Using an antimalarial in mosquitoes overcomes Anopheles and Plasmodium resistance to malaria control strategies

Douglas G. Paton1*, Alexandra S. Probst1, Erica Ma1, Kelsey L. Adams1, W. Robert Shaw1, Naresh Singh1, Selina Bopp1, Sarah K. Volkman1, Domombele F. S. Hien2, Prislaure S. L. Paré2, Rakiswéné S. Yerbanga2, Abdoulaye Diabaté2, Roch K. Dabiré2, Thierry Lefèvre3,4,5, Dyann F. Wirth1, Flaminia Catteruccia1*

1 Department of Immunology and Infectious Diseases, Harvard TH Chan School of Public Health, Boston, United States of America, 2 Institut de Recherche en Sciences de la Santé/Centre Muraz, Bobo-Dioulasso, Burkina Faso, 3 MIVEGEC, IRD, CNRS, University of Montpellier, Montpellier, France, 4 Laboratoire mixte international sur les vecteurs (LAMIVEC T), Bobo Dioulasso, Burkina Faso, 5 Centre de Recherche en Écologie et Évolution de la Santé (CREES), Montpellier, France

* dpaton@hsph.harvard.edu (DGP); fcatter@hsph.harvard.edu (FC)

Abstract

The spread of insecticide resistance in Anopheles mosquitoes and drug resistance in Plasmodium parasites is contributing to a global resurgence of malaria, making the generation of control tools that can overcome these roadblocks an urgent public health priority. We recently showed that the transmission of Plasmodium falciparum parasites can be efficiently blocked when exposing Anopheles gambiae females to antimalarials deposited on a treated surface, with no negative consequences on major components of mosquito fitness. Here, we demonstrate this approach can overcome the hurdles of insecticide resistance in mosquitoes and drug resistant in parasites. We show that the transmission-blocking efficacy of mosquito-targeted antimalarials is maintained when field-derived, insecticide resistant Anopheles are exposed to the potent cytochrome b inhibitor atovaquone, demonstrating that this drug escapes insecticide resistance mechanisms that could potentially interfere with its function. Moreover, this approach prevents transmission of field-derived, artemisinin resistant P.falciparum parasites (Kelch13 C580Y mutant), proving that this strategy could be used to prevent the spread of parasite mutations that induce resistance to front-line antimalarials. Atovaquone is also highly effective at limiting parasite development when ingested by mosquitoes in sugar solutions, including in ongoing infections. These data support the use of mosquito-targeted antimalarials as a promising tool to complement and extend the efficacy of current malaria control interventions.

Author summary

Effective control of malaria is hampered by resistance to vector-targeted insecticides and parasite-targeted drugs. This situation is exacerbated by a critical lack of chemical diversity in both interventions and, as such, new interventions are urgently needed. Recent
laboratory studies have shown that an alternative approach based on treating Anopheles mosquitoes directly with antimalarial compounds can make mosquitoes incapable of transmitting the Plasmodium parasites that cause malaria. While promising, showing that mosquito-targeted antimalarials remain effective against wild parasites and mosquitoes, including drug- and insecticide-resistant populations in malaria-endemic countries, is crucial to the future viability of this approach. In this study, carried out in the US and Burkina Faso, we show that insecticide-resistance mechanisms found in highly resistant, natural Anopheles mosquito populations do not interfere with the transmission blocking activity of tarsal exposure to the antimalarial atovaquone, and that mosquito-targeted antimalarial exposure can block transmission of parasites resistant to the main therapeutic antimalarial drug artemisinin. By combining lab, and field-based studies in this way we have demonstrated that this novel approach can be effective in areas where conventional control measures are no longer as effective.

Introduction

Human malaria, a parasitic disease caused by unicellular eukaryotic Plasmodium parasites and spread through the bite of Anopheles mosquitoes, remains a substantial cause of global morbidity and mortality [1]. Malaria control programs rely on both preventative measures focused on mosquito control and on therapeutic measures based on the use of antimalarial drugs. Mosquito-targeted interventions are the most effective tools at reducing the transmission of Plasmodium parasites, with long-lasting insecticide-impregnated nets (LLINs) and indoor residuals spraying (IRS) as primary methods for malaria prevention. LLINs alone are predicted to have contributed to 68% of malaria cases averted between 2000 and 2015 [2]. Alongside these preventative interventions, artemisinin combination therapies (ACT) have been the cornerstone of human malaria treatment since their widespread introduction at the beginning of this century [3,4] and have contributed substantially to the reduction in malaria mortality and morbidity observed since then [2].

Despite sizeable investment, malaria control and elimination efforts are, however, faltering due to reduced operational effectiveness of these key control tools, largely caused by mosquito resistance to insecticides and parasite resistance to drugs [5–8]. In the malaria hyperendemic regions of southern Mali and southwest Burkina Faso, for example, resistance to pyrethroids is extreme [9], driven by multifactorial and synergistic resistance mechanisms including enhanced metabolic detoxification through upregulated cytochrome P450s (metabolic resistance), and reduced tarsal uptake through cuticular thickening (cuticular resistance) [10–13]. Similarly, the emergence and spread of artemisinin resistance to sub-Saharan Africa—a region where as many as 93% of annual malaria deaths occur [1]—is a major concern. Until recently, resistance to these first line antimalarials was limited geographically to the Greater Mekong Subregion (GMS), however de novo mutations in PfKelch13 associated with in vitro resistance have now been detected in Uganda, Tanzania, and Rwanda [8,14–19]. Of further concern is the recent invasion and spread of the Asian vector Anopheles stephensi to the horn of Africa [20], as this mosquito species is highly competent for the transmission of P. falciparum parasites endemic to the GMS [21] and therefore, invasive populations may facilitate the spread of parasites harboring artemisinin resistance mutations from Asia to Africa. Besides insecticide resistance, an additional hurdle to malaria elimination is represented by residual malaria. Defined as malaria transmission in the presence of universal effective LLIN coverage, residual malaria is driven by mosquitoes that exhibit outdoor or daytime biting preferences and is a
considerable hurdle to malaria eradication efforts [22]. Increased focus on residual malaria has stimulated interest in the use of attractive toxic, or targeted [23], sugar baits (ATSBs) to attract and kill adult mosquitoes irrespective of blood-feeding behavior, which in field trials have shown some promise as a tool for suppressing vector populations [24,25].

Thus, a control strategy that prevents insecticide resistant Anopheles populations from transmitting malaria parasites, including parasite strains carrying drug resistance mutations, regardless of mosquito feeding behavior and without imposing strong selective pressure on mosquitoes could overcome the limitations of current mosquito-targeted interventions. In an effort to generate such a strategy, we recently demonstrated that transmission of P. falciparum parasites can be prevented when Anopheles gambiae are exposed to the antimalarial atovaquone (ATQ) through direct contact—analogous to the mode of insecticide exposure on LLINs or IRS [26]. Contact with ATQ-coated surfaces completely abrogated parasite development when it occurred around the time of infection (between 24 hours (h) before and 12 h post feeding on P. falciparum-infected blood), preventing onward transmission of the parasite. Importantly, mathematical models based on these results showed that integrating antimalarial ingredients into existing mosquito-targeted interventions could considerably reduce malaria transmission in areas of widespread insecticide resistance, empowering our best malaria prevention tools [26].

Here we show that targeting P. falciparum with antimalarials during its development in the Anopheles female circumvents the hurdles of insecticide and drug resistance, providing a critical addition to the malaria elimination toolkit. Parasite development is substantially reduced when wild, as well as recently lab adapted Anopheles coluzzii (a sibling species of An. gambiae) that are highly resistant to pyrethroids are exposed to ATQ prior to feeding on blood taken from P. falciparum-infected donors in Burkina Faso. ATQ is also fully active against field-derived P. falciparum parasites from Cambodia that are resistant to artemisinin. When using distinct drug targets in humans and mosquitoes, this method is therefore capable of both overcoming insecticide resistance mechanisms and stopping transmission of parasite mutations that confer resistance to frontline antimalarials. Finally, we show that delivering ATQ via sugar solutions causes a striking reduction in both parasite numbers and growth, proving that antimalarials could also be incorporated into interventions that target outdoor malaria transmission, such as ATSB. Targeting Plasmodium parasites in the mosquito vector is therefore a promising strategy that circumvents key limitations of current malaria control and preventative interventions.

Results

Exposure to ATQ substantially reduces infection with field P. falciparum isolates in insecticide resistant An. coluzzii

To determine whether antimalarial exposure can maintain efficacy in insecticide-resistant mosquitoes, we took An. coluzzii collected as pupae from larval breeding sites in Bama, Burkina Faso and reared them to adults at the IRSS, Burkina Faso. Adult mosquitoes were infected using P. falciparum gametocyte positive blood obtained from a malaria infected human donor on the day of infection. The An. coluzzii mosquitoes endemic to this part of Burkina Faso—hereafter named AcVK5—are highly resistant to pyrethroids [9,11,27]. AcVK5 mosquitoes were exposed to ATQ for 6 minutes (min) at two concentrations (100 μmol- or 1 mmol/m²) or a mock-treated blank control surface prior to feeding on infectious blood samples. Infection outcomes were assayed at 7 days (d) post infectious blood meal (pIBM) by dissection of the mosquito midgut to determine the prevalence and intensity of parasite oocysts (Fig 1A). Control-exposed AcVK5 females were robustly infected with 81.3% harboring at least one P.
falciparum oocyst, and median infection intensity in infected females of 19 oocysts per midgut.

In contrast, AcVK5 mosquitoes exposed to either dose of ATQ had significantly reduced *P. falciparum* infection both in terms of prevalence and intensity (Fig 1B). At the highest
concentration, we observed a 99% overall reduction in infection relative to the control (84.6% reduction in prevalence and 94.8% reduction in median intensity), while at the lower dose inhibition of infection reached 96% overall (65.9% reduction in prevalence and 89.5% reduction in median intensity). These results demonstrate that direct tarsal antimalarial exposure, for instance incorporating antimalarials in LLINs and IRS, can effectively block transmission of circulating west African *P. falciparum* parasites in highly insecticide resistant endemic *Anopheles* mosquitoes. Although our previous findings had shown that both ATQ doses tested above are capable of complete inhibition of parasite transmission using the combination of standard *P. falciparum* (NF54) and insecticide-susceptible *Anopheles* (G3) populations [26], our results confirm that parasite development can be considerably impaired when exposing mosquitoes to antimalarials.

The observation of few parasites surviving exposure may indicate that parasite or mosquito factors in our Burkinabe populations could be reducing the efficacy of ATQ in this assay, potentially including interference from extant insecticide resistance mechanisms in AcVK5 mosquitoes, or reduced ATQ drug sensitivity in *P. falciparum* in this region. To test these possibilities, we initially assayed the efficacy of ATQ against insecticide resistant, Burkina Faso-derived *An. coluzzii* (hereafter Bama-R) with the lab standard *P. falciparum* strain NF54. Reared under pyrethroid selective pressure under otherwise standard laboratory conditions, Bama-R have maintained the parental trait of 100% resistance to permethrin at the WHO discriminating concentration (DC, 696 μmol/m²) and exhibit appreciable acute survival at five times this dose (S1B Fig). Bama-R mosquitoes are segregating for the *kdr* mutation in *para* conferring target site resistance (S1C Fig), but constitutively overexpress CytochromeP450 genes associated with both metabolic resistance through enhanced small molecule detoxification (S1D Fig [28]), and cuticular thickening [27] Bama-R females were exposed to the maximal effective concentration for tarsal ATQ (EC₉₉, 100 μmol/m², 6 min [26]) or to a vehicle control immediately preceding infection, and parasite prevalence and intensity were determined at 7 d pIBM. While control females were highly infected, with a median of 12 oocysts per infected midgut and 81.25% overall prevalence of infection, no oocysts were observed in females exposed to ATQ, suggesting that insecticide-resistance mechanisms found in highly resistant, natural *Anopheles* populations do not interfere with the transmission blocking activity of ATQ (Fig 1C).

Next, we established an *in vitro* *P. falciparum* culture from a polyclonal isolate (P5) collected from a gametocytemic donor from Burkina Faso [29]) and infected the laboratory standard, insecticide susceptible mosquito strain An. *gambiae* (G3). P5 development was 100% suppressed in females treated with the EC₉₉ of ATQ (Fig 1D) such that zero oocysts were observed in midguts, compared to heavy infections—both in terms of infection intensity (median 59.5 oocysts per midgut) and infection prevalence (95.7%)—in controls. Delivery of ATQ to mosquitoes is therefore fully effective against field-derived *P. falciparum* isolates currently circulating in West Africa.

**ATQ prevents the transmission of an artemisinin-resistant *P. falciparum* isolate from the GMS**

Given the results obtained with field-derived parasites from Africa, we next tested the ability of ATQ to kill artemisinin resistant parasites from the GMS, where mutations conferring artemisinin resistance occur in a high proportion of *P. falciparum* isolates, constituting a major public health threat. We reasoned that these experiments would also allow us to test the concept of directly targeting drug resistant *P. falciparum* during mosquito development, removing resistance mutations from the parasite population. To this
end, we used a Cambodian *P. falciparum* patient clone (KH001_029 [5], hereafter ART29) carrying the C580Y mutation in PfK13 conferring resistance to artemisinin (Fig 2A). We used the major Asian malaria vector *An. stephensi* for these experiments as initial tests with *An. gambiae* did not produce appreciable infections (S2 Fig). ART29 generated robust infections in control, mock-exposed *An. stephensi*, (median 16 oocysts per midgut, 100% prevalence of infection). Conversely, no oocysts were detected in females exposed to ATQ prior to infection (100 μmol/m², 6 min) (Fig 2B). These data show that mosquito exposure to antimalarials, such as by incorporation in bed nets, indoor residual sprays (or other contact methods such as eaves tubes [30], could be an effective strategy for reducing the spread of artemisinin resistance both within and between malaria endemic areas, including sub-Saharan Africa.

Fig 2. Exposing *An. stephensi* to ATQ blocks an artemisinin-resistant *P. falciparum* patient isolate from Cambodia. (a) Experimental scheme. (b) Transmission of artemisinin resistant *P. falciparum* (ART29) is completely blocked when *An. stephensi* females (Anst-S) are exposed to ATQ for 6 min to the maximal effective concentration (EC₉₀) for insecticide-susceptible mosquitoes. In ATQ exposed mosquitoes, prevalence (indicated by pie charts) was zero despite robust infection in mock-exposed controls (Chi², n = 158, df = 1, X² = 156, p < 0.0001). Median lines and values are indicated, “n” indicates the number of independent samples. To isolate Oocyst Prevalence and Oocyst Intensity, midgut samples with zero oocysts have been excluded from intensity analysis. Statistical significance is indicated where relevant as follows: ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001; **** = p < 0.0001.

https://doi.org/10.1371/journal.ppat.1010609.g002
ATQ exposure during an ongoing infection delays oocyst growth and decreases sporozoite prevalence

In the field, mosquitoes that contact an antimalarial compound through mosquito-targeted interventions may harbor parasites from a previous blood meal that have already traversed the midgut lumen and formed oocysts. We therefore investigated the effects of ATQ on parasites in which oocyst development is already underway, exposing G3 mosquitoes 6d pIBM (NF54, Fig 3A). In contrast to females exposed before infection, ATQ had no effect on the prevalence or intensity of infection, as measured at 10d pIBM, suggesting ATQ’s inhibitory effect manifests differently in oocysts compared to zygote or ookinetes (Fig 3B). However, when we measured the size of the developing oocysts, we observed a significant, 45% decrease in the mean oocyst cross-sectional area (Fig 3C). Oocyst size is a good proxy for rate of growth [31] and as such, when we sampled mosquitoes at a later time point when sporozoite invasion of salivary glands has already occurred (14 d pIBM), we observed a 33% reduction in the prevalence of sporozoites in the salivary glands of ATQ-treated females (Fig 3D). Similar results were obtained when ATQ exposure instead occurred at 3 d pIBM (S3 Fig). Taken together, these results suggest that ATQ exposure after oocyst formation has a partial cytostatic effect on *P. falciparum.*

Fig 3. Sporozoite prevalence is significantly reduced after tarsal ATQ exposure during oocyst development when *An. gambiae* (G3) are infected with *P. falciparum* (NF54). (a) Experimental scheme. (b) There was no effect of 6 d pIBM ATQ exposure on either prevalence (indicated by pie charts) or intensity (indicated by points) of infection determined at 10 d pIBM. Prevalence: Chi$^2$, n = 136, df = 1, $X^2$ = 0.733, p = 0.3919, intensity: Mann-Whitney, n = 114, df = 1, $U$ = 1482, p = 0.4335. (c) ATQ exposure at 6 d pIBM significantly reduced the median cross-sectional area of oocysts relative to control (n = 114, df = 1, U = 531, p < 0.0001). (d) The prevalence, but not the median intensity of sporozoites in salivary glands was significantly reduced in mosquitoes exposed to ATQ at 6 d pIBM (Chi$^2$, n = 125, df = 1, $X^2$ = 7.190, p = 0.0073). Median lines and values are indicated, "n" indicates the number of independent samples. To isolate Oocyst Prevalence and Oocyst Intensity, midgut samples with zero oocysts have been excluded from intensity analysis. Statistical significance is indicated where relevant as follows: ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

https://doi.org/10.1371/journal.ppat.1010609.g003
Ingestion of an ATQ-glucose solution blocks the establishment of *P. falciparum* infection

To determine whether anti-parasitic compounds in sugar could suppress *Plasmodium* development in the mosquito, we began by testing the efficacy of mosquito ATQ-glucose ingestion against the transmission of *P. falciparum* parasites isolated from gametocyteemic donor blood samples collected from gametocyte carriers in Nasso, near Bobo Dioulasso, Burkina Faso. Adult female *An. gambiae*, collected as pupae from larval breeding sites, were denied sugar for 24 h, then given access to an ATQ-treated sugar solution (100 μM ATQ/0.5% v/v DMSO/10% w/v glucose) *ad libitum* for the 24 h preceding infection (Fig 4A). We observed a striking, 85% reduction in the prevalence of wild *P. falciparum* infection in female mosquitoes that had access to ATQ-glucose prior to infection (Fig 4B). Importantly, median mosquito survival between control and ATQ treatment groups was not significantly different (S4 Fig), confirming previous findings that atovaquone is not toxic to mosquitoes at parasiticidal concentrations [26]. This implies that other *Plasmodium*-specific inhibitors would therefore not impose selective pressures leading to resistance mechanisms in the mosquito.

Using the same conditions with *in vitro* cultured *P. falciparum* (NF54) and lab-adapted *An. gambiae* (G3) resulted in a remarkably similar infection outcome, with a 92.5% reduction in oocyst prevalence in female *An. gambiae* given access to ATQ-glucose solution relative to controls (Fig 4C). When ATQ concentration was reduced to 10- and 100-fold ATQ dilutions, we observed progressively reduced, dose dependent effects on prevalence (Fig 4D).

**ATQ ingestion during an ongoing *P. falciparum* infection impairs sporogony**

As mosquitoes may often visit a sugar bait after acquiring an infectious blood meal, we also investigated the impact of ATQ ingestion on ongoing *P. falciparum* (NF54) infections, providing ATQ-treated sugar to G3 females continuously from 2 d pIBM to the end of the experiment at 14 d pIBM. By 2d pIBM, ookinetes have escaped the midgut lumen and formed oocysts on the midgut basal lamina (Fig 5A). As based on our previous results (Fig 3) we expected a possible cytostatic effect on oocyst growth, we performed a sampling time course to capture oocyst development through mid- to late sporogony under continual ATQ ingestion (7d, 10d and 14 d pIBM). We also counted salivary gland sporozoites, the end point of parasite development in the mosquito, at 14 d pIBM. In agreement with our previous results, we observed no change in oocyst prevalence and intensity because of ATQ ingestion (S5 Fig). However, we observed an 80.8% decrease in oocyst cross-sectional area relative to controls at 7 d pIBM, which persisted at later time points (89% and 76.3% decreases at 10- and 14 d pIBM, respectively) (Fig 5B). By 14 d pIBM, ATQ-exposed oocysts had a similar size to 7 d pIBM control oocysts, suggesting a remarkable suppression of growth. Inspection of DAPI-stained infected midguts revealed a stark decrease in the number of nuclear foci, with a single, diffuse DNA signal compared to many condensed foci in oocysts in controls (Fig 5C). Strikingly, we detected no sporozoites in the salivary glands of mosquitoes given access to ATQ-glucose 14 d pIBM, despite robust infection in controls (Fig 5D). Combined, these data point to a strong suppression of oocyst growth, DNA replication and sporozoite differentiation after ATQ ingestion, suggesting that besides preventing new mosquito infection, this delivery method would be extremely effective at curbing ongoing infections and transmission.

**Discussion**

In this study we demonstrate the strong potential of incorporating antimalarials into LLINs, IRS and ATSBs to stop transmission of endemic parasites by insecticide-resistant *Anopheles*. 
Our proof of principle compound ATQ was very effective at killing a polyclonal, field-derived parasite isolate from sub-Saharan Africa, showing that an antimalarial-based LLIN or IRS could suppress transmission even in areas of malaria hyperendemicity, where pyrethroid
resistance in *Anopheles* mosquitoes is exceptionally high, and multiple parasite haplotypes coexists [32,33].

Tarsal ATQ exposure against wild *An. coluzzii* (AcVK5) collected as pupae from breeding sites was able to strongly suppress the transmission of *P. falciparum* isolates circulating in children. Interestingly, in these infections, carried out in Burkina Faso, we observed a small number of “breakthrough” oocysts at ATQ doses that are non-permissive in tests using, respectively, lab-adapted and susceptible NF54 parasites and G3 mosquitoes [26]. We reasoned that this marginal reduction in efficacy could be due to extant insecticide resistance circulating...
in wild *An. gambiae* s.l populations in Burkina Faso, including the wild AcVK5 mosquitoes used for these experiments. While clearly target-site resistance mechanisms are unlikely to impact the activity of antimalarials like ATQ, both cuticular and metabolic resistance could potentially interfere with function by limiting compound uptake and stability, respectively. The CYP450 monoxygenases associated with pyrethroid metabolic detoxification in *An. gambiae* s.l. have been shown to confer some degree of resistance to a structurally and functionally diverse array of compounds including the insecticide DDT, the juvenile hormone agonist pyriproxyfen [34], and several arthropod mitochondrial complex I inhibitors including the otherwise promising insecticide fenpyroximate [35]. Similarly, cuticular resistance, where the waxy exocuticle of the mosquito has thickened as an adaptation to insecticide pressure [11], could slow or eliminate uptake of other small molecules. However, tarsal ATQ (at the EC\textsubscript{99}, 100 \(\mu\text{mol/m}^2\) for 6 min) completely abrogated parasite infection in Bama-R mosquitoes, which are derived from the parental AcVK5 population and exhibit a similar, high level of insecticide resistance combining the additive effects of metabolic and cuticular resistance components (S1 Fig, [27]). This observation suggests that mosquito pyrethroid resistance status, or other vector factors, did not affect tarsal antimalarial efficacy in our experiments.

Similarly, in the reciprocal experiment, transmission of the culture-adapted Burkinabe *P. falciparum* isolate P5 was blocked in an insecticide susceptible *An. gambiae* lab strain (G3), again indicating that parasite factors are likely not responsible for the observed reduction in efficacy. Clinically-induced resistance to ATQ in *P. falciparum* is associated with mutation at position 268 of the mitochondrial gene cytochrome \(b\) [36], while \textit{in vitro} selection typically results in mutation elsewhere in the gene [37–39]. ATQ is not in clinical use in sub-Saharan Africa, so it is unlikely that ATQ resistance-conferring mutations are circulating in Burkina Faso at any appreciable frequency. However, while certain ATQ-resistance mutations induce a transmission defect in both *Plasmodium berghei* and *P. falciparum*—resulting in failure to establish infection in mosquitoes [38] there are contradictory findings in the literature [39], and it has been shown that naturally occurring ATQ resistance-conferring mutations can persist at ultra-low frequency in parasite populations through mitochondrial heteroplasmy [40].

Thus while the Burkinabe isolate (P5) used in our lab-based studies was susceptible to ATQ \textit{in vitro}, and Sanger sequencing of cytochrome \(b\) from both P5 asexual blood stages and oocysts showed that these parasites are wild-type, we cannot rule out the possibility that either cryptic parasite factors, lost during the short P5 laboratory adaptation period, or specific vector-parasite interactions in wild populations are responsible for the observed reduction in ATQ efficacy in our Burkina-based experiments. The observed decrease in *P. falciparum* numbers remains extremely significant (above 96% total inhibition at 100 \(\mu\text{mol/m}^2\)). Taken together, these data demonstrate that mosquito-targeted antimalarial exposure can bypass currently circulating insecticide resistance mechanisms, maintaining activity even in areas where conventional insecticides have ceased to be effective—an essential trait of any new mosquito-targeted tool.

ATQ exposure also killed artemisinin-resistant *P. falciparum* parasite ART29 in *An. stephensi*. \textit{De novo} PfK13 mutations associated with \textit{in vitro} resistance, already highly prevalent in Cambodia and other part of the GMS, have now been detected in Uganda, Tanzania and Rwanda [14–19] and the recent invasion and spread of *An. stephensi* to the horn of Africa [20] may facilitate the spread of these parasites. Widespread artemisinin resistance in Africa, a region where malaria prevention is already challenging due to insecticide resistance, would be a major public health concern. Although our results were somewhat expected given ART29 parasites are ATQ-sensitive \textit{in vitro} during asexual development [41], they are an important proof of concept, both that drug-based mosquito-targeted interventions could be developed to specifically contain and eliminate parasite haplotypes conferring resistance to human
antimalarial therapeutics, and that tarsal uptake of antimalarials function similarly in *An. stepheni*. Antimalarial pressure directed at different drug targets in the human and mosquito life stages could effectively suppress the spread of resistance mutations selected in either host and to either target, allowing the possibility for close integration of human- and mosquito-targeted antimalarial interventions in the future. Importantly, mosquito-targeted antimalarials attack the parasite during an extreme population bottleneck and in a non-cyclical stage in its life cycle [42–44], reducing the probability of selection of both extant and *de novo* resistance mutations during mosquito-stage drug challenge compared to treatment during the human asexual cycle. Nevertheless, even the possibility for selection of resistance during parasite sporogonic development means, as a fundamental principle, any mosquito-targeted antimalarial compound integrated into LLIN or IRS must not share a mode of action with current human antimalarial therapeutics. Thus, despite its efficacy in our studies, ATQ could not be responsibly incorporated into a mosquito-targeted intervention due to its use as a human prophylactic and therapeutic drug [45], making identification of additional active compounds in diverse mode of action classes a priority. Crucial to this effort will be further studies to understand the kinetics of small molecule absorption, distribution, metabolism, and excretion (ADME) in mosquitoes.

Ingestion of sugar solutions containing ATQ before *P. falciparum* infection blocked transmission in both field-derived and *in vitro* cultured *P. falciparum*. Such close agreement between these experiments, carried out at different sites with different parasites and mosquitoes, is a testament to the promise of this strategy, demonstrating its high effectiveness in spite of the inherent variability of mosquito sugar feeding behavior [46]. Furthermore, continued mosquito access to ATQ-sugar post infection reduced oocyst growth resulting in absence of salivary gland sporozoites. These results support the use of antimalarials in ATSB-like strategies to reduce residual malaria transmission carried out by mosquitoes that predominantly rest and feed outside and thereby avoid both LLINs and IRS. While current proposed ATSB designs rely on insecticidal ingredients [24,25,47], the use of antimalarials in sugar baits could act as a more specific and environmentally benign paradigm for this promising intervention should suitable antimalarial ingredients be identified [48–50]. Future studies to assay mosquito choice, repellency behavior, and total drug ingestion when both treated and untreated sugar sources are presented will be important to ensure any end product can compete with natural sugar-nectar sources.

ATQ uptake via tarsal contact at 3- or 6-days post infection also significantly reduced oocyst growth, resulting in an appreciable reduction in the proportion of salivary gland sporozoite-positive mosquitoes. *P. falciparum* is therefore also vulnerable to inhibition through tarsal exposure during the oocyst stage, which is by far the longest developmental stage in the mosquito, taking between 7–10 days depending on the frequency of blood feeding [31], and thus is the parasite life stage most likely to encounter a mosquito-targeted intervention. These data closely match studies in *P. berghei* from the mid-1990s showing that presence of ATQ in a second blood meal during oocyst development reduced oocysts size and salivary gland sporozoite burden. Interestingly, in contrast to our findings, the authors observed a reduction in oocyst numbers after 2nd blood meal/ATQ ingestion. Whether this reduction is due to biological differences between *P. falciparum* and *P. berghei*, interactions between drug kinetics and blood digestion, or another effect would be interesting to investigate. Nevertheless, the ability to stall or kill oocysts and sporozoites is a highly desirable quality for mosquito-targeted antimalarials.

ATQ targets the ubiquinol oxidation (Qo) site of cytochrome b [51], a key element of the mitochondrial electron transport chain (mtETC) which has dual roles in both ATP generation through oxidative phosphorylation and DNA replication through ubiquinone-mediated redox
of dihydroorotate dehydrogenase [52]. Thus, inhibition of either DNA replication or ATP production, or both, could explain the cytostatic effect observed here. Consistent with our findings, previous studies have shown that disruption of ATP production through knock-out of components of the tricarboxylic acid cycle in *P. falciparum* [53] and mtETC in *P. berghei* [54–56] can cause oocyst arrest. Moreover, chemical inhibition of *P. vivax* DNA replication during sporogony was also sporontocidal [57], suggesting mitochondrial inhibitors could also be utilized to prevent transmission of these widespread human malaria parasites. Identifying the specific mechanism by which ATQ and other mtETC inhibitors affect sporogonic development in *Plasmodium* is an interesting area for further study.

Although at an early stage, mosquito-targeted antimalarials have the potential to be an effective element to drive malaria incidence down. To this end, identifying more compounds with strong antiparasitic activity during the mosquito stages of *P. falciparum* development—and in particular compounds with sporogony-specific activity—will be a crucial next step, and one that should leverage the extensive libraries of known antimalarials. Indeed, one of the key strengths of this approach is the potential to exploit and repurpose compounds that are otherwise unsuitable for human therapeutic use, whether due to toxicity, poor bioavailability, poor kinetics, or other limiting factors. By integrating human and mosquito-based interventions, this strategy will extend and protect the efficacy of human therapeutics and vector control strategies, giving malaria control efforts renewed vigor.

**Materials and methods**

**Ethics statement**

Procedures for collecting *P. falciparum* gametocyte-positive blood were approved via the IRSS IRB (project registration number: 031-2016/CEIRES), the Harvard TH Chan School of Public Health IRB (IRB19-2043) and Harvard University Provost’s Office.

**Mosquito lines, insecticide resistance selection and husbandry**

*Anopheles* spp. mosquito populations used in this study were: 1) wild *A. coluzzii* captured as pupae from breeding sites, described below. 2) Laboratory-reared *A. gambiae* obtained from an outbred colony established in 2019 and repeatedly replenished with F1 from wild-caught females collected in Soumouso (11°23′14″N, 4°24′42″W). 3) *A. gambiae* G3 (“G3”), a highly lab-adapted, insecticide-susceptible strain competent for *P. falciparum* of African origin. 4) *A. stephensi* (Anst-S), a similarly lab-adapted, insecticide-susceptible strain competent for *P. falciparum* of both African and Asian origin received as a gift from The Institute of Molecular Medicine, University of Lisbon, Portugal. 5) *A. coluzzii*. Bama-R (“Bama-R”) a colony established through hybridization of the F1 progeny of female *A. coluzzii* collected from Vallee du Kou, Burkina Faso, with our G3 colony. Since establishment, Bama-R has been kept under frequent permethrin selection pressure and exhibits a consistent pyrethroid resistance phenotype.

At the time of this study, (F17-20) Bama-R females where highly resistant to pyrethroids, exhibiting 97% survival in standard WHO insecticide resistance assays—briefly, 1 h exposure to the WHO discriminating concentration (DC, 275 mg/m²) of permethrin-impregnated papers with mortality scored at 24 h post-exposure—and 43% survival at 5x the DC. Except for selection of resistance in Bama-R, all mosquito colonies were maintained identically at 26 °C ± 2 °C and 80% ± 10% relative humidity (RH). Larvae were cultured in 2-liter (l) catering pans in 500 ml distilled water (dH₂O) under an optimized density and feeding regimen. At the onset of pupation, pupae were separated from larvae using a vacuum aspirator, collected in dH₂O, and placed in a 30x30x30 cm cage (Bugdorm, Megaview Science Co, Ltd, Thailand). After emergence, adult mosquitoes had access to separate sources of 10% glucose (Sigma
Aldrich, US) and dH₂O ad libitum. For colony maintenance, 5–7-day-old adults were provided a blood meal of donated human blood using an artificial membrane feeding system (Hemotek, UK). For mosquito colony 2) females were maintained on rabbit blood by direct feeding (protocol approved by the national committee of Burkina Faso; IRB registration #00004738 and FWA 00007038) and adult males and females fed with a 5% glucose solution. Larvae were reared at a density of about 300 first-instar larvae in 700 ml of water in plastic trays and fed with Tetramin Baby Fish Food (Tetrawerke, Melle, Germany).

**P. falciparum strains and culture**

*P. falciparum* strains used in this study were: 1) *P. falciparum* NF54. NF54 is the drug-susceptible standard strain for mosquito transmission studies, obtained from BEI Resources in 2014. This parasite culture was received through a material transfer agreement (MTA) with the laboratory of Dr. Carolina Barillas-Mury. 2) *P. falciparum* P5. P5 is polyclonal (n = 3, KMMM, KMKM, RMMM), as determined by MSP1 PCR genotyping following standard procedures [29], and has been culture-adapted from a blood sample contributed by a gametocytemic malaria donor in Burkina Faso in 2017. 3) *P. falciparum* KH001_029 “ART29”. ART29 is a *P. falciparum* monogenomic parasite isolate obtained from an infected human patient in Pusrat, Cambodia (KH1 clade) between 2011 and 2013 as part of the TRAC I initiative [5]. This parasite carries the *PfK13* mutation C580Y associated with resistance to artemisinin. ART29 has clear phenotypic artemisinin resistance, both as determined by *in vivo* clearance time (11.8 h) and *in vitro* ring-stage survival (22.6%) [58], alongside resistance to other antimalarial drugs, including mefloquine and chloroquine, but is not resistant to piperaquine or ATQ [41]. These parasites generate robust infections in *An. stephensi* mosquitoes, but not *An. gambiae* (S2 Fig).

For mosquito infection with gametocytemic donor blood samples, *P. falciparum* samples were collected and prepared for infection as described previously [59]. Briefly, *P. falciparum* gametocyte-positive whole blood samples were collected from 5–13-year-old donors from the villages surrounding Bobo Dioulasso, Burkina Faso as part of a separate study. Red blood cells were isolated by centrifugation of a 4 ml aliquot of donor blood followed by resuspension in *Plasmodium* naïve human AB serum. Blood samples were then provided to mosquitoes using a custom blown, water heated glass feeder. For mosquito infection with *in vitro* cultured parasites, females were transferred to a secure malaria infection facility and provided a 14–21 d post-induction stage V *P. falciparum* gametocyte culture using a custom blown, water heated glass feeder. For all infection experiments, within 24 h of infection, partially engorged or unfed mosquitoes were collected by vacuum aspiration and discarded. To determine oocyst burden, between 7 and 14 d pIBM, infected mosquitoes were collected by vacuum aspiration, and dissected to isolate the midgut. The oocyst burden was determined after staining midguts with 0.2% w/v mercurchrome and examination under a 40x air objective inverted compound light microscope (Olympus, US). For sporozoites, at 14 d pIBM infected mosquitoes were collected by vacuum aspiration and beheaded. The mosquito salivary glands were extracted into RPMI media by applying pressure to the lateral thorax. *P. falciparum* sporozoites were isolated by homogenization and centrifugation of salivary gland material, followed by resuspension in a known volume of RPMI. Sporozoites were counted using a disposable haemocytometer under a 20x air objective inverted compound light microscope (Olympus, US). All *P. falciparum* strains were cultured and induced to form gametocytes using standard protocols [60,61]. All strains have been confirmed to be *P. falciparum* by PCR followed by DNA sequencing of the amplified products [62] and have been confirmed free of mycoplasma infection.
Pre- and post-infection tarsal contact infection assays

Tarsal exposure plates were prepared as described previously [26]. Briefly, for 100 μmol/m² plate concentrations, 0.1 ml of this solution of a 0.1% w/v solution of ATQ in acetone was diluted with 1 ml additional acetone and spread onto a 6 cm diameter (0.002628 m²) glass petri dish. For 1 mmol/m², 1 ml of 0.1% w/v ATQ/acetone solution was added directly to the plate. Plates dried for a minimum of 4 h, with agitation on a lateral shaker at room temperature. For pre-infection exposure, 30 min prior to infection, 3–5 d old virgin female mosquitoes were incubated on either ATQ-coated plates, or an acetone treated control, for 6 min. To prevent crowding and agitation, a maximum of 25 mosquitoes were exposed per plate, with all exposures occurring in parallel. For post-infection exposure, due to biosafety considerations, compound exposures were carried out in serial, with a maximum of 10 infected mosquitoes per plate.

Pre- and post-infection sugar feeding infection assays

For experiments involving compound-treated sugar solutions, 20 mM stock solutions of each active ingredient (AI) were prepared in 100% DMSO. Each 20 mM stock solution was diluted 200-fold in 10% w/v glucose to achieve the final working concentration of 100 μM AI/0.5% DMSO/10% w/v glucose. For pre-infection sugar feeding experiments, 2–4 d post-emergence females were denied access to glucose or water for 24 h and then provided access to either the test solution, or a control solution of 0.5% v/v DMSO/10% glucose, for 24 h. After this time had elapsed, all mosquitoes were provided with an infectious P. falciparum blood meal as described above. Infected mosquitoes were provided with untreated 10% w/v glucose ad libitum for the remainder of the experiment. For post-infection sugar-feeding experiments, 3–5 d post-emergence female mosquitoes were infected with P. falciparum and denied access to glucose or water for 48 h pIBM. After this time, infected mosquitoes were continuously provided either 100 μM ATQ/0.5% DMSO/10% w/v glucose or 0.5% DMSO/10% w/v glucose as control, ad libitum. Sugar feeders were replaced every 48 h for the remainder of the experiment, up to 14 d pIBM.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism v8.4.2 for MacOSX (GraphPad Software Inc., USA) and JMP Pro 15 (SAS Corp. US).

Data generated from donor isolated gametocytes

For infections with donor isolated gametocytes (Figs 1(B) and 4(B)), prevalence and intensity of infection were analyzed using more complex statistics to account for between-replicate effects of different human gametocyte donors. For prevalence: we constructed a General Linear Model as follows, independent variable/y “Infected?” (Two-level, categorical (yes/no)), with cofactors “Treatment” (Two-level, categorical (Control/ATQ)) and “Gametocyte Donor” (Two-level, categorical (Donor 1/Donor 2)), we also included the interaction term Treatment*Gametocyte Donor to detect higher level effects. As the output was categorical, the GLM model was run with a binomial distribution and logit link-function. To achieve the best model fit, we iteratively removed cofactors from the model, and selected the model output with the lowest corrected Akaike information criterion (AICc). In all cases, the best model fit included both cofactors, but excluded the interaction term. For intensity: Independent variable “Oocyst Count” (Continuous, positive integer) with cofactors “Treatment” (Two-level, categorical (Control/ATQ)) and “Gametocyte Donor” (Two-level, categorical (Donor 1/Donor 2)), and
the interaction term ‘Treatment’ Gametocyte Donor. To account for the overdispersion typical of parasite count data, we again took an iterative approach to model construction. Data were analyzed using both a GLM using a Poisson distribution, (link function: log; overdispersion parameter estimated by Pearson Chi-square/DF) and Generalized Regression with a Negative Binomial distribution. Relative model quality was determined by comparison of AICc for each distribution function (For GLM, with and without the overdispersion correction) and by iterative removal of cofactors. The highest quality model fit was an overdispersion-corrected Poisson/Log GLM with both cofactors but without the interaction term.

Data generated from in vitro experiments

For all other infections, differences in prevalence were analyzed by Chi². In experiments where both treatment groups had individuals that produced >0 oocysts, differences in median oocyst burden between groups (intensity of infection) was analyzed using a Mann-Whitney Mean Ranks test. For multiple comparisons, differences in prevalence between multiple groups were determined using pairwise Chi² corrected for multiple comparisons (Bonferroni). Similarly, multiple comparisons of intensity were carried out using a Kruskal-Wallis test with Dunn’s post hoc.

Supporting information

S1 Fig. An. gambiae Bama-R mosquitoes are highly resistant to permethrin. (a) Experimental scheme. (b) G3 females were 0% resistant at the DC, while 97% of exposed Bama-R females survived at the same dose, and 43% survived exposure to 5xDC, indicating extreme permethrin resistance. Mean survival ± SEM from 3 replicates is indicated. (c) Bama-R mosquitoes are segregating for the kdr allele conferring target site resistance to pyrethroids. Allele frequency at generation F17 was 51.7% (n = 75), indicating that the observed resistance phenotype is the result of additional modalities. (d) qPCR analysis shows that key cytochrome P450 genes associated with metabolic insecticide resistance are constitutively upregulated in Bama-R females compared to a susceptible control (G3). Median expression level normalized to the Anopheles housekeeping gene rpl19 are shown, error bars represent the interquartile range (IQR). Expression levels for Cyp6P3 and Cyp6Z2 were significantly elevated compared to a phenotypically susceptible control (Cyp6P3, Mann-Whitney, n = 10, df = 1, U = 0, p = 0.0079; Cyp6Z2, Mann-Whitney, n = 10, df = 1, U = 2, p = 0.0317) while Cyp6M2 was not significantly upregulated. Statistical significance is indicated where relevant as follows: ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

(TIF)

S2 Fig. Development of the artemisinin-resistant P. falciparum parasite ART29 in An. stephensi and An. gambiae. Female G3 (An. gambiae) and Anst (An. stephensi) were provided with a blood meal containing mature ART29 gametocytes. Outcome of infection was determined at 7 d pIBM by oocyst count. While ART29 exhibited poor infectivity in An. gambiae (23.8% infection prevalence), they established robust infections in An. stephensi (100% infection prevalence).

(TIF)

S3 Fig. The proportion of An. gambiae mosquitoes with salivary gland sporozoites is significantly reduced after tarsal ATQ exposure at 3 d pIBM. (a) Experimental scheme. (b) There was no effect of 3 d pIBM ATQ exposure on either prevalence (indicated by pie charts) or intensity (indicated by points) of infection determined at 10 d pIBM. Prevalence: Chi², n = 95, df = 1, X² = 0.540, p = 0.4623, intensity: Mann-Whitney, n = 80, df = 1, U = 717.5,
p = 0.4530. (c) ATQ exposure at 3 d pIBM significantly reduced the median cross-sectional area of oocysts at 7 d pIBM relative to control (Chi², n = 50, df = 1, U = 110, p < 0.0001). (d) The prevalence, but not the median intensity of \textit{P. falciparum} sporozoites in mosquito salivary glands was significantly reduced in mosquitoes exposed to ATQ at 3 d pIBM (Chi², n = 144, df = 1, X² = 9.995, p = 0.0016). Median lines and values are indicated, “n” indicates the number of independent samples. To isolate Oocyst/Sporozoite Prevalence and Oocyst/Sporozoite intensity, midgut samples with zero oocysts have been excluded from intensity analysis. Statistical significance is indicated where relevant as follows: ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

(TIF)

S4 Fig. AcVK5 survival and infectivity after ingestion of ATQ/glucose. (a) Survival prior to and following 24 h access to 100 μM/10% w/v Glucose/0.5 v/v DMSO (4 d post emergence, orange arrow), followed by \textit{P. falciparum} (donor blood) infection (5 d post emergence, red arrow). Ingestion of ATQ/glucose had no impact on the survival of AcVK5 mosquitoes relative to a control group provided with 10% w/v glucose/0.5% v/v DMSO (Log-Rank Survival, n = 1374, df = 1, X² = 1.3795, p = 0.2402).

(TIF)

S5 Fig. Ingestion of an ATQ-glucose solution after \textit{P. falciparum} infection has no effect on oocyst prevalence or intensity. There was no difference relative to controls in either intensity (n = 68, df = 1, U = 495.5, p = 0.3257) or prevalence (n = 81, df = 1, X² = 2.441, p = 0.1182) of oocysts at 10 d pIBM in females with continued access to 100 μM ATQ/0.5% DMSO/10% w/v glucose from 2 d pIBM—14 d pIBM. Median lines and values are indicated, “n” indicates the number of independent samples. To isolate Oocyst Prevalence and Oocyst Intensity, midgut samples with zero oocysts have been excluded from intensity analysis. Statistical significance is indicated where relevant as follows: ns = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

(TIF)

Author Contributions

Conceptualization: Douglas G. Paton, Alexandra S. Probst, Dyann F. Wirth, Flaminia Catteruccia.

Formal analysis: Douglas G. Paton.

Funding acquisition: Thierry Lefèvre, Dyann F. Wirth, Flaminia Catteruccia.

Investigation: Douglas G. Paton, Alexandra S. Probst, Erica Ma, Kelsey L. Adams, W. Robert Shaw, Naresh Singh, Selina Bopp, Domombele F. S. Hien, Prislaure S. L. Paré, Rakiswendé S. Yerbanga.

Methodology: Douglas G. Paton, Alexandra S. Probst, Naresh Singh, Domombele F. S. Hien, Thierry Lefèvre.

Project administration: Douglas G. Paton, Flaminia Catteruccia.

Resources: W. Robert Shaw, Naresh Singh, Selina Bopp, Sarah K. Volkman, Abdoulaye Diabaté, Roch K. Dabiré, Thierry Lefèvre, Dyann F. Wirth.

Supervision: Douglas G. Paton, Rakiswendé S. Yerbanga, Abdoulaye Diabaté, Roch K. Dabiré, Thierry Lefèvre, Dyann F. Wirth, Flaminia Catteruccia.

Visualization: Douglas G. Paton.
Writing – original draft: Douglas G. Paton, Flaminia Catteruccia.

Writing – review & editing: Douglas G. Paton, Alexandra S. Probst, Rakiswendé S. Yerbanga, Thierry Lefèvre, Flaminia Catteruccia.

References

1. World Malaria Report 2020. World Health Organization. 2020.

2. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. Nature. 2015; 526(7572):207–11. https://doi.org/10.1038/nature15535 PMID: 26375008; PubMed Central PMCID: PMC4820050.

3. Lin JT, Juliano JJ, Wongsrichanalai C. Drug-Resistant Malaria: The Era of ACT. Curr Infect Dis Rep. 2010; 12(3):165–73. Epub 2011/02/11. https://doi.org/10.1007/s11908-010-0099-y PMID: 21308525; PubMed Central PMCID: PMC3058555.

4. Ogbonna A, Uneke CJ. Artemisinin-based combination therapy for uncomplicated malaria in sub-Saharan Africa: the efficacy, safety, resistance and policy implementation since Abuja 2000. Trans R Soc Trop Med Hyg. 2008; 102(7):621–7. Epub 2008/05/24. https://doi.org/10.1016/j.trstmh.2008.03.024 PMID: 18499204.

5. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, et al. Spread of artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med. 2014; 371(5):411–23. Epub 2014/07/31. https://doi.org/10.1056/NEJMoa1314981 PMID: 25075834; PubMed Central PMCID: PMC4143591.

6. Staedke SG, Gonahasa S, Dorsey G, Kamya MR, Maiteki-Sebuzi C, Lynd A, et al. Effect of long-lasting insecticidal nets with and without piperonyl butoxide on malaria indicators in Uganda (LLINEUP): a pragmatic, cluster-randomised trial embedded in a national LLIN distribution campaign. Lancet. 2020; 395(10232):1292–303. Epub 2020/04/20. https://doi.org/10.1016/S0140-6736(20)30214-2 PMID: 32305094; PubMed Central PMCID: PMC7181182.

7. Amaratunga C, Lim P, Suon S, Sreng S, Mao S, Sopha C, et al. Dihydroartemisinin-piperaquine resistance in Plasmodium falciparum malaria in Cambodia: a multisite prospective cohort study, The Lancet Infectious diseases. 2016; 16(3):357–65. https://doi.org/10.1016/S1473-3099(15)00487-9 PMID: 26774243; PubMed Central PMCID: PMC4792715.

8. Baikagala B, Fukuda N, Ikeda M, Katuro OT, Tachibana SI, Yamauchi M, et al. Evidence of Artemisinin-Resistant Malaria in Africa. N Engl J Med. 2021; 385(13):1163–71. Epub 2021/09/23. https://doi.org/10.1056/NEJMoa2101746 PMID: 34551228.

9. Toé KH, Jones CM, N’Fale S, Ismail HM, Dabiré RK, Ranson H. Increased Pyrethroid Resistance in Malaria Vectors and Decreased Bed Net Effectiveness, Burkina Faso. Emerging Infectious Diseases. 2014; 20(10):1691–6. https://doi.org/10.3201/eid2010.140619 PMC4193182. PMID: 25279965.

10. Samantidis GR, Panteleri R, Denecke R, Kounadi S, Christou I, Nauen R, et al. ‘What I cannot create, I do not understand’: functionally validated synergism of metabolic and target site insecticide resistance. Proc Biol Sci. 2020; 287(1927):20200838. Epub 2020/05/27. https://doi.org/10.1098/rspb.2020.0838 PMID: 32453986; PubMed Central PMCID: PMC7287358.

11. Balabanidou V, Kampouraki A, MacLean M, Blomquist GJ, Tittiger C, Juarez MP, et al. Cytochrome P450 associated with insecticide resistance catalyzes cuticular hydrocarbon production in Anopheles gambiae. Proc Natl Acad Sci U S A. 2016; 113(33):9268–73. https://doi.org/10.1073/pnas.1608295113 PMID: 27439866; PubMed Central PMCID: PMC4995928.

12. Ranson H, Jensen B, Vulule JM, Wang X, Hemingway J, Collins FH. Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan Anopheles gambiae associated with resistance to DDT and pyrethroids. Insect Mol Biol. 2000; 9(5):491–7. Epub 2000/10/13. https://doi.org/10.1046/j.1365-2583.2000.00209.x PMID: 11029667.

13. Stevenson BJ, Bibby J, Pignatelli P, Muangnoicharoen S, O’Neill PM, Lian LY, et al. Cytochrome P450 6M2 from the malaria vector Anopheles gambiae metabolizes pyrethroids: Sequential metabolism of deltamethrin revealed. Insect Biochem Mol Biol. 2011; 41(7):492–502. Epub 2011/02/18. https://doi.org/10.1016/j.ibmb.2011.02.003 PMID: 21324359.

14. Tacoli C, Gai PP, Bayingana C, Stiff K, Geus D, Ndoli J, et al. Artemisinin Resistance-Associated K13 Polymorphisms of Plasmodium falciparum in Southern Rwanda, 2010–2015. Am J Trop Med Hyg. 2016; 95(5):1090–3. Epub 2016/11/04. https://doi.org/10.4269/ajtmh.16-0483 PMID: 27573632; PubMed Central PMCID: PMC5094222.

15. Bwire GM, Ngasala B, Mikomangwa WP, Kilonzi M, Kamuhabwa AAR. Detection of mutations associated with artemisinin resistance at k13-propeller gene and a near complete return of chloroquine susceptible falciparum malaria in Southeast of Tanzania. Sci Rep. 2020; 10(1):3500. Epub 2020/02/28.
Antimalarials in mosquitoes overcome resistance to malaria control strategies

https://doi.org/10.1371/journal.ppat.1001069

16. Uwimana A, Legrand E, Stokes BH, Ndikumana JM, Warsame M, Umulisa N, et al. Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda. Nat Med. 2020; 26(10):1602–8. Epub 2020/08/05. https://doi.org/10.1038/s41591-020-1005-2 PMID: 32747827; PubMed Central PMCID: PMC7541349.

17. Asua V, Conrad MD, Aydemir O, Duvalsaaint M, Legac J, Duarte E, et al. Changing Prevalence of Potential Mediators of Aminooquinoline, Antifolate, and Artemisinin Resistance Across Uganda. J Infect Dis. 2021; 223(6):985–94. Epub 2020/11/05. https://doi.org/10.1093/infdis/jiaa687 PMID: 33146722; PubMed Central PMCID: PMC8006419.

18. Uwimana A, Umulisa N, Venkatesan M, Svigel SS, Zhou Z, Munyaneza T, et al. Association of Plasmodium falciparum kelch13 R561H genotypes with delayed parasite clearance in Rwanda: an open-label, single-arm, multicentre, therapeutic efficacy study. The Lancet Infectious diseases. 2021; 21(8):1120–8. Epub 2021/04/18. https://doi.org/10.1016/S1473-3099(21)00142-0 PMID: 33864981.

19. Straimer J, Gandhi P, Renner KC, Schmitt EK. High prevalence of P. falciparum K13 mutations in Rwanda is associated with slow parasite clearance after treatment with artemether-lumefantrine. J Infect Dis. 2021. Epub 2021/07/04. https://doi.org/10.1093/infdis/jiaa352 PMID: 34216470.

20. Sinka ME, Pironon S, Massey NC, Longbottom J, Hemingway J, Moyes CL, et al. A new malaria vector in Africa: Predicting the expansion range of Anopheles stephensi and identifying the urban populations at risk. Proceedings of the National Academy of Sciences of the United States of America. 2020; 117 (40):24900–8. Epub 2020/09/16. https://doi.org/10.1073/pnas.2003976117 PMID: 32929026; PubMed Central PMCID: PMC7547157.

21. St Laurent B, Miller B, Burton TA, Amarantunga C, Men S, Sovannaroth S, et al. Artemisinin-resistant Plasmodium falciparum clinical isolates can infect diverse mosquito vectors of Southeast Asia and Africa. Nat Commun. 2015; 6:8614. Epub 2015/10/21. https://doi.org/10.1038/ncomms9614 PMID: 26485448; PubMed Central PMCID: PMC4616032.

22. Sherrard-Smith E, Skarp JE, Beale AD, Forndael C, Norris LC, Moore SJ, et al. Mosquito feeding behavior and how it influences residual malaria transmission across Africa. Proceedings of the National Academy of Sciences of the United States of America. 2019; 116(30):15086–95. Epub 2019/07/10. https://doi.org/10.1073/pnas.1820646116 PMID: 31285346; PubMed Central PMCID: PMC6660788.

23. Fraser KJ, Mwandigha L, Traore SF, Traore MM, Doumbia S, Junnila A, et al. Estimating the potential impact of Attractive Targeted Sugar Baits (ATSBs) as a new vector control tool for Plasmodium falciparum malaria. Malar J. 2021; 20(1):151. Epub 2021/03/19. https://doi.org/10.1186/s12936-021-03684-4 PMID: 33731111; PubMed Central PMCID: PMC7968277.

24. Muller GC, Beier JC, Traore SF, Toure MB, Traore MM, Bah S, et al. Successful field trial of attractive toxic sugar bait (ATSB) plant-spraying methods against malaria vectors in the Anopheles gambiae complex in Mali, West Africa. Malar J. 2010; 9:210. Epub 2010/07/29. https://doi.org/10.1186/1475-2875-9-210 PMID: 2063142; PubMed Central PMCID: PMC2914067.

25. Beier JC, Muller GC, Gu W, Arheart KL, Schlein Y. Attractive toxic sugar bait (ATSB) methods decimate populations of Anopheles malaria vectors in arid environments regardless of the local availability of favoured sugar-source blossoms. Malar J. 2012; 11:31. https://doi.org/10.1186/1475-2875-11-31 PMID: 22291755; PubMed Central PMCID: PMC3293779.

26. Paton DG, Childs LM, Itoe MA, Holmdahl IE, Buckee CO, Catteruccia F. Exposing Anopheles mosquitoes to antimalarials blocks Plasmodium parasite transmission. Nature. 2019; 567(7747):239–43. Epub 2019/07/10. https://doi.org/10.1038/s41586-019-0973-1 PMID: 30814727.

27. Adams KL, Sawadogo SP, Ngnjan C, Niang A, Paton DG, Robert Shaw W, et al. Cuticular hydrocarbons are associated with mating success and insecticide resistance in malaria vectors. Commun Biol. 2021; 4(1):911. Epub 2021/07/28. https://doi.org/10.1038/s42023-021-02343-1 PMID: 34312484; PubMed Central PMCID: PMC8313523.

28. Kwiatkowska RM, Platt N, Poupardin R, Irving H, Dabire RK, Mitchell S, et al. Dissecting the mechanisms responsible for the multiple insecticide resistance phenotype in Anopheles gambiae s.s., M form, from Vallée du Kou, Burkina Faso. Gene. 2013; 519(1):98–106. Epub 2013/02/06. https://doi.org/10.1016/j.gene.2013.01.036 PMID: 23380570; PubMed Central PMCID: PMC3611593.

29. Kaneko O, Kimura M, Kawamoto F, Ferreira MU, Tanabe K. Plasmodium falciparum: allelic variation in the merozoite surface protein 1 gene in wild isolates from southern Vietnam. Exp Parasitol. 1997; 86 (1):45–57. https://doi.org/10.1006/expar.1997.4147 PMID: 9149240.

30. Knola BG, Farenhorst M, Andriessen R, Smetseilaar J, Suer RA, Osinga AJ, et al. Eave tubes for malaria control in Africa: an introduction. Malar J. 2016; 15(1):404. Epub 2016/08/16. https://doi.org/10.1186/s12936-016-1452-x PMID: 27915308; PubMed Central PMCID: PMC4982263.
31. Shaw WR, Holmdahl IE, Itoe MA, Werling K, Marquette K, Paton DG, et al. Multiple blood feeding in mosquitoes shortens the Plasmodium falciparum incubation period and increases malaria transmission potential. PLoS Pathog. 2020; 16(12):e1009131. PMID: 33382824; PubMed Central PMCID: PMC7774842.

32. Soulama I, Nebie I, Ouedraogo A, Gansane A, Diarra A, Tione AB, et al. Plasmodium falciparum genotyping diversity in symptomatic malaria of children living in an urban and a rural setting in Burkina Faso. Malar J. 2009; 8:135. Epub 2009/06/24. https://doi.org/10.1186/1475-2875-8-135 PMID: 19545390; PubMed Central PMCID:PMC7205376.

33. Sondo P, Derra K, Rouamba T, Nakanabo Diallo S, Taconet P, Kazienga A, et al. Determinants of Plasmodium falciparum multiplicity of infection and genetic diversity in Burkina Faso. Parasit Vectors. 2020; 13(1):427. Epub 2020/08/21. https://doi.org/10.1186/s13071-020-04302-z PMID: 32819420; PubMed Central PMCID:PMC7441709.

34. Mitchell SN, Stevenson BJ, Muller P, Wilding CS, Eguy-Yawson A, Field SG, et al. Identification and validation of a gene causing cross-resistance between insecticide classes in Anopheles gambiae from Ghana. Proc Natl Acad Sci U S A. 2012; 109(16):6147–52. https://doi.org/10.1073/pnas.1203452109 PMID: 22460795; PubMed Central PMCID:PMC3341073.

35. Lees RS, Ismail HM, Logan RAE, Malone D, Davies R, Anthousi A, et al. New insecticide screening platforms indicate that Mitochondrial Complex I inhibitors are susceptible to cross-resistance by mosquito P450s that metabolise pyrethroids. Sci Rep. 2020; 10(1):16232. https://doi.org/10.1038/s41598-020-73267-x PMID: 33004954; PubMed Central PMCID:PMC7530702.

36. Musset L, Bouchaud O, Matheron S, Massias L, Le Bras J. Clinical atovaquone-proguanil resistance of Plasmodium falciparum associated with cytochrome b codon 268 mutations. Microbes Infect. 2006; 8(11):2599–604. https://doi.org/10.1016/j.micinf.2006.07.011 PMID: 16962361.

37. Bopp SE, Magistrad o P, Wong W, Schaffner SF, Mukherje e A, Lim P, et al. Plasmepsin II-III copy number forms indicate that Mitochondrial Complex I inhibitors are susceptible to cross-resistance by mosquito P450s that metabolise pyrethroids. Sci Rep. 2020; 10(1):16232. https://doi.org/10.1038/s41598-020-73267-x PMID: 33004954; PubMed Central PMCID:PMC7530702.

38. Goodman CD, Siregar JE, Molland V, Vega-Rodriguez J, Syafrud din D, Matsu oka H, et al. Parasites resistant to the antimalarial atovaquone fail to transmit by mosquitoes. Science. 2016; 352(6283):349–53. https://doi.org/10.1126/science.aad9279 PMID: 27081071; PubMed Central PMCID:PMC5149070.

39. Bopp SE, Manary MJ, Bright AT, Johnston GL, Dharia NV, Luna FL, et al. Mitotic evolution of Plasmodium falciparum shows a stable core genome but recombination in antigen families. PLoS Genet. 2013; 9(2):e1003293. https://doi.org/10.1371/journal.pgen.1003293 PMID: 23408914; PubMed Central PMCID:PMC3567157.

40. Siegel S, Rivera A, Adapa SR, Wang C, Adapa SR, Macnaghten D, Matsuoka H, et al. Parasites resistant to the antimalarial atovaquone fail to transmit by mosquitoes. Science. 2016; 352(6283):349–53. https://doi.org/10.1126/science.aad9279 PMID: 27081071; PubMed Central PMCID:PMC5149070.

41. Siegel S, Rivero A, Adapa SR, Wang C, Manetsch R, Jiang RHY, et al. Mitochondrial heteroplasmy is responsible for Atovaquone drug resistance in Plasmodium falciparum. bioRxiv. 2017:232033. https://doi.org/10.1101/232033.

42. Bopp S, Magistrado P, Wong W, Schaffner SF, Mukherjee A, Lim P, et al. Plasmepsin II-III copy number accounts for bimodal piperazine resistance among Cambodian Plasmodium falciparum. Nat Commun. 2018; 9(1):1769. Epub 2018/05/04. https://doi.org/10.1038/s41477-018-04104-z PMID: 29720620; PubMed Central PMCID:PMC5931971.

43. Graumans W, Jacobs E, Bousema T, Sinnis P. When Is a Plasmodium-Infected Mosquito an Infectious Mosquito? Trends Parasitol. 2020; 36(8):705–16. https://doi.org/10.1016/j.pt.2020.05.011 PMID: 32620501; PubMed Central PMCID:PMC7386819.

44. Sinden RE, Billingsley PF. Plasmodium invasion of mosquito cells: hawk or dove? Trends Parasitol. 2001; 17(5):209–12. https://doi.org/10.1016/s1471-4922(01)01928-6 PMID: 11323288.

45. Smith RC, Vega-Rodriguez J, Jacobs-Lor ena M. The Plasmodium bottleneck: malaria parasite losses in the mosquito vector. Mem Inst Oswaldo Cruz. 2014; 109(5):644–61. https://doi.org/10.1590/0074-0276130597 PMID: 25185005; PubMed Central PMCID:PMC415648.

46. Burrows J, Fidock DA, Miller RS, Rees S. Blocking Plasmodium Development in Mosquitoes: A Powerful New Approach for Expanding Malaria Control Efforts. Am J Trop Med Hyg. 2019; 102(4):734–5. Epub 2019/07/03. https://doi.org/10.4269/ajtmh.19-0318 PMID: 31264564; PubMed Central PMCID:PMC6779223.

47. Kessler S, Vilman M, Guerin PM. The sugar meal of the African malaria mosquito Anopheles gambiae and how deterrent compounds interfere with it: a behavioural and neurophysiological study. J Exp Biol. 2013; 216(Pt 7):1292–306. Epub 2012/12/25. https://doi.org/10.1242/jeb.076588 PMID: 23264482.

48. Qualls WA, Muller GC, Traore SF, Traore MM, Arheart KL, Doumbia S, et al. Indoor use of attractive toxic sugar bait (ATSB) to effectively control malaria vectors in Mali, West Africa. Malar J. 2015; 14:1301.
