Potential Metabolic Resistance Mechanisms to Ivermectin in Anopheles Gambiae: A Synergist Bioassay Study

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Abstract

Background Despite remarkable success obtained with current malaria vector control strategies in the last 15 years, additional innovative measures will be needed to achieve the ambitious goals set for 2030 by the World Health Organization (WHO). New tools will need to address insecticide resistance and residual transmission as key challenges. Endectocides such as ivermectin are drugs that kill mosquitoes which feed on treated subjects. Mass administration of ivermectin can effectively target outdoor and early biting vectors, complementing the still effective conventional tools. Although this approach has garnered attention, development of ivermectin resistance is a potential pitfall. Herein, we evaluate the potential role of xenobiotic pumps and cytochrome P450 enzymes in protecting mosquitoes against ivermectin by active efflux and metabolic detoxification, respectively.

Methods We determined the lethal-concentration 50 for ivermectin in colonized *Anopheles gambiae*, then we used chemical inhibitors and inducers of xenobiotic pumps and cytochrome P450 enzymes in combination with ivermectin to probe the mechanism of ivermectin detoxification.

Results Dual inhibition of xenobiotic pumps and cytochromes have a synergistic effect with ivermectin, greatly increasing mosquito mortality. Inhibition of xenobiotic pumps alone had no effect on ivermectin-induced mortality. Induction of xenobiotic pumps and cytochromes may confer partial protection from ivermectin.

Conclusion there is a clear pathway for development of ivermectin resistance in malaria vectors. Detoxification mechanisms mediated by cytochrome P450 enzymes are more important than xenobiotic pumps in protecting mosquitoes against ivermectin.

Introduction

Since the turn of the century, there have been made significant advances against malaria; the global malaria mortality rate has reduced by more than 50%, saving more than 6.8 million lives [1,2]. Two vector control measures are mainly responsible for this success; (1) use of insecticide-treated nets and (2) indoor residual spraying, both of which are insecticide-based and home-centered[3]. The continuous use and reliance on insecticides has put selective pressure on the mosquitoes, radically changing the vector species´ distribution and behavior [4]. This allows malaria transmission to continue by shifting to times and spaces unprotected by the current vector control measures, most noticeably early biting, and/or outdoor biting [5]. Moreover, the selection pressure has yielded mosquito populations that are resistant to the current insecticides used in malaria vector control [6].

The new challenges in vector control has called for the development of new tools with the capabilities to circumvent the new challenges [7]. One of the proposed tools that is currently under evaluation is the use of endectocides [8]. Endectocides are antiparasitic drugs with activity against endoparasites and ectoparasites such as mosquitoes which feed on treated humans or animals. Importantly, endectocides

can target exophilic and exophagic vectors, tackling the problem of residual transmission, while perfectly complementing the indoor vector control measures [9].

Owing to its excellent safety profile and activity against most malaria vectors, ivermectin is the leading endectocide candidate for malaria control [10,11]. In addition, ivermectin is extensively used for the control of neglected tropical diseases (NTDs) that overlap with malaria in endemic areas, potentially increasing the cost-effectiveness of implementing ivermectin mass drug administration (MDA) [12,13].

Several of the effects ivermectin has on malaria vectors point towards a low risk and slow speed for the development of resistance. These include (a) its mechanism of action, agonizing glutamate-gated chloride channels, that differs from all currently approved public health insecticides [14]; (b) direct delivery to the midgut with a blood meal that can bypass resistance associated with cuticular mechanisms [15]; (c) marked reduction in fertility and fecundity of malaria vectors exposed to sub-lethal concentrations [16,17]. Although development of ivermectin resistance in malaria vectors could take long, it is inevitable as it has already been reported in other arthropods [18]. Moreover, considering that ivermectin MDAs for NTDs are ongoing in the past 30 years, these could have potentially exposed malaria vectors to mostly sub-lethal ivermectin concentrations that could enhance the process of resistance development [19]. Therefore, it is important to have an early and thorough understanding of potential mosquito detoxification mechanisms for ivermectin. This will be crucial for early development of approaches that could delay, counter, or detect ivermectin resistance.

Herein, we evaluated the potential detoxification mechanisms involved in the response to ivermectin in the *Anopheles gambiae* s.s. mosquito. We probed two general mechanisms of detoxification; (1) metabolic detoxification mediated by enzymes specifically cytochrome P450s and (2) detoxification by excretion facilitated by ATP binding cassette (ABC) transporters, specifically the P-glycoprotein (P-gp). The increased activity of cytochrome P450 enzymes leads to increased bio-degradation of the toxins while an increased activity of the P-glycoprotein leads to increased excretion of xenobiotics. Both mechanisms reduce the toxic effects of the compounds by decreasing the insects' systemic exposure to the toxic compounds [20]. Both detoxification mechanisms have been implicated in resistance to insecticides used for the control of malaria vectors with metabolic detoxification being the most common mechanism [21,22]. Elevated levels of cytochrome P450s (CYP), esterases, and glutathione S-transferases (GSTs) are associated with resistance to pyrethroids, organophospates and DDT, respectively [22]. Though both mechanisms are known to contribute to ivermectin resistance in other arthropods, their potential contribution to ivermectin resistance in mosquitoes has not been thoroughly explored [23–25].

In this study, we assessed the interaction of different chemical inhibitors and inducers of cytochrome P450 and P-glycoproteins with ivermectin in *An. gambiae* s.s. (Kilifi strain) mosquitoes. Our main questions were whether and how the inhibitors and inducers of cytochrome P450 and P-gp affect the ivermectin-induced mosquito mortality.

Materials And Methods

Experimental design

The experiments were conducted in two phases. In phase one, an ivermectin dose-finding experiment was performed using triplicate batches of 50 female mosquitoes (3–5 days old) with the aim of identifying the 10-day LC_{50} of ivermectin for our colony (Fig. 1). The 10-day period was chosen based on the minimum extrinsic incubation period of *Plasmodium falciparum* parasites i.e. mosquitoes dying before 10 days are unlikely to become infectious [26]. We tested five different concentrations of ivermectin spanning +/- 20–40% of the 5-day LC_{50} described by Kobylinski et al. [27], namely 4 ng/ml, 8 ng/ml, 12 ng/ml, 16 ng/ml, and 20 ng/ml.

In Phase two, following the identification of a concentration of ivermectin yielding about 50% mortality in 10 days, we evaluated (a) the effect on mosquito mortality of CYP and/or P-gp inhibitors and inducers alone at different concentrations and (b) the effect of combining ivermectin with different doses of CYP and/or P-gp inhibiters and inducers (Fig. 2).

For voriconazole, ritonavir, cobicistat, cycloporine A, elacridar and rifampicin the concentrations tested were based on the maximum blood concentration reached in humans after a single dose (C_{max}) as reported in the literature (Table 1). Four concentrations corresponding to C_{max}, 75% C_{max}, 50% C_{max} and 25% C_{max} were evaluated. Hereafter, C_{max} concentration is referred to as A, 75% C_{max} as B, 50% C_{max} as C, and 25% C_{max} as D.

Given that mosquitoes were to be exposed in batches to the different drugs and combinations, the study was considered cluster-randomized in which the batch exposed to any drug was the unit of randomization and the mosquito was the analysis unit. The sample size was adjusted for a cluster effect. A 50% increase in ivermectin-driven 10-day mosquito mortality was considered of potential public health value. According to the method of Hayes and Bennett [28], using three replicas of 50 mosquitoes per group, gives the study 80% power at 5% significance level to detect a 50% increase in 10-day mortality from 50–75% by adding the synergist. This uses an intra-cluster correlation coefficient of 0.06 described before for mosquito colonies [29]. These calculations are confirmed using the formula of Gangnon and Kosorok [30] that shows a design effect of 3.34 with a 70% possibility of observing mortality within 10 days.

Table 1
Synergists and corresponding doses used for the experiments

Drug	Mechanism of action	D_A (C_{max})	D_B (75% of C_{max})	D_C (50% of C_{max})	D_D (25% of C_{max})
Cobicistat [31]	Dual CYP/P-gp inhibitor*	990 ng/ml	742 ng/ml	495 ng/ml	247 ng/ml
Cyclosporine A [32]	Selective P-gp inhibitor†	1,802 ng/ml	1,351 ng/ml	901 ng/ml	450 ng/ml
Elacridar [33]	Selective P-gp inhibitor	160 ng/ml	120 ng/ml	80 ng/ml	40 ng/ml
Rifampicin [34]	Dual CYP/P-gp inducer	7,000 ng/ml	5,250 ng/ml	3,500 ng/ml	1,750 ng/ml
Ritonavir [35]	Dual CYP/P-gp inhibitor	11,000 ng/ml	8,250 ng/ml	5,500 ng/ml	2,750 ng/ml
Voriconazole [36]	Dual CYP/P-gp inhibitor	3,667 ng/ml	2,750 ng/ml	1,833 ng/ml	916 ng/ml

* with effect markedly skewed towards CYP inhibition, †negligible effect on CYPs

Mosquitoes

Throughout the study we used *Anopheles gambiae* s.s. Kilifi strain maintained in KEMRI Wellcome Trust Research Programme in Kilifi, Kenya insectary. The mosquitoes were maintained at 28 °C and 80% relative humidity at a 12-h light:12-h dark photoperiod. Adult mosquitoes were fed with *ad libitum* 10% glucose solution via impregnated cotton wool while larvae were fed with Tetramine fish flakes.

Experimental drugs

Based on their mechanism of action in humans, voriconazole, ritonavir and cobicistat were classified as dual CYP/P-gp inhibitors [18, 19], cyclosporine-A and elacridar were classified as P-gp specific inhibitors [37], and rifampicin was classified as a dual CYP/P-gp inducer [38]. The choice of inhibitors and inducers used in the present study was based on their ability to act as substrates for the cytochrome P450 CYP3A4 which is the major enzyme involved in ivermectin metabolism in humans [39].

Ivermectin, voriconazole, ritonavir, cobicistat, cyclosporine A, elacridar and rifampicin were obtained from Sigma Aldrich (Spain). The active pharmacological ingredients were dissolved in Dimethyl sulfoxide (DMSO) to prepare stock solutions for all compounds. Aliquots of the prepared solutions were frozen at -20 °C. For each experiment, the stock solutions were diluted in phosphate buffered saline (PBS) to achieve the desired concentration.

Membrane blood feeding

For blood feeding, we used certified drug-free cattle blood defibrinated using the Reynold's method [40]. Briefly, after blood collection the blood was gently shaken for 5–10 minutes in a 250 ml glass bottle containing a copper wire arranged as an elongated spiral with two outer loops inside. The wire was then removed taking with it fibrin that became bound to it [40]. The defibrinated blood was mixed with the drugs at appropriate concentration to a total volume of 6 ml. In the case of the control, the blood was mixed with PBS only.

Fifty 2-5-day-old blood-naïve female mosquitoes were transferred from stock cages to mosquito holding cups (1,000 cm^3) and starved of water and glucose for 6–8 hours before blood feeding. The holding cups were covered by untreated net with a lateral aspiration hole covered with a double layer of dental dam. Blood feeding was done using an inverted cup technique [41]. The blood was placed on the bottom surface of a paper cup (500 ml capacity) and covered by a thinly stretched Parafilm membrane. The membrane was secured with masking tape, the cup was inverted and filled with warm water (approximately 38 °C). The cups were then held over the netting material of the holding cups and mosquitoes were allowed to feed. Mosquito feeding was done in the dark for a period of 30–60 minutes. Visually unfed mosquitoes were removed and only fully engorged mosquitoes kept in the holding cages for follow-up and maintained at standard insectary conditions. Mortality was monitored every 24 hours for 10 days by counting and removing dead mosquitoes. At least three replicates were performed for every drug or drug combination tested. The position of the cages was rotated daily.

Data collection and statistical analysis

Daily mortality data were entered in Excel sheets. Survival Kaplan-Meier and Cox´s regression analyses were performed in Addinsoft's XLSTAT ® Version 2018.5 (New York, NY, USA) and GNU R (R Core Team [2020] R: A language and environment for statistical computing, version 3.6.3, R Foundation for Statistical Computing, Vienna, Austria, https://www.R-project.org). Comparisons of survival patterns were done with Log-rank test using a 5% significance. When the overall p-value was less than 0.05 pairwise comparisons was performed and Bonferroni correction used to correct for multiple comparisons.

Results

Ivermectin induces dose dependent but delayed mortality in A. gambiae

We first determined the 10-day LC_{50} of ivermectin in our colony of *An. gambiae* s.s. The effect of ivermectin on mosquito survival was dose dependent (Fig. 3). The 8 ng/ml concentration was the only one that resulted in approximately 50% mortality in 10 days showing a mortality of 46.49% (Table 2). Therefore, this is the concentration of ivermectin that was chosen and used in phase two experiments.

Table 2
Mosquito survival after ingestion of multiple concentrations of ivermectin

Ivermectin concentration	Mean Survival Time (95% CI)	% Mortality
0 ng/ml (control)	8.1 (7.6–8.5)	20
4 ng/ml	8.1 (7.5–8.6)	38.1
8 ng/ml	7.3 (6.8–7.9)	46.5
12 ng/ml	6.0 (5.5–6.5)	71.1
16 ng/ml	5.6 (5.2–6.1)	75.4
20 ng/ml	4.5 (4.1–4.9)	88.5

Mortality after consumption of ivermectin did not occur immediately and was negligible for the first three days. Instead, mosquito mortality occurred in excess between day four and six. Most of the mosquitoes surviving after day six remained alive until day 10. These tendencies were observed across all the concentrations of ivermectin with exception of control group indicating that the three days mortality observed on day four to six was driven by ivermectin and not by mosquito ageing.

Dual CYP/ P-gp inhibitors have a synergistic effect on ivermectin-induced mosquito mortality

We next investigated whether the dual inhibition of both CYP and P-gp affected ivermectin induced mortality. For these experiments, we used three drugs; ritonavir, voriconazole and cobicistat that are known to have a dual CYP/P-gp inhibition.

Firstly, we excluded the possibility that any observed differences in mortality observed by combining ivermectin with a CYP/P-gp inhibitor was because of additive mortality caused by the CYP/P-gp inhibitors themselves. No significant differences in mortality were observed between mosquitoes that consumed CYP/P-gp inhibitors alone and those that did not irrespective of the dose used (Fig. 4A, 4C and 5A).

In contrast, differences in mortality were observed between mosquitoes that consumed Ivermectin alone and those that consumed ivermectin combined with a dual CYP/P-gp inhibitor. Notably, experiments conducted with ritonavir and voriconazole show enhanced ivermectin-induced mortality and dose-dependent synergism (Fig. 4B and 4D). Unlike voriconazole, the synergistic effect of ritonavir saturated at the second lowest concentration (concentration C). Additionally, when ivermectin was combined with ritonavir or voriconazole an increase on percentage of mortality and decrease of mean survival time of the mosquitoes was observed (Table 3).

Table 3
Synergist drugs combined with ivermectin

Drug and Concentration	Mean Survival Time (95% CI)	% Mortality
Voriconazole		
Control	8.5 (8.0-9.1)	21.9
Ivermectin	8.0 (7.5–8.5)	35.0
IVM + VOR A	5.4 (4.9–5.9)	59.8
IVM + VOR B	5.7 (5.3-6.0)	44.9
IVM + VOR C	7.6 (7.0-8.1)	42.2
IVM + VOR D	7.4 (6.9–7.8)	34.7
Ritonavir		
Control	8.0 (7.5–8.4)	18.3
Ivermectin	8.2 (7.7–8.8)	30.3
IVM + RIF A	6.8 (6.1–7.5)	53.7
IVM + RIF B	6.6 (5.8–7.4)	51.8
IVM + RIF C	6.7 (6.1–7.3)	55.2
IVM + RIF D	7.3 (6.7-8.0)	42.3
Cobicistat		
Control	7.1 (6.8–7.5)	18.5
Ivermectin	7.8 (7.1–8.4)	45.6
IVM + COB A	8.6 (8.1–9.1)	23.6
IVM + COB B	8.2 (7.8–8.7)	29.6
IVM + COB C	7.5 (6.9–8.1)	39.0
IVM + COB D	8.1 (7.6–8.6)	30.8
Elacridar		
Control	9.5 (9.3–9.8)	12.2
Ivermectin	7.1 (6.5–7.6)	54.6
IVM + ELA A	6.9 (6.4–7.4)	62.3
IVM + ELA B	7.3 (6.8–7.8)	49.6
IVM + ELA C	6.5 (6.1-7.0)	51.4

Drug and Concentration	Mean Survival Time (95% CI)	% Mortality
IVM + ELA D	7.0 (6.5–7.4)	56.1
Cyclosporine A		
Control	9.0 (8.3–9.6)	15.6
Ivermectin	9.0 (8.5–9.6)	21.8
IVM + CYC A	7.8 (6.8–8.8)	34.7
IVM + CYC B	7.7 (6.8–8.6)	38.1
IVM + CYC C	7.7 (7.0-8.4)	20.9
IVM + CYC D	8.5 (7.9–9.2)	33.3
Rifampicin		
Control	7.2 (6.6–7.7)	39.1
Ivermectin	6.7 (6.0-7.5)	53.4
IVM + RIF A	6.9 (6.2–7.6)	49.5
IVM + RIF B	6.4 (5.6–7.1)	61.3
IVM + RIF C	8.2 (7.6–8.9)	31.5
IVM + RIF D	6.4 (5.8-7-0)	66.3

Cobicistat, an exceptional antagonistic effect

Cobicistat alone had no effect on mosquito mortality regardless of the concentration used (Fig. 5A). Despite being a dual CYP/P-gp inhibitor, cobicistat showed an antagonistic effect on ivermectin-induced mortality when combined with ivermectin. High cobicistat concentrations protected the mosquitoes from ivermectin toxicity with a higher survival probability recorded in cobicistat combined with ivermectin than ivermectin alone (Fig. 5B). Additionally, when combined with ivermectin, cobicistat showed a lower percentage mortality in comparison to ivermectin alone (Table 3).

Inhibition of P-gp alone does not affect ivermectin-induced mosquito mortality

Following the observation of an effect of CYP/P-gp inhibition on ivermectin-induced mortality, we next assessed the effects of only inhibiting P-gp transporters. For this, we used elacridar and cyclosporine A which are predominantly p-gp inhibitors in vivo. Similarly, to the dual CYP/P-gp inhibitors, the selective P-gp inhibitors alone did not cause significant mortality at all doses tested (Fig. 6A and 6C).

Moreover, when combined with ivermectin both elacridar and cyclosporine A showed no effect on ivermectin-induced mortality suggesting that P-gp inhibitors do not synergize with ivermectin to increase mortality (Fig. 6B and 6D).

Simultaneous induction of cytochrome P450 and P-gp may confer modest protection from ivermectin-induced mortality

We evaluated the effect of rifampicin which is a dual CYP/P-gp inducer on mosquito mortality. No significant difference was observed in the survival of mosquitoes feeding on rifampicin alone at the different doses tested (Fig. 7A).

However, when combined with ivermectin, rifampicin showed antagonism at low concentrations by enhancing ivermectin- induced mortality (Table 3 and Fig. 7B).

Discussion

Susceptibility to ivermectin has been shown to vary among mosquito species as well as among mosquito strains of the same species [27,42,43]. Depending on the time frame which survival is monitored, *An. gambiae* have been shown to have an LC50 of 19.8, 15.9 and 22.4 ng/ml when survival is monitored for 9, 7 and 5 days, respectively [27,44,45]. In our case by monitoring survival for 10 days we achieved 46.49% mortality with a concentration of 8 ng/ml. Though the levels of ivermectin in the blood drop rapidly, a concentration above 8 ng/ml can be maintained for close to 36 hours following an ivermectin dose in humans [14,27]. Our results are aligned with the those of Smit *et al*. in which even very low ivermectin concentrations can increase mosquito mortality if the follow up period encompasses the usual lifespan [46].

At the used doses, ivermectin-induced mortality in mosquitoes is delayed by 2–3 days. One potential explanation is the time taken for ivermectin to be absorbed from the midgut, as faster lethality onsets has been observed when ivermectin is directly injected into the midgut than when it is taken as part of a blood meal [42]. The second plausible explanation for the delayed mortality is the involvement of ivermectin metabolites rather than the parent compound in causing mosquito mortality. Presently, there is accumulating evidence suggesting the involvement of ivermectin metabolites in causing mosquito mortality though the specific metabolites are yet to be identified [46,47]. However, even before the onset of lethality that is measurable with a 10-day follow up, ivermectin can potentially affect mosquito mortality in the wild due to its effects on locomotion [48]. Simultaneously, a reduction in locomotion abilities would affect the vectorial capacity regardless of mosquito mortality, which can in turn further reduce malaria transmission.

Ivermectin-induced mortality is greatly dependent on attaining high systemic levels of ivermectin in the mosquito. The exposure to ivermectin is determined by the mosquito's detoxification capacity. Generally, in insects, detoxification processes involve metabolic enzymes such as cytochrome P450, esterase and Glutathione-S-transferases (GSTs) together with efflux pumps like the P-gp [49]. In this study, we investigated whether and how cytochrome P450s and P-gp transporters affected ivermectin-induced mortality in mosquitoes. Our results demonstrate that the simultaneous inhibition of cytochrome P450s and P-gp transporters enhances ivermectin-dependent mortality in a dose dependent manner indicating synergism. However, this only happened selectively when ritonavir or voriconazole was used. Unexpectedly, the use of cobicistat which is also a dual CYP/P-gp inhibitor rendered some protection from ivermectin. Cobicistat is a structural analogue of ritonavir but unlike ritonavir which is known to inhibit and induce multiple CYPs, cobicistat more selectively inhibits CYP3A4 [50]. Though, ritonavir and cobicistat are considered clinically equivalent, the small difference in ritonavir's ability to induce CYPs could result in differences in drug to drug interaction [51,52]. The induction of CYPs could possibly lead to ivermectin metabolism making ivermectin metabolites available. In addition to ivermectin parent compound, ivermectin metabolites have also been suggested to contribute to mosquito mortality [46,47].

Despite dual CYP/P-gp inhibitors showing an effect on ivermectin-induced mortality, P-gp selective inhibitors did not have a measurable effect. Taken together our results suggest that detoxification mechanisms mediated by CYPs are more important in ivermectin detoxification. This is contrary to what has been reported in mosquito larval stages where Buss *et al*. demonstrated that inhibition of P-gp using verapamil leads to increased toxicity in *Culex* mosquitoes [23]. Collectively both findings suggest heterogeneity in detoxification mechanisms in different stages of mosquito development. As a holometabolous insect, the changes between the immature stages (larvae and pupae) and the adult stage are characterized by differences in diet, habitat, morphology, physiology and behavior. These differences could potentially lead to differences in evolution of protective mechanisms [53]. Larval stages are known to be more prone to developing insecticide resistance compared to adults [53]. Whether the P-gp mediated detoxification reported in *Culex* larvae is additive or alternative to CYP mediated detoxification in larvae warrants to be investigated. Larval habitats are often exposed to ivermectin through contamination of aquatic habitats with excreta from treated livestock. The stability of ivermectin in water for long periods increases the exposure of the larvae to ivermectin and could potentially accelerate the development of resistance [54]. It is important to understand the mechanisms behind larval resistance to ivermectin and whether they contribute to ivermectin resistance in adults.

Our results warrant the investigation of selective CYP inhibitors for the ability to synergize ivermectin-induced mortality including piperonyl butoxide (PBO). This will help answer the question whether CYP inhibition independent of P-gp inhibition could still have practical implications.

The involvement of CYPs in ivermectin metabolism could potentially lead to cross-resistance between ivermectin and current insecticides used in vector control. Previously Deus *et al*. has demonstrated that pyrethroid resistant *Ae. aegypti* have higher tolerance to ivermectin [43]. This could potentially affect

ivermectin susceptibility in mosquito populations already showing metabolic resistance to insecticides and highlights the need to investigate the impact of insecticide resistance on susceptibility to ivermectin. Notably, the current recommendation to tackle metabolic resistance to insecticides is the use of piperonyl butoxide (PBO) [55]. PBO which is an inhibitor of CYP450 enzymes and currently in use by incorporation into pyrethroid-LLINs, could also enhance ivermectin-induced mortality. However, there is needed to first evaluate whether it synergizes ivermectin-induced mortality.

Nevertheless, ivermectin remains a good alternative to mosquito populations whose mode of resistance is via point mutations since this resistance mechanism is not analogous between the current insecticides and ivermectin [56]. While resistance to insecticides is caused by mutations in the sodium channel, acetylcholinesterase or GABA receptor genes, ivermectin resistance in other arthropods is associated with mutations on the Glutamate-gated chloride channels (GluCls) [57,58].

In the case ivermectin is to be used in mosquito populations with metabolic resistance to the current insecticides, our study provides insights on the possibility of ivermectin cross resistance with other insecticides. Our results suggest that detoxification mechanisms mediated by cytochrome P450 enzymes are more important in ivermectin resistance compared to detoxification mediated by efflux pumps.

Declarations

Ethics approval

This work does not involve humans or vertebrates and did not require IRB approbal

Consent for publication

Not applicable

Data availability

All study data is contained within this manuscript and the supplementary material

Competing interests

No competing interests were disclosed

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Author contributions

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Formal analysis: CCh, PN, CK

Funding acquisition: CCh

Investigation: PN, TBD, MM, MW, MMt

Methodology: CCh, MM, MW

Supervision: MM, CCh

Writing - original draft: PN, CK, CCh

Writing - review & editing: all authors contributed, reviewed and approved the last draft.

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Figures

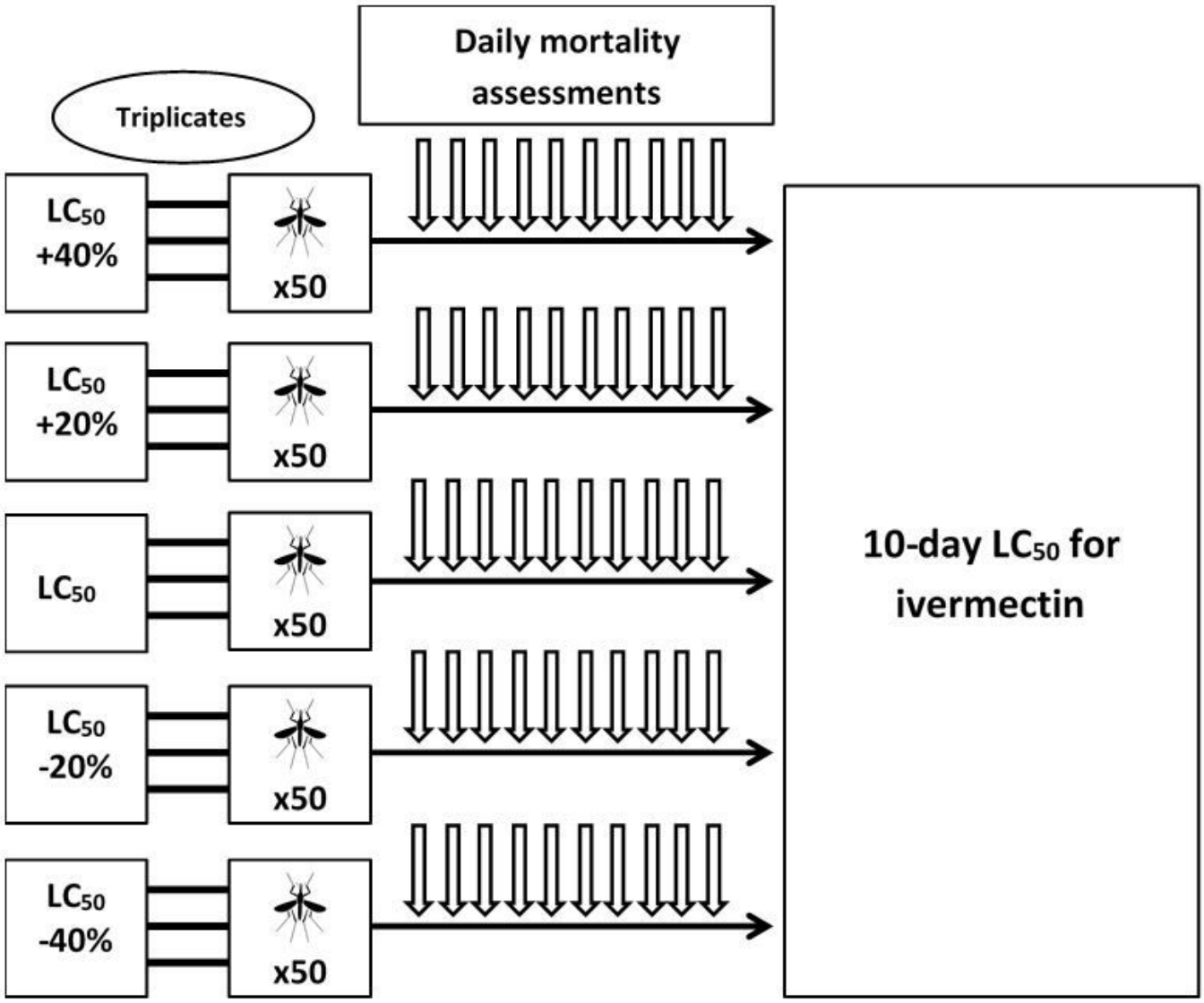


Figure 1

Schematic representation of phase one experiments. Dose-finding study for ivermectin´s 10-day insecticidal concentration LC50 in our Anopheles gambiae s.s. colony. LC50: insecticidal concentration 50

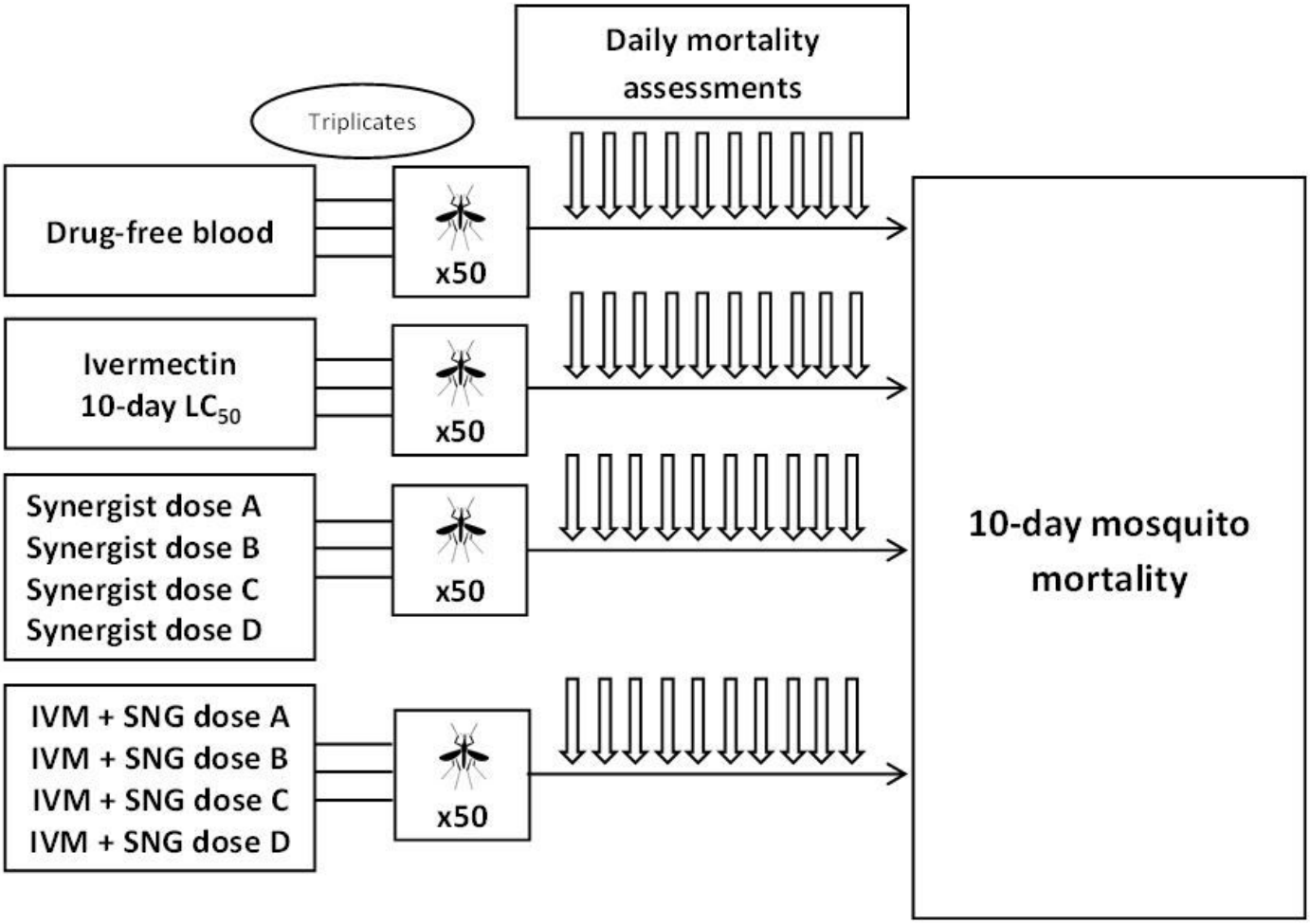
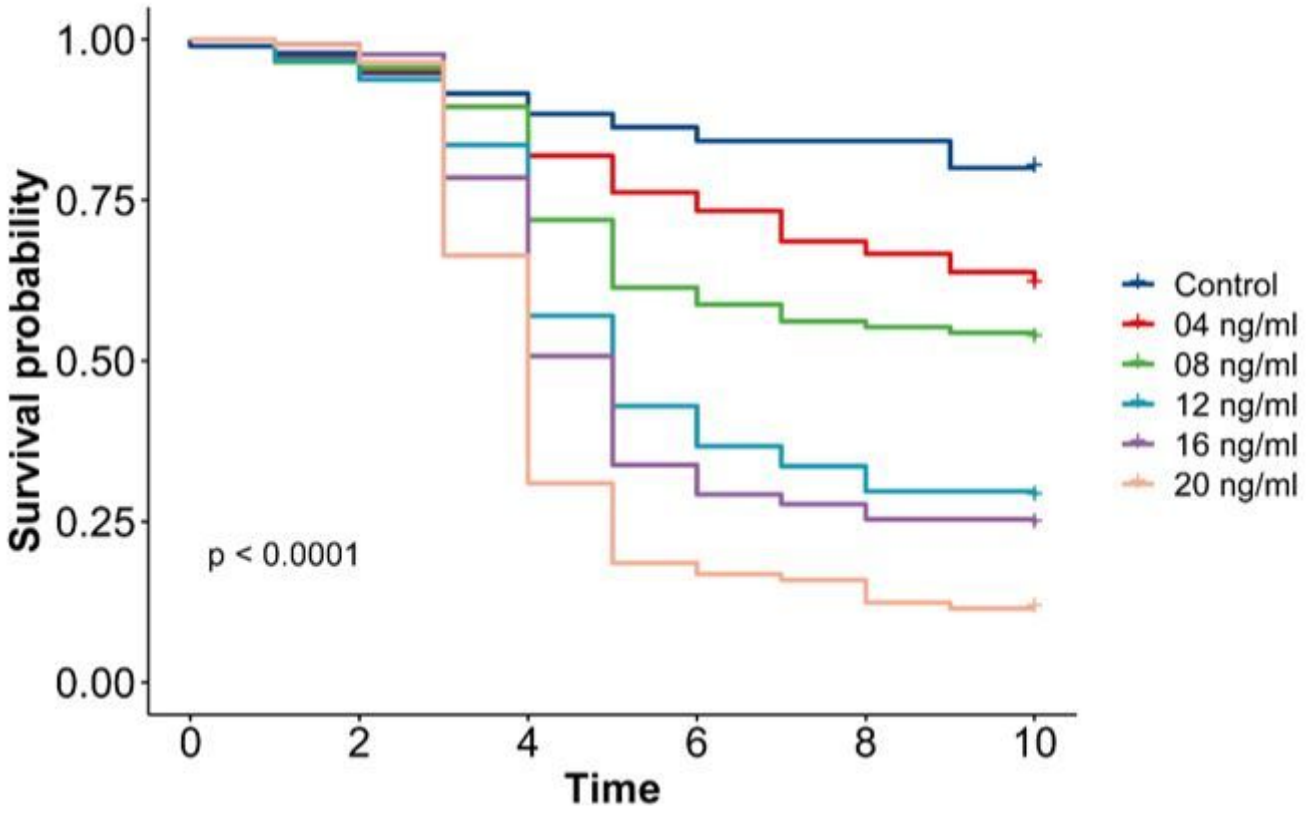


Figure 2

Schematic representation of phase two experiments. Assessment of the 10-day mosquito mortality after feeding on blood containing different concentrations of CYP/P-gp inhibitors and inducers, either alone or in combination with ivermectin at the 10-day IC50 dose determined in phase one.



	Control	04 ng/ml	08 ng/ml	12 ng/ml	16 ng/ml
04 ng/ml	**				
08 ng/ml	***				
12 ng/ml	***	***	***		
16 ng/ml	***	***	***		
20 ng/ml	***	***	***	***	**

Figure 3

Ivermectin induces dose dependent but delayed mortality in An. gambiae. Daily survival probability of An. gambiae mosquitoes after ingesting blood containing different concentration of ivermectin. Table shows pairwise comparisons of survival in different concentrations with significance level indicated by asterisk; $p < 0.01$, ***, $p < 0.001$. The 10-day LC50 is between 8 and 12 ng/ml for this strain in this insectary.

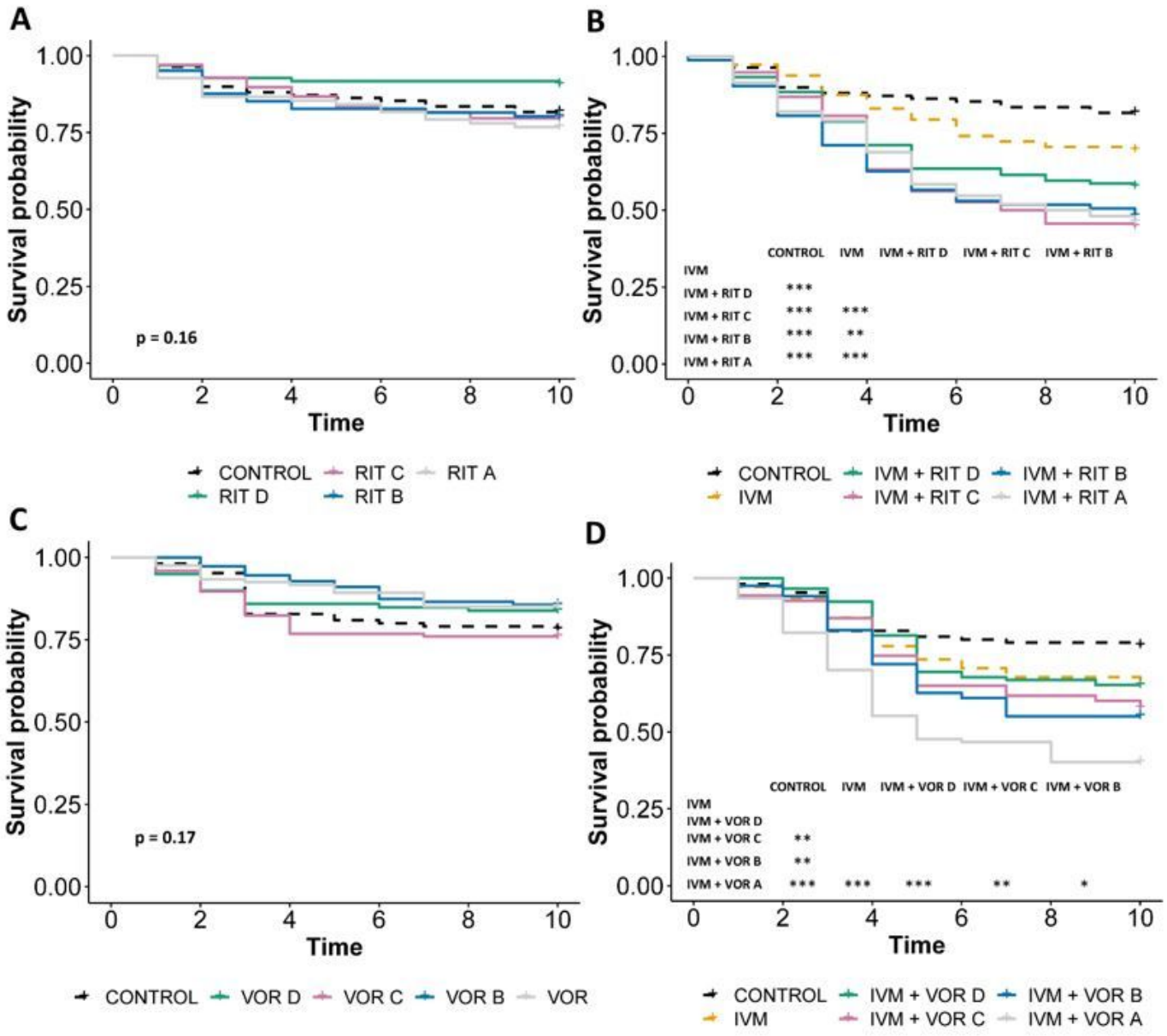


Figure 4

Synergistic effect of dual CYP/ P-gp inhibitors on ivermectin-induced mosquito mortality. Daily survival probability of An. gambiae mosquitoes after imbibing blood containing (A) varied concentrations ritonavir (RIT), (B) ivermectin (IVM) mixed with varied concentrations ritonavir, (C) varied concentrations voriconazole, and (D) ivermectin mixed with varied concentrations of voriconazole. When the overall p-value was less than 0.05 pairwise comparisons was performed and the significance level indicated; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Concentration A > B > C > D.

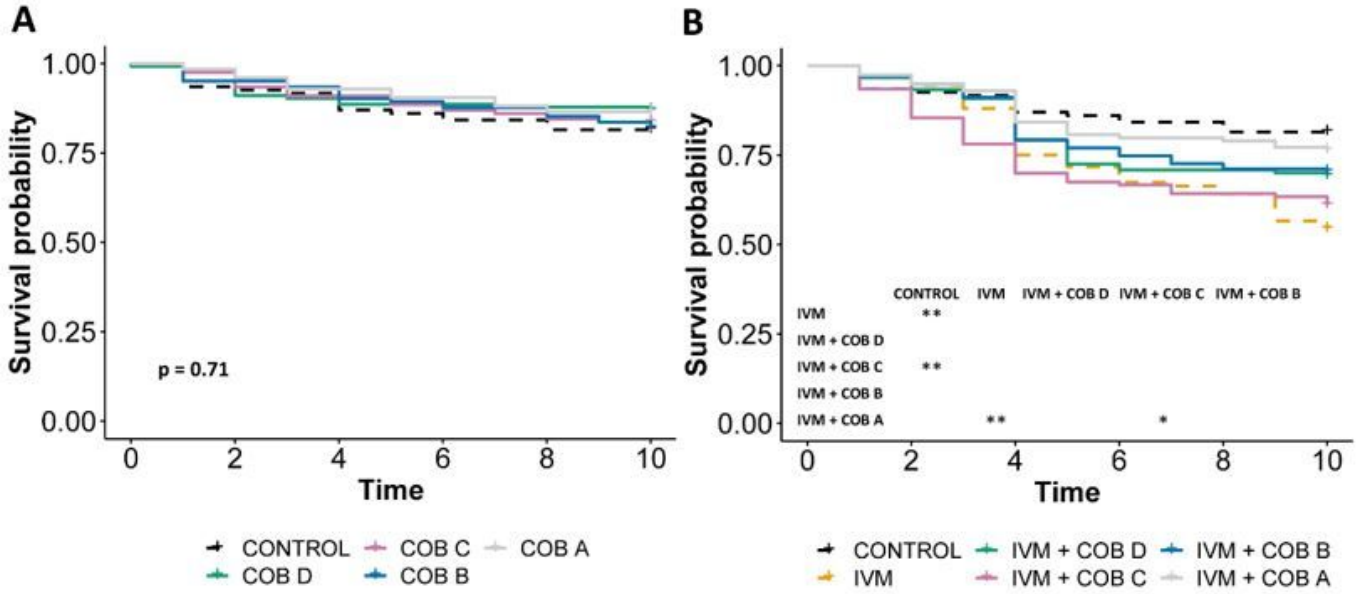


Figure 5

Cobicistat appears to antagonize the ivermectin-induced mosquito mortality. Daily survival probability of An. Gambiae mosquitoes after ingesting blood containing (A) varied concentrations cobicistat COB, (B) ivermectin (IVM) mixed with varied concentrations cobicistat. When the overall p-value was less than 0.05 pairwise comparisons were performed and the significance level indicated; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Concentration A > B > C > D.

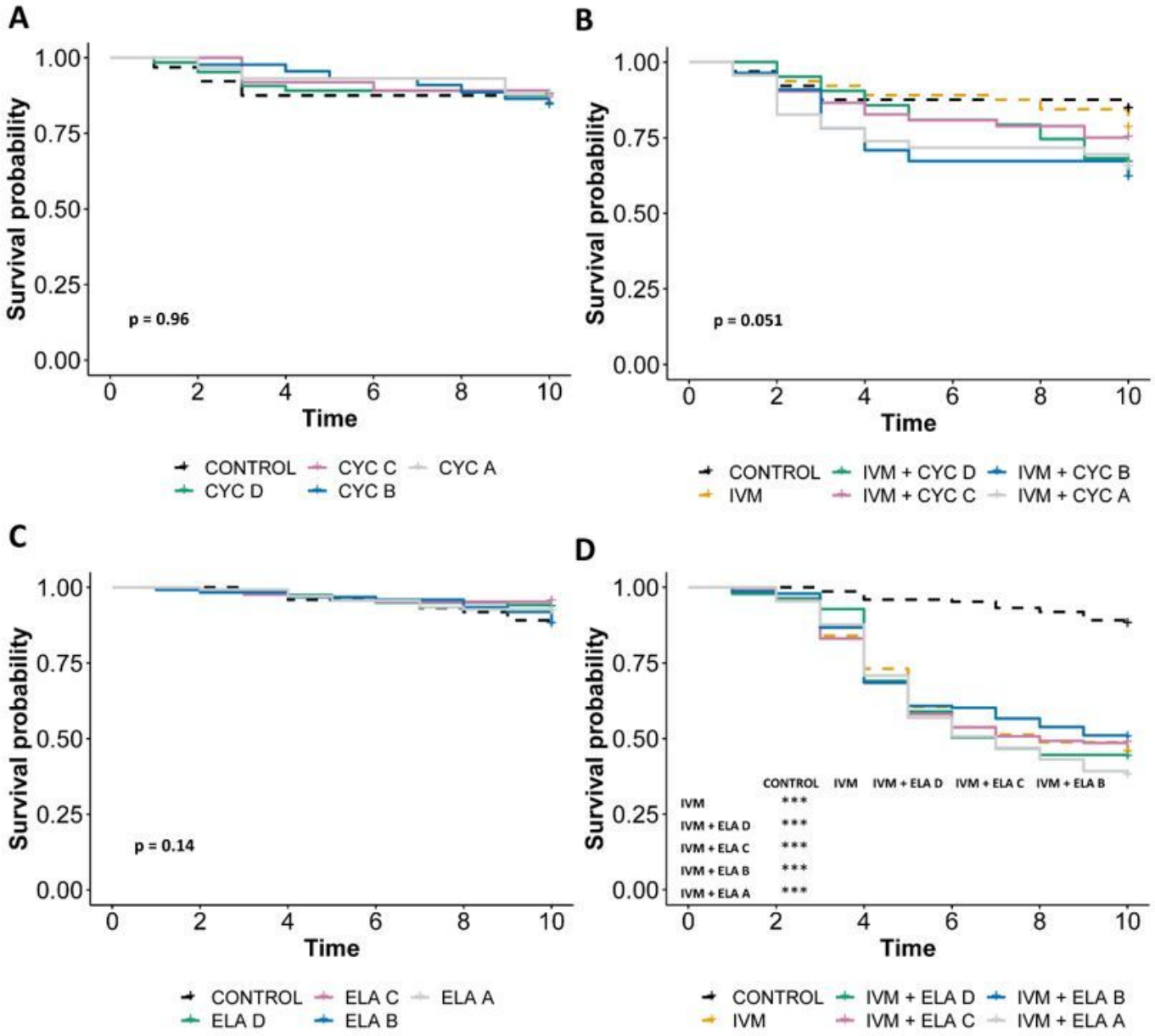


Figure 6

Inhibition of P-gp alone does not affect ivermectin-induced mosquito mortality. Daily survival probability of An. Gambiae mosquitoes after ingesting blood containing (A) varied concentrations cyclosporine A (CYC), (B) ivermectin (IVM) mixed with varied concentrations cyclosporine A (C) varied concentrations elacridar (ELA), (D) ivermectin mixed with varied concentrations elacridar. When the overall p-value was less than 0.05 pairwise comparisons were performed and the significance level indicated; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Concentration A > B > C > D.

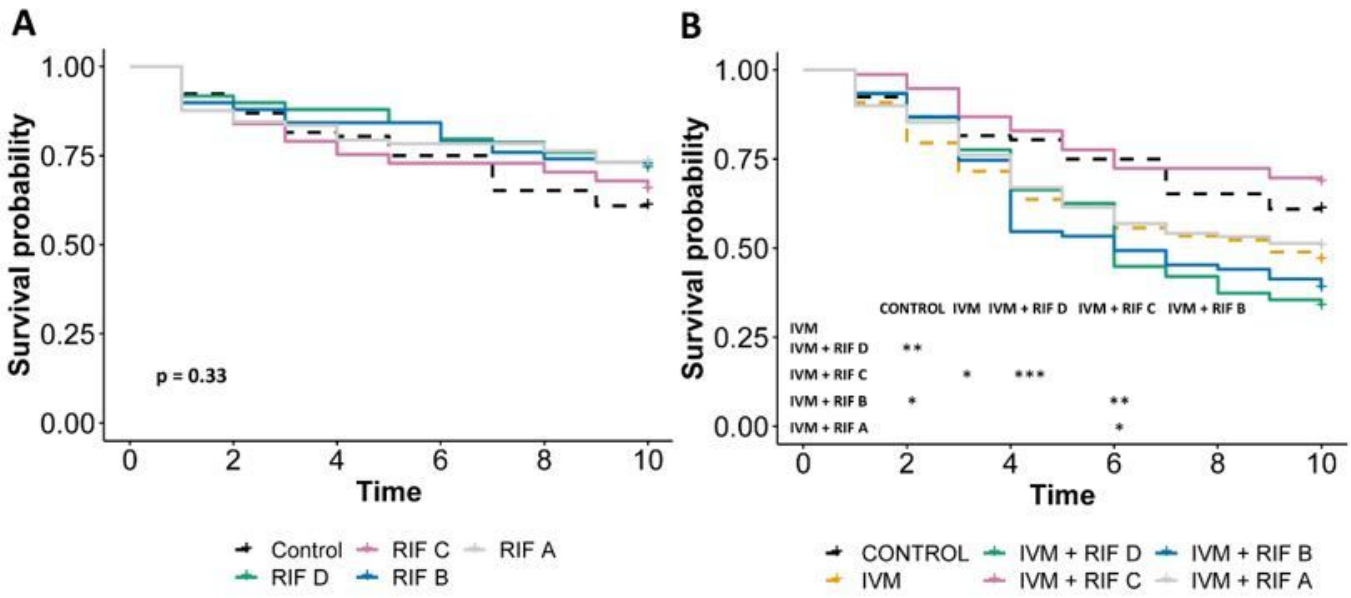


Figure 7

Simultaneous induction of cytochrome P450 and P-gp transporters may confer modest protection from ivermectin-induced mortality. Daily survival probability of An. Gambiae mosquitoes after ingesting blood containing (A) varied concentrations rifampicin (RIF), (B) ivermectin (IVM) mixed with varied concentrations rifampicin. When the overall p-value was less than 0.05 pairwise comparisons were performed and the significance level indicated; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Concentration A > B > C > D.

Supplementary Files

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