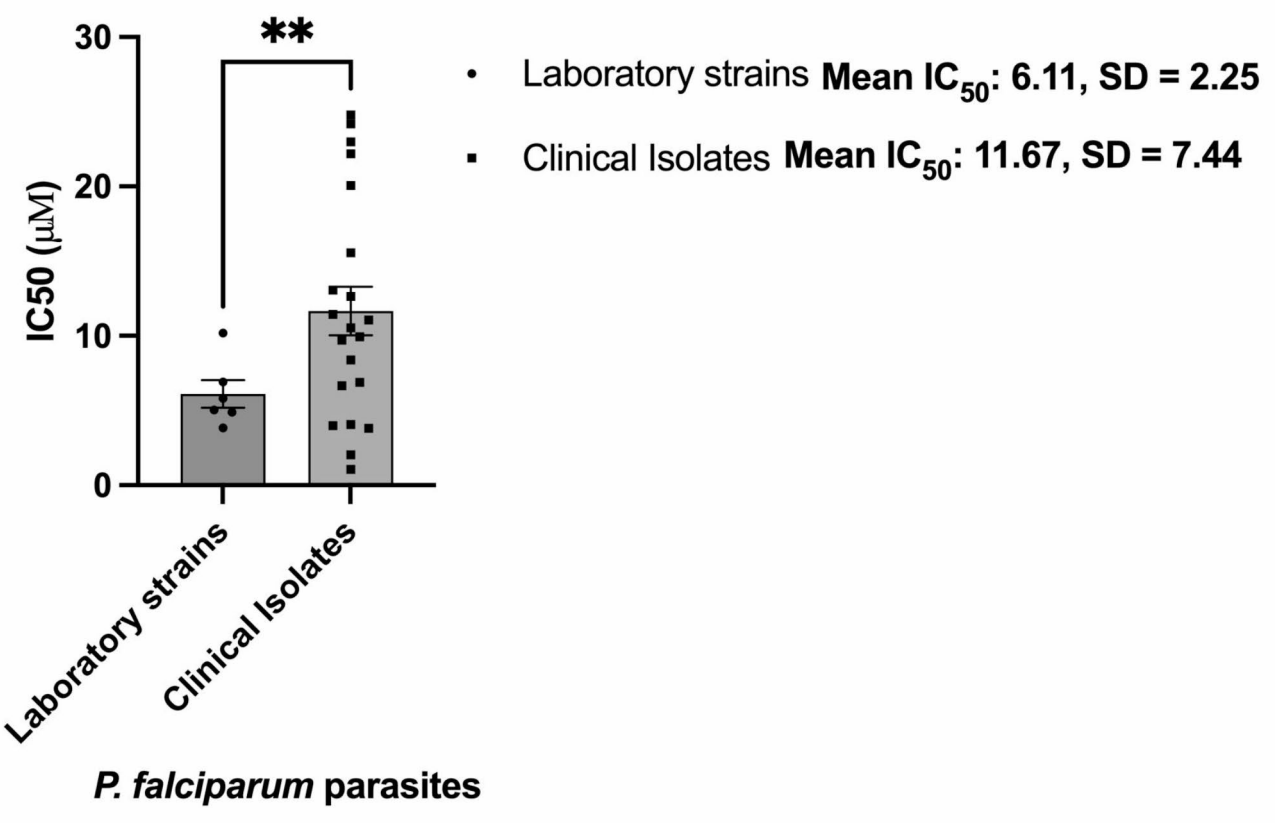

Fig. 1. A plot of the mean IC_{50} values of gossypol against the laboratory and clinical isolates of *P. falciparum*. The clinical isolates were generally less sensitive to gossypol compared to the laboratory strains of *P. falciparum* parasites (Unpaired t-test, $*p < 0.05$). Error bars denote SEM. The overall average (both clinical isolates and laboratory strains) average IC_{50} was 10.46 μM.

unequal variances ($p = 0.015$); hence, Welch's correction was conducted. The difference in response between the laboratory strains and the clinical isolates was 5.557 ±1.865 (95% CI: 1.714–9.400), indicating a substantial difference in susceptibility between the two groups. Arguably, our data also suggested gossypol was about twice as potent against the laboratory strains compared to the clinical isolates.

Selection of Dd2 *P. falciparum* parasites with reduced susceptibility to gossypol

Following the validation of the antimalarial activity of gossypol against the laboratory and clinical isolates of *P. falciparum*, we further selected parasites with reduced susceptibility to gossypol using the *P. falciparum* Dd2 strain. The Dd2 strain was chosen due to its intrinsic resistance to multiple antimalarial drugs[24], making it an ideal model for studying the genetic and biochemical mechanisms driving antimalarial resistance. Investigating resistance in Dd2 parasites not only deepens our understanding of how *P. falciparum* develops drug resistance but also helps identify new therapeutic targets and elucidate gossypol's mechanism of action. To achieve this objective, Dd2 parasites were cultured in media supplemented with gossypol at sub-IC_{50} concentrations, starting at 0.5 μM for two weeks, followed by 2.5 μM for three weeks. These concentrations were below the baseline IC_{50} for Dd2 (5.049 μM). No significant changes in IC_{50} values were observed during this period (data not shown). Following this, the concentration of gossypol was increased to 3.5 μM, and the culture was monitored continuously. After three months, the IC_{50} values for two independent replicates (Dd2_3.5 μM_1 and Dd2_3.5 μM_2) had decreased to 2.599 μM and 2.970 μM, respectively, representing nearly half the initial IC_{50} (Fig. 2 and Supplementary Fig. S6). Interestingly, after four months of continuous culture, the IC_{50} values increased to 10.860 μM and 11.480 μM, respectively (Fig. 2B). After five months, we observed an increase in susceptibility, with IC_{50} values for Dd2_3.5 μM_1 and Dd2_3.5 μM_2 decreasing to 6.645 μM and 7.684 μM, respectively (Fig. 2B). However, after six months, the IC_{50} values increased again to 14.940 μM and 15.670 μM for the two replicates. Surprisingly, after seven months of culturing, the IC_{50} values for Dd2_3.5 μM_1 and Dd2_3.5 μM_2 decreased once more to 2.519 μM and 4.651 μM, respectively(Fig. 2B). The IC_{50} values after six months were nearly three times higher than the starting values (5.049 μM) and about six times higher than the values recorded at three-months (Fig. 2A and B). This pattern of fluctuating susceptibility, with an increase in IC_{50}(14.940 μM and 15.670 μM) after six months compared to the parental strain, suggests the emergence of Dd2 parasites with reduced susceptibility to gossypol (Dd2_3.5 μM). These results represent the first report of a dynamic sensitivity response in malaria parasites exposed to gossypol over time, where susceptibility both increases and decreases throughout the selection process. The potency of standard antimalarials against the Dd2_3.5 μM

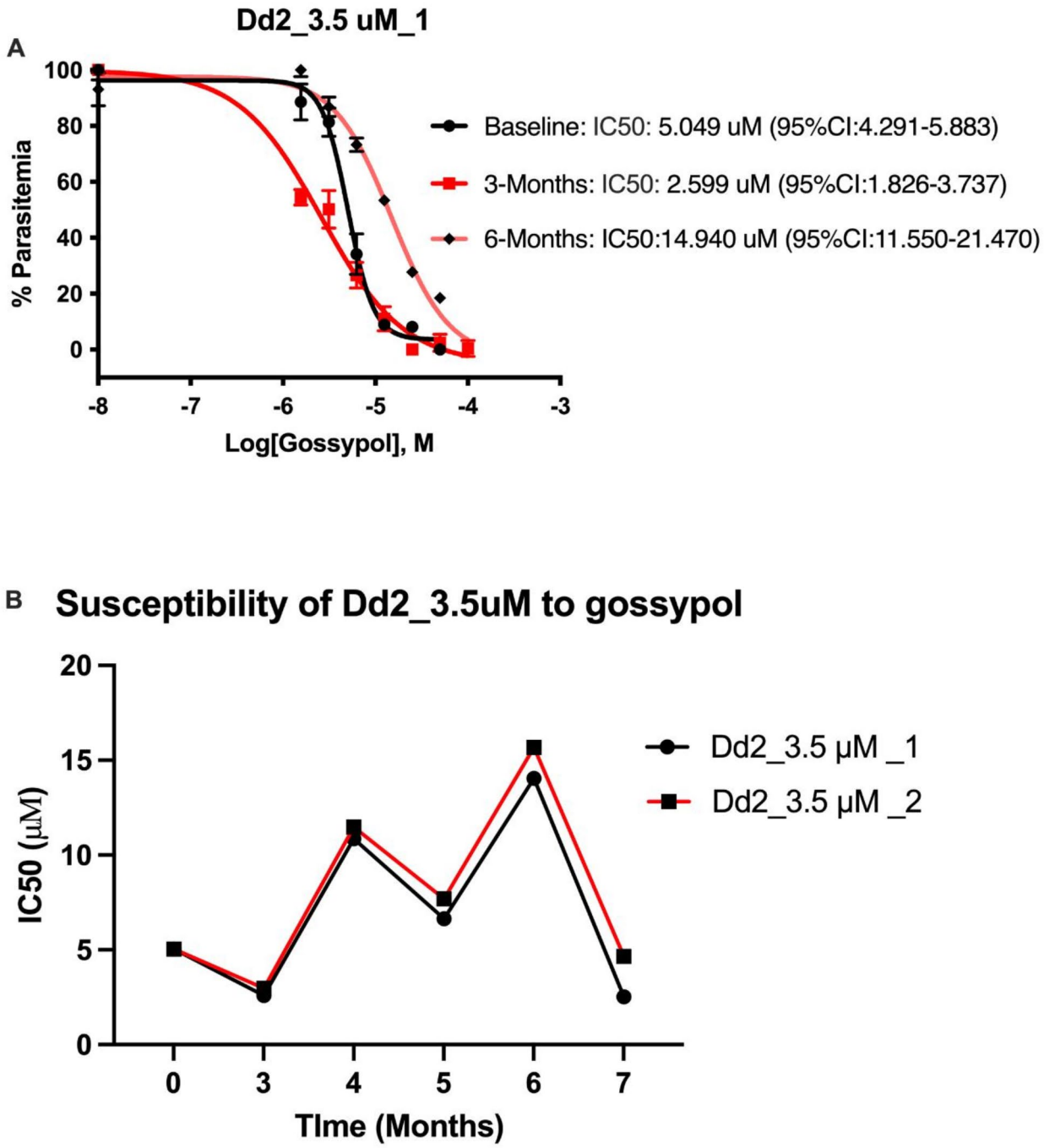

Fig. 2. Selection of Dd2 *P. falciparum* parasites with reduced susceptibility (Dd2_3.5 µM) to gossypol. (**A**) Dose-response curves showing the susceptibility of Dd2_3.5 µM_1 parasites at baseline (IC_{50} values of 5.049 µM (95% CI: 4.291–5.883)), three months (IC_{50} values of 2.599 µM (95% CI: 1.826–3.737)) and finally at six months (14.94 µM (95% CI: 11.55–21.47) ; (**B**) Susceptibility pattern of the Dd2_3.5 µM parasites (two replicates) during the in vitro selection process.

parasites was also similar to those previously reported for Dd2 parasites against chloroquine and artemisinin-based compounds[25], suggesting these parasites were not contaminated. Furthermore, molecular analyses of the Dd2_3.5 µM parasites for the presence or absence of the *P. falciparum* histidine-rich protein 2 and 3 (P*f*HRP2 and P*f*HRP3) (Supplementary Fig. S4 and Table S.2) confirmed that they were not contaminated with other *P. falciparum* strains[26].

Dd2_3.5 µM parasites screened against standard antimalarial drugs

To determine the existence of cross-resistance between gossypol and existing antimalarials, we screened Dd2_3.5 µM parasites against chloroquine and dihydroartemisinin (DHA) (Fig. 3). The difference in IC_{50} values observed between Dd2_3.5 µM (2.686 nM) and the parental Dd2 strain (9.126 nM) (Fig. 3A) was about 6.44 nM, which suggested that Dd2_3.5 µM parasites were more sensitive to DHA compared to the Dd2 parental strain. Again, the Dd2_3.5 µM parasites were almost two times more sensitive to chloroquine (249.90 nM) compared to the parental Dd2 strain (425.20 nM) (Fig. 3B), which also alluded to the fact that the Dd2_3.5 µM parasites had higher sensitivity to chloroquine compared to the parental Dd2 strain. Therefore, based on these observations, our data suggests that the Dd2_3.5 µM parasites were generally more sensitive to chloroquine and DHA than their parental Dd2 strains. This data further suggests the existence of no observable cross-resistance between gossypol, DHA and chloroquine. Additionally, the Dd2_3.5 µM parasites and the parental Dd2 strains were sensitive to DHA (IC_{50} value < 12 nM) and resistant to chloroquine (IC_{50} value > 100 nM) further suggests no contamination with other strains in the process of culturing them (Fig. 3A-C).

Response of Dd2_3.5 µM parasites to MMV006087, MMV085203 and MMV008956

In our previous study on the Malaria box compounds, we identified MMV006087, MMV085203 and MMV008956 (Supplementary Fig. S1) as the most potent compounds against four clinical isolates of *P. falciparum*[23]. For this study, we also tested for the existence of cross-resistance between gossypol and the three Malaria box compounds. This was to examine if gossypol had cross-resistance to potentially new antimalarial compounds. To accomplish this aim, we screened Dd2_3.5 µM parasites against the three Malaria box compounds and compared the IC_{50} values to that of the parental Dd2 strain (Fig. 4). The Dd2_3.5 µM parasites were about five times more sensitive to MMV006087 compared to the parental Dd2 strain (IC_{50} values of 18.43 nM and 92.38 nM, respectively) (Fig. 4A). The Dd2_3.5 µM parasites were also about five times more sensitive to the MMV085203 (IC_{50} values of 140.60 nM) compared to the parental strain with an IC_{50} value of 649.50 nM (Fig. 4B). For MMV008956 against Dd2_3.5 µM parasites and parental Dd2 strain, IC_{50} values of 139.50 nM and 280.90 nM were observed, respectively (Fig. 4C). Therefore, based on the difference in IC_{50} values, the Dd2_3.5 µM parasites were more sensitive to the three Malaria box compounds compared to their parental strains. Additionally, it was observed that MMV006087 was more potent against the Dd2_3.5 µM parasites and Dd2 parental strains compared to MMV008956 and MMV085203.

Determination of copy number variation in the clinical isolates and Dd2_3.5 µM parasites

Studies have suggested that mutations and copy number variations in genes such as *pfcrt* and *pfmdr1*mediating malarial drug resistance can modulate the sensitivity of the parasites to antimalarial drugs[27]. Therefore, we first evaluated copy number variations in the *pfmdr1* and *pfcrt* genes in the clinical isolates (Fig. 5). From our results, there were no differences in copy number of the *pfcrt* gene in all the clinical isolates (data not shown), which was not surprising as copy number variations in this gene have not been previously reported in *P. falciparum* parasites. We also observed that almost all the clinical isolates had *pfmdr1* gene copy numbers of one except isolate 14400, which had a copy number of two (Fig. 5). Although this was interesting, the increased *pfmdr1* gene copy number in this clinical isolate did not seem to modulate its sensitivity to gossypol relative to the other clinical isolates that had *pfmdr1* gene copy number of one. Additionally, we determined if copy number variation in the *pfmdr1* and *pfcrt* genes were responsible for the reduced sensitivity in the Dd2_3.5 µM parasites (Fig. 5). This was achieved by evaluating and comparing the copy numbers in these genes in the Dd2_3.5 µM parasites and their parental strains. From our data, we observed no differences in copy numbers of *pfmdr1* and *pfcrt* genes between the Dd2_3.5 µM parasites and the parental line (Fig. 5). These results may suggest that there could be other genetic signatures that might explain the variations in the sensitivity observed in the clinical isolates and the Dd2_3.5 µM parasites.

Evaluating the effect of multiple clones in clinical isolates on their susceptibility to antimalarial compounds

In this study, 17 of the clinical isolates were successfully genotyped using the MSP1 and MSP2 genotyping techniques. For the *msp1* gene, there were more monoclonal infections (67%) compared to polyclonal infections (32%) (Table 2). The K1 allele was the most prevalent (65%), followed by the RO33 (41%) and then MAD20 (30%). In the *msp2* gene, there were also more monoclonal infections (77%) than polyclonal infections (23%) (Table 2). The 3D7 allele was the most prevalent (88%), followed by the FC27 (53%). A comparison of the monoclonal and polyclonal infection frequencies between MSP1 and MSP2 showed no statistically significant difference (Fisher's Exact Test, $p = 0.41$), indicating similar rates of single and multiple genotype infections between the two genes. The average multiplicity of infection (MOI) for MSP1 was 2.0 and 1.8 for MSP2, with individual samples showing between 1 and 4 alleles per sample (Table 2). Thus, a moderate level of genetic diversity was observed within the clinical isolates sampled. Following this, we further evaluated the effect of multiple *P. falciparum* genotypes on the susceptibility of the clinical isolates to gossypol. Since all the isolates contained at least two genotypes, we compared the mean IC_{50} of isolates with two genotypes to those with three or more genotypes. From the data (Fig. 6), the mean IC_{50} value for isolates with two genotypes was 11.04 µM and 13.14 µM for those with three or more genotypes. An unpaired t-test was subsequently conducted to compare the mean IC_{50} values between the clinical isolates with two clones ($n = 10$) and those with more than two clones ($n = 7$). The susceptibility of the clinical isolates with two clones against gossypol (Mean = 11.04, SD = 7.37) was not significantly different from those with more than two clones (Mean = 13.14, SD = 6.70), $t(15) = 0.589$, $p < 0.565$). The F test confirmed equal variances ($p = 0.845$), hence, a standard t-test was conducted. The difference in susceptibility of the parasites with two clones to those with more than two clones was 2.064 ±3.505 (95% CI: −5.409-9.543), indicating no substantial difference in susceptibility between the two groups.

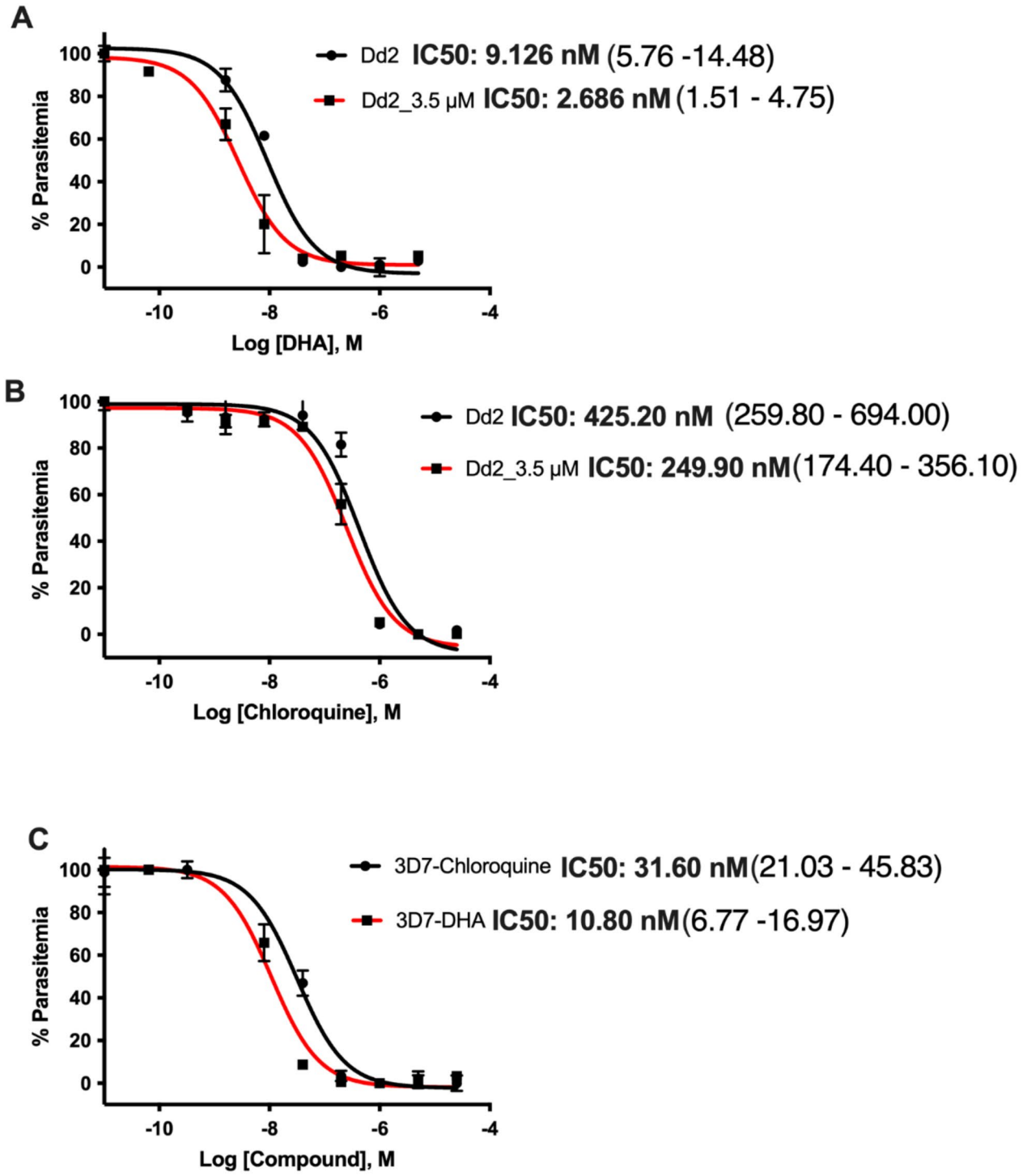

Fig. 3. Dose-response curves for the Dd2_3.5 μM parasites, Dd2 parental strain and 3D7 parental strains of *P. falciparum* against standard antimalarial drugs. (**A**) Dose-response curves showing the response of chloroquine against Dd2_3.5 μM and Dd2 parental strain; (**B**) Dose-response curves showing the response of dihydroartemisinin (DHA) against Dd2_3.5 μM and Dd2 parental strain and (**C**) Dose-response curves showing the response of DHA and chloroquine against 3D7. Each data point represents the mean ± SEM ($n = 2$). The plot shows the percentage parasitemia against the log of the concentration of the compound.

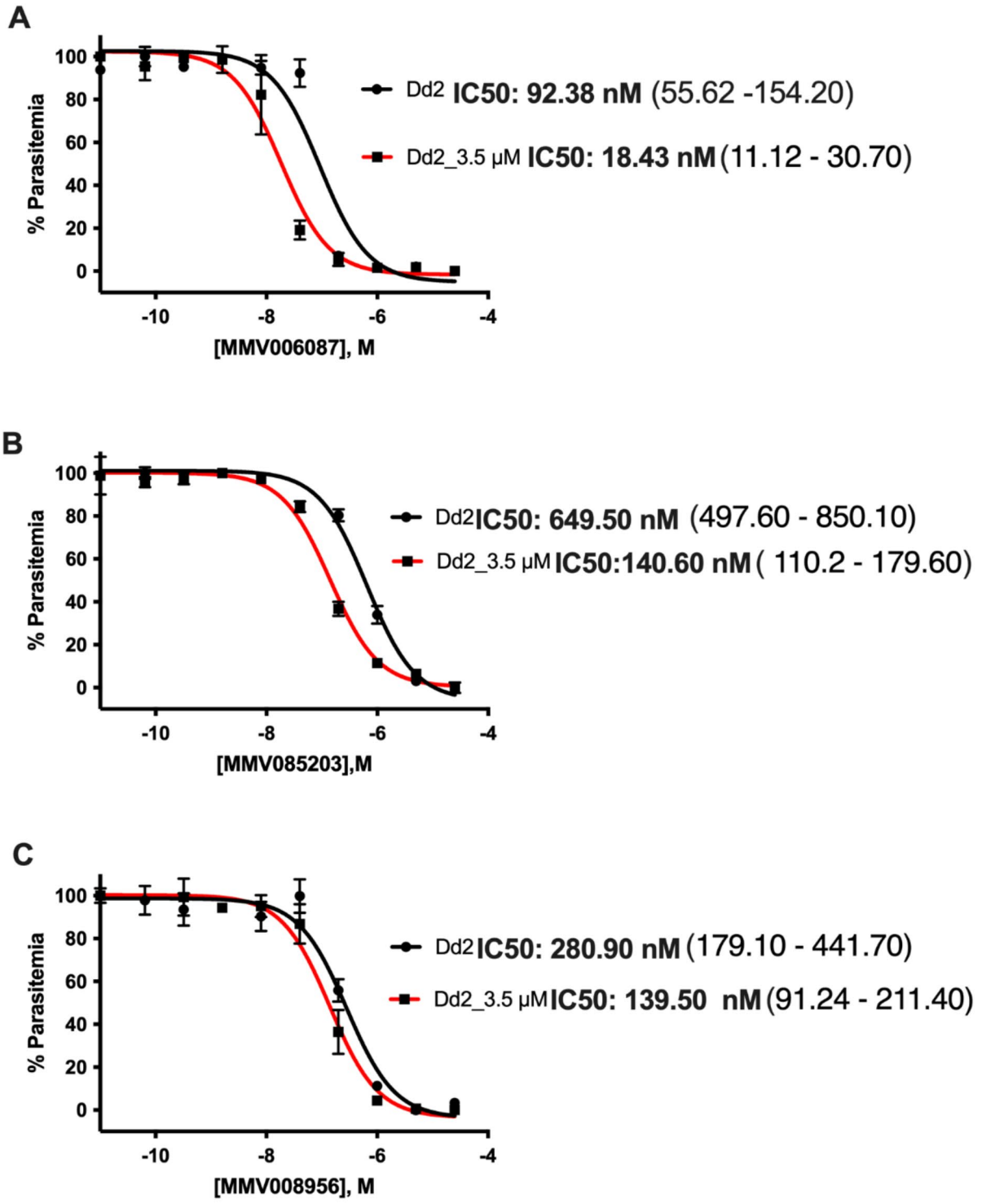

Fig. 4. Dose-response curves for Dd2_3.5 μM against three Malaria box compounds. Panels A–C, are the dose-response curves showing the response of Dd2_3.5 μM parasites against (**A**)MMV006087, (**B**) MMV085203 and (**C**)MMV008956, at concentrations from 0.064 nM to 25 μM. Each data point represents the mean ± SEM ($n = 2$). The plot shows the percentage parasitemia against the log of the concentration of the compound.

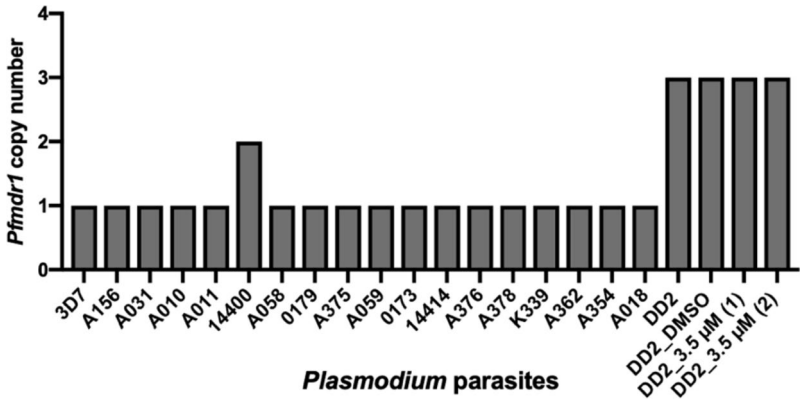

Fig. 5. Graphs showing *pfmdr1* and *pfcrt* copy number variation in Dd2 parental strain (Dd2), Dd2_3,5 µM parasites, 3D7 and clinical isolates. **A** All the clinical isolates had one *pfmdr1* copy number, except for the clinical isolate 14400, which had a copy number of two. There was also no difference in *pfmdr1* copy number between Dd2 parental strain and Dd2_3,5 µM parasites.

Gene	Allele	Allele frequency ($n=17$)	Monoclonal Infection ($n=17$)	Polyclonal Infection ($n=17$)	MOI ($n=17$)
msp1			23	11	2.0
	K1	11			
	RO33	5			
	MAD20	7			
	K1 + RO33	3			
	K1 + MAD20	3			
	RO33 + MAD20	3			
	K1 + RO33 + MAD20	2			
msp2			24	7	1.8
	3D7	15			
	FC27	9			
	3D7 + FC27	7			

Table 2. Allelic diversity and multiplicity of infection (MOI) in the clinical isolates using MSP1 and MSP2 genotyping.

Discussion

The limited number of partially effective malaria vaccines coupled with growing reports of resistance to the frontline antimalarial drug, especially in sub-Saharan Africa, has created the need for new antimalarial drugs with novel targets and mechanisms of action[4,6,7,9,13,28]. In this regard, it was important to take a further look at gossypol, which has historically been shown to possess antimalarial activity[18]. Previous studies have demonstrated that gossypol is effective against *Plasmodium falciparum* at concentrations above 1 µM, with IC_{50}values of 10 µM against chloroquine-resistant and chloroquine-susceptible strains[18]. However, despite its potential, the toxic effects of gossypol—primarily due to its aldehyde functional groups—have limited its development as a viable antimalarial treatment[29,30]. Fortunately, derivatives of gossypol have been demonstrated to be less toxic and have similar potency as the parent compound[29,30]. Based on these findings, we found it prudent to further explore the potency of gossypol using laboratory strains and clinical isolates of *P. falciparum*.

Our study confirmed that the potency of gossypol against laboratory strains of *P. falciparum* was consistent with previous research[18], which not only reaffirmed the compound's potential but also validated our growth inhibition assays. While earlier investigations predominantly focused on laboratory-adapted strains, the efficacy of gossypol against clinical isolates remained unclear. Therefore, it was imperative to adequately validate the potency of gossypol using the clinical isolates. Clinical isolates offer a more accurate representation of the parasite population encountered in endemic regions, and our previous studies have highlighted the significance of including them in antimalarial compound screening efforts[23]. The significance of thorough evaluation of the potency compounds before clinical use is highlighted in a study by Arrowsmith et al.[31]. After analysing 108 reports of Phase II failures from 2008 to 2010 for new drugs and major new indications of existing drugs, the authors found that 51% of the clinical failure of drugs resulted from insufficient efficacy[32]. The study further indicated that the failure arising from insufficient efficacy could be due to a lack of sufficient evidence to prove the efficacy advantage of the compounds over an existing or more advanced drug[32]. Our findings revealed that the efficacy of gossypol against half of the clinical isolates screened was similar to data from earlier studies[19,29].

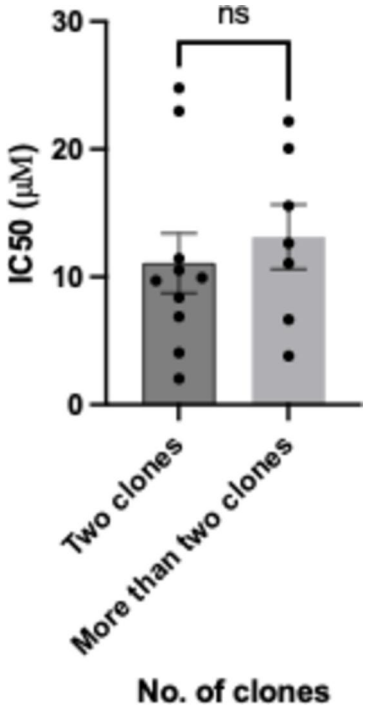

Fig. 6. Comparing the susceptibility of clinical isolates with two clones to those with more than two clones. The IC_{50} values of the clinical isolates with two *P. falciparum* genotypes were not significantly different from those with more than two genotypes (Unpaired t-test, $p = 0.56$). Error bars denote SEM.

However, several clinical isolates exhibited almost two-fold less susceptibility to gossypol compared to the laboratory strains, substantiating the need to include clinical isolates in antimalarial drug screening activities. To our knowledge, this is the largest screen of gossypol against this number of laboratory strains and clinical isolates of *P. falciparum* in a single study.

Several studies have shown that clinical isolates harbour multiple *P. falciparum* clones with different drug-resistance backgrounds, and this has the potential to affect the efficacy of antimalarial drug treatment outcomes[33,34]. Our study showed that clinical isolates with two genotypes were more susceptible to gossypol than those with more than two genotypes, though the difference was not statistically significant. This may suggest that factors beyond genotype number, such as specific genetic polymorphisms, including mutations, could influence gossypol sensitivity. Following this, we also examined the potential contribution of polymorphisms in the *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) and the chloroquine resistance transporter (*pfcrt*) gene, which has been shown to modulate sensitivity to antimalarials[27,35]. Interestingly, all clinical isolates had the same number of *pfcrt* and *pfmdr1* gene copies, except for one isolate with two *pfmdr1* copies, but this did not affect its sensitivity to gossypol. These findings could suggest that other genetic or, potentially, epigenetic factors could be responsible for the observed differences in gossypol sensitivity.

During the resistance selection process, we observed varied susceptibility patterns of the Dd2_3.5 μM parasites in response to gossypol, with reduced susceptibility to the compounds after six months. The continuous exposure of the *P. falciparum* parasites to gossypol likely produced a heterogeneous parasite population with varying levels of susceptibility or resistance[36–38]. This could result from competitive suppression between parasites more sensitive to gossypol and those less sensitive[39,40]. Furthermore, studies indicate that artemisinin induces a subpopulation of *P. falciparum* parasites with decreased susceptibility to artemisinin within a clonal population into quiescence[41,42]. Other studies have also implicated the role of epigenetic mechanisms mediating drug resistance in *P. falciparum* within the isogenic parasite population, where only a subset of parasites can survive drug pressure[43,44]. For instance, inhibition of the histone acetyltransferase P*f*GCN5 in artemisinin-resistant parasites was shown to increase their sensitivity to artemisinin treatment[45]. In line with these studies, we hypothesize that short-term epigenetic or metabolic changes might have occurred during the selection period, leading to altered susceptibility of the Dd2_3.5 μM parasites to gossypol. Thus, our next step will involve isolating and characterizing the drug susceptibility phenotypes of the different clones within the heterogeneous parasite population. Additionally, whole-genome and epigenetic analyses will be conducted to identify genetic and epigenetic signatures that may explain the variation in gossypol sensitivity.

Our study also explored the potential for cross-resistance, which occurs when resistance to one drug confers resistance to other drugs with similar mechanisms of action[4]. Interestingly, the Dd2_3.5 μM parasites in our study were more sensitive to chloroquine, DHA, and the three Malaria box compounds, suggesting that gossypol did not have cross-resistance to these compounds. Additionally, this data may indicate that gossypol could enhance the efficacy of other drugs. While the absence of cross-resistance to chloroquine, DHA, and the Malaria box compounds in the Dd2_3.5 μM parasites suggests that gossypol may not share resistance pathways with

Antimalaria Drug (s)	Resistant Strain	Resistance-Associated Gene (s)
SD	3D7	*dhps*
CQ, QN, PM and SD	Dd2	*Pfcrt*, *Pfmdr* copy number variation
CQ, SD, MFQ and PM	W2mef	*Pfcrt*, *dhps*, *Pfmdr* copy number variation
MFQ	NF54	*Pfmdr* copy number variation
CQ, PM, QN and CG	7G8	*Pfcrt, dhfr*,
CQ	GB4	*Pfcrt*

Table 3. Summary of antimalarial drug resistance and the associated genes in laboratory *P. falciparum* strains. Adapted and modified from a previous study[53]. CQ, chloroquine; CG, cycloguanil; SD, sulfadoxine; PM, pyrimethamine; MFQ, mefloquine; QN, quinine; *Plasmodium falciparum* chloroquine resistance transporter, PfCRT; Plasmodium multidrug resistance, PfMDR; dihydropteroate synthase, DHPS; dihydrofolate reductase, DHFR.

these compounds, we are unable to rule out the possibility of a gossypol-specific resistance mechanism. Thus, further studies in the form of target deconvolution and genetic analyses will be necessary to confirm whether gossypol acts on a novel target or whether resistance mechanisms are unique to gossypol itself.

In conclusion, our study confirmed the potency of gossypol against *P. falciparum* laboratory strains and clinical isolates, although the clinical isolates generally show reduced susceptibility to gossypol. The absence of cross-resistance with other antimalarials further suggests that gossypol may target a unique pathway, making it a promising candidate for future therapeutic development. Future studies will involve whole-genome and epigenetic investigations to explore the mechanisms of action and resistance to gossypol, as well as identify potentially new antimalarial drug targets.

Materials and methods

P. falciparum parasites and parasite culture

For this study, *P. falciparum* laboratory-adapted parasites, 3D7, W2mef, Dd2, GB4, 7G8 and K1 and twenty-one randomly selected clinical isolates (Table 1) were cultured using standard methods[46,47] with slight modifications. The laboratory strains were cultured with RPMI-1640 (Sigma) that was supplemented with 0.5% AlbuMAX II (Gibco), 20 mg hypoxanthine, 2 g sodium bicarbonate (Sigma). For the clinical isolates, the media was further modified with 2% normal human serum (PAN-Biotech, Germany). The cultures were maintained at 37 °C using human group O^+ erythrocytes at 4% hematocrit and mixed gas composed of 93% nitrogen (N_2), 5% carbon dioxide (CO_2), and 2% oxygen (O_2) (Air Liquide, Birmingham, United Kingdom). The laboratory strains (Table 3) used for this study each have different drug-resistant phenotypes. The clinical isolates used for this study are archived samples collected for a study on erythrocyte invasion mechanisms (EIM)[48,49]. These samples were processed upon arrival from the field, cryopreserved using the glycerolyte method[50]and stored in liquid nitrogen tanks[48]for future use. Details regarding ethical clearance and study sites for the samples were previously described[48]. The samples were thawed using serial NaCl solutions[50] whenever there was the need to set assays. The selection of 21 clinical isolates was based on parasites that adapted well to *in vitro* culture conditions at the time of the study. This number allowed us to perform an initial screening to evaluate the efficacy of gossypol against a range of diverse *P. falciparum* isolates representing various genetic backgrounds. For the drug assays, *P. falciparum* parasites were cultured to > 5% parasitemia of ring-stage parasites. Using 5% Sorbitol treatment, a synchronised culture of ring-stage parasites[51]was obtained and diluted to 1% parasitemia and 2% haematocrit for conducting the growth inhibition assays as previously described[23]. In order not to lose some of the clones in the clinical isolates during synchronization, the assays for the clinical isolates were set up with cultures containing > 90% ring-stage parasites.

Selection of parasites with reduced susceptibility to gossypol (Dd2_3.5 µM)

A step-wise selection process was used to select parasites with reduced susceptibility to gossypol using the Dd2 laboratory strains (Dd2_3.5 µM) based on established approaches[39–52]. Briefly, the parasites were cultured in vitro at a starting parasitemia of 5% with media containing 0.5 µM of gossypol for two weeks and subsequently cultured in media containing 2.5 µM gossypol for three weeks. The concentration of gossypol was increased to 3.5 µM gossypol and used to culture the parasites from the 2.5 µM gossypol for a further seven months. Microscopy and growth inhibitory assays were conducted at least once every month to monitor and assess the emergence of parasites with reduced susceptibility to gossypol[23]. The cultures were maintained daily with media containing gossypol at the appropriate concentration. Two biological replicates (Dd2_3.5 µM_1 and Dd2_3.5 µM_2) were used during the selection process. Before setting up the in vitro growth inhibitory assays, the parasites were washed thrice with RPMI to wash off the gossypol-containing media.

In vitro drug susceptibility assay

To evaluate the potency of gossypol against the laboratory, clinical isolates and Dd2_3.5 µM parasites, a stock of 10 mM gossypol (Sigma-Aldrich, USA) was serially diluted and screened against the parasites at final-well concentrations between 1.56 µM to 100 µM as previously described[23]. The growth inhibition assays were set up in triplicate wells with a total volume of 100 µL consisting of 10 µL of gossypol and 90 µL parasite culture at 1% parasitemia and 2% haematocrit in a 96-well plate. RPMI containing 1% DMSO was used as a negative

control, and uninfected erythrocytes at 2% haematocrit were used to correct for background fluorescence. The parasitemia corresponding to each culture well after 72 h of incubation was determined by removing 80 µL of the supernatant and adding 80 µL of 0.2× SYBR Green I (Invitrogen, USA) stain in lysis buffer (20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% (W/V) saponin and 0.08% (V/V) Triton X-100)) based on established methods[54]. The plates were then incubated in the dark at 37ºC for a minimum of 30 min, and the total fluorescence from each of the wells was determined with the Varioskan Lux multimode microplate fluorescent plate reader (ThermoFisher Scientific, USA) at an excitation of 485 nm, emission of 520 nM and gain at 100.

Data analysis

The data acquired from the fluorescent plate reader were initially processed using Microsoft Excel software (Version 16.89.1) by subtracting background fluorescence from all values. After processing, the data were imported into GraphPad Prism (Version 9.01) for further analysis. Sigmoidal dose-response curves were generated, and IC_{50} values were estimated for each assay. Briefly, the compound's concentrations were transformed to a logarithmic scale. The fluorescence readings corresponding to parasitemia were then normalized by setting the lowest mean in each data set to 0% and the highest mean to 100%. Given the typical sigmoidal (S-shaped) nature of dose-response curves, a non-linear regression analysis was performed using the four-parameter logistic model (4PL). This model was selected because it provides key information about the minimum and maximum response levels, the IC_{50} value, and the slope of the curve. To assess the quality of the curve fitted to the data, the R-squared value, a statistical test for goodness-of-fit, was calculated for each data set. In this study, R-squared values greater than 0.9 were considered to represent a good fit for the dose-response curves. The IC_{50} values were derived from the fitted dose-response curves, and each data point is presented as the mean ± standard error of the mean (SEM) from two independent experiments, each conducted in triplicate. The IC_{50} values and their corresponding 95% confidence intervals (CI) were used to compare the efficacy of gossypol in laboratory strains, clinical isolates, and the Dd2_3.5 µM parasites. Additionally, the GraphPad Prism software was also used to test for statistically significant differences in mean IC_{50} values. An unpaired (independent) t-test was used to determine whether there was a statistically significant difference between the mean IC_{50} values of the laboratory strains and the clinical isolates or clinical isolates with different clones. Before performing the t-test, the assumptions of normality and homogeneity of variances were evaluated. The Shapiro-Wilk test was used to assess the normality of the data in each group. The F test for equality of variances was employed to check for homogeneity of variances. If the F test indicated that the variances were not equal, Welch's t-test, which does not assume equal variances, was used to correct for this violation.

Evaluating copy number variation in pfcrt and pfmdr1 genes

To understand the differences in the response of the *P. falciparum* parasites to gossypol, the copy numbers in the *pfcrt* and *pfmdr1*genes were determined using a SyBr green-based real-time quantitative polymerase chain reaction assay (RT-qPCR)[55]. Each sample was set up with a total reaction volume of 10 µL comprising 1X Luna Universal qPCR Master Mix (New England Biolabs, Hitchin, UK), 0.2 µM of each of the primers, and 2 µL of DNA template in triplicates on the QuantStudio5 system (Applied Biosystems, Waltham, MA). The cycling conditions for the assay were an initial amplification at 50ºC for 2 min, 95ºC for 1 min, 40 cycles at 95ºC for 30 s, 54ºC for 40 s and 60ºC for 1 min. The specificity of the resulting amplicons was analyzed using the melt curve. The endogenous control was the *P. falciparum* Beta-tubulin gene. DNA from the 3D7 and Dd2 laboratory strains was also used as reference samples, and a non-template control was also included in each run. The Ct values for each gene of interest (*pfcrt* and *pfmdr1*) were normalized to the Ct values of the reference gene, yielding ΔCt. The ΔCt values were then compared to a control strain known to have a single copy of *pfcrt* and *pfmdr1* to obtain ΔΔCt. The delta-delta Ct (2—ΔΔCt) method was used to estimate the relative copy numbers of the *pfcrt* and *pfmdr1*genes[38]. For each sample, the copy number was determined as the mean value from the triplicate qPCR reactions. Copy number results were expressed as approximations (e.g., 1.0, 2.0, 3.0 copies), with minor variations (e.g., 0.8 or 2.2) rounded up to the nearest whole number for clarity.

Genotyping parasites for multiclonality

The different parasite genotypes in the clinical isolates and Dd2_3.5 µM parasites were identified by analysis of polymorphic genetic loci of the merozoite surface protein genes *msp1* and *msp2*[56]. Briefly, genomic DNA was extracted from seventeen (17) of the clinical isolates of *P. falciparum* parasites (four of the clinical isolates were lost at the time of this experiment) and amplified in a polymerase chain reaction (PCR) with primers targeting conserved regions of *msp1* block 2 and *msp2* block 3 (Table S1). In a nested PCR reaction, the DNA from the first reaction was amplified with primer sets targeting specific allelic families of *msp1* (MAD20, K1 and RO33) and *msp2* (3D7/IC and FC27). Genomic DNA from laboratory strains was used as a positive control and a template-free control was also included as a negative control for all the reactions. The size of the PCR product was determined using a 100 bp DNA ladder (Supplementary Fig. S5) and the polymorphisms in each allelic family of the *msp1* and *msp2* genes were determined as previously described[57]. The allele frequencies and multiplicity of infection (MOI) were determined by computing the number of unique alleles in the *msp1* and *msp2* genes. A MOI of one (1) indicates monoclonal infection and greater than one (>1) indicates polyclonal infection. The proportion of mono- and polyclonal infections was computed and compared between MSP1 and MSP2 using the Fisher's Exact test.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Received: 18 August 2024; Accepted: 6 January 2025
Published online: 09 January 2025

References

1. WHO. in WHO global malaria programme: world malaria report 2010 204–204. (2010).
2. WHO. *World Malaria Report 2014*Vol. 23 (World Health Organization, 2014).
3. Organization, W. H. *World Malaria Report 2023* (World Health Organization, 2023).
4. WHO. World malaria report 2019. Geneva: World Health Organization. Licence: CC BY-NC-SA 3.0 IGO. *WHO* (2019). (2019).
5. WHO. World Malaria report 2015. Geneva: World Health Organization. *WHO* (2015). (2015).
6. Dondorp, A. M. et al. Artemisinin resistance in Plasmodium Falciparum malaria. *N Engl. J. Med.* **361**, 455–467. https://doi.org/10.1056/NEJMoa0808859 (2009).
7. Imwong, M. et al. The spread of artemisinin-resistant Plasmodium falciparum in the Greater Mekong subregion: a molecular epidemiology observational study. *Lancet Infect. Dis.* **17**, 491–497. https://doi.org/10.1016/S1473-3099(17)30048-8 (2017).
8. WHO. *World Malaria Report 2022* (World Health Organization, 2022).
9. Mihreteab, S. et al. Increasing prevalence of artemisinin-resistant HRP2-negative malaria in Eritrea. *N. Engl. J. Med.* **389**, 1191–1202 (2023).
10. Uwimana, A. et al. Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium Falciparum kelch13 R561H mutant parasites in Rwanda. *Nat. Med.* **26**, 1602–1608 (2020).
11. Asua, V. et al. Changing prevalence of potential mediators of aminoquinoline, antifolate, and artemisinin resistance across Uganda. *J. Infect. Dis.* **223**, 985–994 (2021).
12. Olotu, A. et al. Seven-year efficacy of RTS,S/AS01 malaria vaccine among young African children. *N Engl. J. Med.* **374**, 2519–2529. https://doi.org/10.1056/NEJMoa1515257 (2016).
13. Coelho, C. H., Doritchamou, J. Y. A., Zaidi, I. & Duffy, P. E. Advances in malaria vaccine development: report from the 2017 malaria vaccine symposium. *NPJ Vaccines* 2, 34 (2017). https://doi.org/10.1038/s41541-017-0035-3
14. Van Voorhis, W. C. et al. Open Source Drug Discovery with the Malaria Box compound Collection for Neglected diseases and Beyond. *PLoS Pathog.* **12**, e1005763. https://doi.org/10.1371/journal.ppat.1005763 (2016).
15. Douglas, R. G., Reinig, M., Neale, M. & Frischknecht, F. Screening for potential prophylactics targeting sporozoite motility through the skin. *Malar. J.* **17**, 1–10 (2018).
16. Feng, L. S. et al. Hybrid molecules with potential in vitro antiplasmodial and in vivo antimalarial activity against drug-resistant Plasmodium falciparum. *Med. Res. Rev.* **40**, 931–971. https://doi.org/10.1002/med.21643 (2020).
17. Schiafino-Ortega, S. et al. 1, 2-Diphenoxiethane salts as potent antiplasmodial agents. *Bioorg. Med. Chem. Lett.* **28**, 2485–2489 (2018).
18. Heidrich, J., Hunsaker, L. & Vander Jagt, D. Gossypol, an antifertility agent, exhibits antimalarial activity in vitro. *IRCS J. Med. Sci.* (1983).
19. Razakantoanina, V., Kim, N., Jaureguiberry, G. & P. P. & Antimalarial activity of new gossypol derivatives. *Parasitol. Res.* **86**, 665–668. https://doi.org/10.1007/pl00008549 (2000).
20. Moh, P., Li, P., Darby, M., Brueggemeier, R. & Lin, Y. Characteristics of covalent gossypol binding to microsomal proteins. *Res. Commun. Chem. Pathol. Pharmacol.* **76**, 305–322 (1992).
21. National, C. G. o. M. A. A. Gossypol-a new antifertility agent for males. *Chin. Med. J.* **4**, 417–428 (1978).
22. Chirawurah, J. D. et al. MMV006087 is a potent Malaria Box compound against Plasmodium Falciparum clinical parasites. *Front. Drug Discovery*. **3**, 1190471 (2023).
23. Chirawurah, J. D. et al. Antimalarial activity of Malaria Box compounds against Plasmodium falciparum clinical isolates. *Int. J. Parasitol. Drugs Drug Resist.* **7**, 399–406. https://doi.org/10.1016/j.ijpddr.2017.10.005 (2017).
24. Chugh, M. et al. Identification and deconvolution of cross-resistance signals from antimalarial compounds using multidrug-resistant Plasmodium falciparum strains. *Antimicrob. Agents Chemother.* **59**, 1110–1118 (2015).
25. Pradines, B., Bertaux, L., Pomares, C., Delaunay, P. & Marty, P. Reduced in vitro susceptibility to artemisinin derivatives associated with multi-resistance in a traveller returning from South-East Asia. *Malar. J.* **10**, 268. https://doi.org/10.1186/1475-2875-10-268 (2011).
26. Walker-Jonah, A., Dolan, S. A., Gwadz, R. W., Panton, L. J. & Wellems, T. E. An RFLP map of the Plasmodium falciparum genome, recombination rates and favored linkage groups in a genetic cross. *Mol. Biochem. Parasitol.* **51**, 313–320. https://doi.org/10.1016/0166-6851(92)90081-t (1992).
27. Sa, J. M. et al. Geographic patterns of Plasmodium Falciparum drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Proc. Natl. Acad. Sci. U S A.* **106**, 18883–18889. https://doi.org/10.1073/pnas.0911317106 (2009).
28. Tse, M. T. Antimalarial drugs: speeding to a new lead. *Nat. Rev. Drug Discov.* **9**, 842. https://doi.org/10.1038/nrd3301 (2010).
29. Royer, R. E., Deck, L. M., Campos, N. M. & Hunsaker, L. A. Vander Jagt, D. L. Biologically active derivatives of gossypol: synthesis and antimalarial activities of peri-acylated gossylic nitriles. *J. Med. Chem.* **29**, 1799–1801. https://doi.org/10.1021/jm00159a043 (1986).
30. VanderJagt, D., Baack, B., Campos, N., Hunsaker, L. & Royer, R. A derivative of gossypol retains antimalarial activity. *IRCS Med. SCIENCE-BIOCHEMISTRY*. **12**, 845–846 (1984).
31. Arrowsmith, J., Miller, P. & Phase II and phase III attrition rates 2011–2012. *Nat. Rev. Drug Discovery*. **12**, 569–570 (2013).
32. Arrowsmith, J. & Phase, I. I. 2008–2010. *Nat. Rev. Drug Discovery* 10 (2011).
33. Bushman, M. et al. Within-host competition and drug resistance in the human malaria parasite Plasmodium Falciparum. *Proc. Biol. Sci.* **283**, 20153038. https://doi.org/10.1098/rspb.2015.3038 (2016).
34. de Roode, J. C., Culleton, R., Bell, A. S. & Read, A. F. Competitive release of drug resistance following drug treatment of mixed Plasmodium chabaudi infections. *Malar. J.* **3**, 33. https://doi.org/10.1186/1475-2875-3-33 (2004).
35. Gresty, K. J. et al. Genetic mutations in pfcrt and pfmdr1 at the time of artemisinin combination therapy introduction in South Pacific islands of Vanuatu and Solomon Islands. *Malar. J.* **13**, 406 (2014).
36. Langreth, S. G., Nguyen-Dinh, P. & Trager, W. Plasmodium Falciparum: merozoite invasion in vitro in the presence of chloroquine. *Exp. Parasitol.* **46**, 235–238 (1978).
37. Lambros, C. & Notsch, J. Plasmodium Falciparum: mefloquine resistance produced in vitro. *Bull. World Health Organ.* **62**, 433 (1984).
38. Brockelman, C., Monkolkeha, S. & Tanariya, P. Decrease in susceptibility of Plasmodium falciparum to mefloquine in continuous culture. *Bull. World Health Organ.* **59**, 249 (1981).
39. Oduola, A. M., Milhous, W., Weatherly, N., Bowdre, J. & Desjardins, R. Plasmodium Falciparum: induction of resistance to mefloquine in cloned strains by continuous drug exposure in vitro. *Exp. Parasitol.* **67**, 354–360 (1988).
40. Bushman, M. et al. Within-host competition and drug resistance in the human malaria parasite Plasmodium Falciparum. *Proc. Royal Soc. B: Biol. Sci.* **283**, 20153038 (2016).
41. Witkowski, B. et al. Increased tolerance to artemisinin in Plasmodium Falciparum is mediated by a quiescence mechanism. *Antimicrob. Agents Chemother.* **54**, 1872–1877 (2010).
42. Yu, X. et al. Ring-stage growth arrest: metabolic basis of artemisinin tolerance in Plasmodium Falciparum. *Iscience* **26** (2023).

43. Mira-Martínez, S. et al. Epigenetic switches in clag3 genes mediate blasticidin S resistance in malaria parasites. *Cell. Microbiol.* **15**, 1913–1923 (2013).
44. Mira-Martínez, S. et al. Identification of antimalarial compounds that require CLAG3 for their uptake by Plasmodium falciparum-infected erythrocytes. *Antimicrob. Agents Chemother.* **63** https://doi.org/10.1128/aac.00052-19 (2019).
45. Rawat, M. et al. Histone acetyltransferase PfGCN5 regulates stress responsive and artemisinin resistance related genes in Plasmodium Falciparum. *Sci. Rep.* **11**, 852 (2021).
46. Trager, W. & Jensen, J. B. Human malaria parasites in continuous culture. *Science* **193**, 673–675. https://doi.org/10.1126/science.781840 (1976).
47. Maier, A. G. & Rug, M. In vitro culturing Plasmodium Falciparum erythrocytic stages. *Malaria: Methods Protocols*, 3–15 (2013).
48. Mensah-Brown, H. E. et al. Analysis of Erythrocyte Invasion mechanisms of Plasmodium Falciparum Clinical isolates across 3 Malaria-endemic areas in Ghana. *J. Infect. Dis.* **212**, 1288–1297. https://doi.org/10.1093/infdis/jiv207 (2015).
49. Mensah-Brown, H. E. et al. Assessing the impact of differences in malaria transmission intensity on clinical and haematological indices in children with malaria. *Malar. J.* **16**, 96. https://doi.org/10.1186/s12936-017-1745-8 (2017).
50. Ljungström, I., Moll, K., Perlmann, H., Scherf, A. & Wahlgren, M. *Methods in malaria research*Citeseer,. (2008).
51. Lambros, C. & Vanderberg, J. P. Synchronization of Plasmodium Falciparum erythrocytic stages in culture. *J. Parasitol.* **65**, 418–420 (1979).
52. Corey, V. C. et al. A broad analysis of resistance development in the malaria parasite. *Nat. Commun.* **7**, 11901. https://doi.org/10.1038/ncomms11901 (2016).
53. Nzila, A. & Mwai, L. In vitro selection of Plasmodium Falciparum drug-resistant parasite lines. *J. Antimicrob. Chemother.* **65**, 390–398. https://doi.org/10.1093/jac/dkp449 (2010).
54. Smilkstein, M., Sriwilaijaroen, N., Kelly, J. X., Wilairat, P. & Riscoe, M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob. Agents Chemother.* **48**, 1803–1806. https://doi.org/10.1128/AAC.48.5.1803-1806.2004 (2004).
55. Ferreira, I. D., Rosario, V. E. & Cravo, P. V. Real-time quantitative PCR with SYBR Green I detection for estimating copy numbers of nine drug resistance candidate genes in Plasmodium Falciparum. *Malar. J.* **5**, 1. https://doi.org/10.1186/1475-2875-5-1 (2006).
56. Snounou, G. et al. Biased distribution of msp1 and msp2 allelic variants in Plasmodium falciparum populations in Thailand. *Trans. R Soc. Trop. Med. Hyg.* **93**, 369–374. https://doi.org/10.1016/s0035-9203(99)90120-7 (1999).
57. Mayengue, P. I., Ndounga, M., Malonga, F. V., Bitemo, M. & Ntoumi, F. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in Plasmodium Falciparum isolates from Brazzaville, Republic of Congo. *Malar. J.* **10**, 276. https://doi.org/10.1186/1475-2875-10-276 (2011).

Acknowledgements

We thank the Medicines for Malaria Venture for giving us the Malaria box compounds for this work and the study participants who provided the samples through the Erythrocyte Invasion Mechanism (EIM) project for their support.

Author contributions

J.D.C., G.A.A. and Y.A. conceived the study; J.D.C., B.A., S.B, S.N.Y. and F.A. performed the experiments; J.D.C., S.B. S.N.Y. and F.A. analysed the data; J.D.C., Y.A., G.A.A., L.A-E. drafted the manuscript. All authors have critically reviewed the manuscript.

Funding

Jersley D. Chirawurah was supported by a DELTAS Africa grant (DEL-15-007: Awandare). The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)'s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa's Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust (107755/Z/15/Z: Awandare) and the UK government. The views expressed in this publication are those of the author(s) and not necessarily those of AAS, NEPAD Agency, Wellcome Trust or the UK government.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-85643-6.

Correspondence and requests for materials should be addressed to G.A.A. or Y.A.