Anopheles mosquito survival and pharmacokinetic modeling show the mosquitocidal activity of nitisinone

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Nitisinone reduces survival of the malaria-transmitting mosquito, *Anopheles gambiae* by blocking tyrosine catabolism

Accessible Summary:

In our search for effective mosquito control agents, we examined nitisinone, an FDA-approved drug used to treat rare metabolic disorders, and compared its mosquitocidal properties to ivermectin. Nitisinone works by targeting a crucial enzyme that mosquitoes need for bloodmeal digestion. When Anopheles mosquitoes - the primary malaria vectors - consume blood containing nitisinone, the drug proves lethal to young, old and insecticide-resistant populations. Both modeling and empirical evidence show nitisinone outperforms ivermectin. Even at low therapeutic doses, nitisinone remains deadly to mosquitoes, highlighting its potential for malaria prevention.

Abstract

One approach to interrupting the transmission of insect-borne diseases that is successfully used in veterinary medicine, is exploiting the ability of antiparasitic drugs to make vertebrate blood toxic for blood-feeding insects. Recent studies have identified 4-hydroxyphenylpyruvate dioxygenase (HPPD), an enzyme of the tyrosine detoxification pathway, is essential for hematophagous arthropods to digest their blood meals. This includes Anopheline mosquitoes, which transmit malaria-causing *Plasmodium* parasites. An FDA-approved HPPD enzyme inhibitor called nitisinone is a drug used to treat rare human inherited disorders of the tyrosine pathway. Here we demonstrate that feeding human blood containing nitisinone to the malaria-transmitting mosquito species, *Anopheles gambiae,* was mosquitocidal to both young and old mosquitoes as well as insecticide-resistant *Anopheles* strains. Pharmacokinetic-pharmacodynamic (PK/PD) modeling of nitisinone's dose-response relationship, when administered at the highest recommended doses for adults and children, demonstrated improved efficacy against mosquitoes compared to the gold-standard endectocidal drug, ivermectin. Furthermore, blood samples from individuals with alkaptonuria (a rare genetic metabolic disorder in the tyrosine degradation pathway), who were taking a daily low dose of 2 mg of nitisinone, was lethal to mosquitoes. Thus, inhibiting the *Anopheles* HPPD enzyme with nitisinone warrants further investigation as a new and complementary intervention for malaria control.

INTRODUCTION

Malaria is one of the leading causes of mortality and morbidity in many lower-middle income countries, with 619,000 deaths estimated in 2021, of which 95% were in sub-Saharan Africa (*1*). Since 2014, progress with malaria elimination has slowed as growing populations place more people at risk (*1, 2*). Transmission of this disease is driven by female *Plasmodium*-infected *Anopheles* mosquitoes and mosquito control accounts for the majority of averted cases (~68%) (*3*). Interventions have historically relied on insecticides (*4*), which help to reduce mosquito survival.

Malaria elimination programs currently face several challenges, including the development of parasite resistance to effective anti-malarial treatments, first observed in Asia (*5, 6*) and more recently in Africa (*7*). Additionally, insecticide resistance in mosquito populations has increased globally at alarming rates (*8, 9*). Furthermore, mosquitoes are evolving behavioral resistance, avoiding indoor environments sprayed with insecticides (*10, 11*) and increasing outdoor feeding (*12, 13*). These adaptations compromise the efficacy of current tools and highlight the urgent need to develop strategies that address residual malaria transmission (*14, 15*).

Endectocides are systemic anti-parasitic drugs that can be safely administered to people or animals to achieve blood concentrations toxic to parasites and, in some cases, to blood-feeding arthropods. Even when insects and ticks ingest sublethal doses, their lifespans are reduced, thereby impairing the development of pathogens (*16*). Thus, endectocidal drugs offer a new mode of action compared to insecticides and provide many operational opportunities, including their potential synergy with mass drug administration programs for antimalarials or seasonal malaria chemoprevention during high transmission seasons.

Presently, the only human endectocide tested in clinical trials is ivermectin, an anti-parasitic drug that has been used since 1987 in mass campaigns to treat lymphatic

filariasis (*17*) and onchocerciasis (*18, 19*). Following decades of observed impact on mosquito mortality (*20, 21*), ivermectin, a potent inhibitor of the insect glutamate-gated channel, has a mosquitocidal effect for up to 28 days in human blood (*22*). Potential benefits of ivermectin administration for malaria control are supported by epidemiological modeling that predicts a reduction in malaria transmission when ivermectin is used in combination with antimalarial therapeutic drugs (*23-27*). A large-scale clinical trial in The Gambia co-administering ivermectin with dihydroartemisinin-piperaquine showed epidemiological effects. However, no entomological impact was confirmed and an additional effect of ivermectin alone was inconclusive (*28*). Currently, we await data from three late-stage ivermectin clinical trials (*29*). Whereas evidence of ivermectin's impact on malaria transmission remains inconclusive, several factors may threaten its potential for large-scale deployment as an endectocidal drug.

Nitisinone is a repurposed herbicide that has been approved to treat the human genetic disorder tyrosinemia type I since 1992 (*30*) and, more recently, alkaptonuria (*31, 32*). This drug is an inhibitor of 4-hydroxyphenylpyruvate dioxygenase (HPPD) and thus targets the tyrosine metabolism pathway (*30*). Tyrosinemia type I and alkaptonuria are both rare autosomal genetic disorders caused by mutations in the fumarylacetoacetate hydrolase (*FAH*) gene (*30*) and the homogentisate 1,2-dioxygenase (*HGD*) (*31*) gene, respectively. Co-morbidities of tyrosinemia type I usually manifest within the first six months of life, often involving renal and neurologic dysfunction (*30*). In contrast, alkaptonuria is less severe but leads to the toxic accumulation of homogentisic acid in bones and cartilage causing early-onset osteoarthritis (*31*). For tyrosinemia type I, treatment requires a daily dose of 1 - 2 mg/kg of nitisinone, whereas only 2 mg/day can effectively manage alkaptonuria (*31, 32*).

Recent research has demonstrated that nitisinone is toxic for triatomine bugs, ticks, tsetse flies and mosquitoes after being fed nitisinone-containing bloodmeals. This toxicity

was possibly linked to the toxic accumulation of tyrosine (*33*, *34, 35, 36*). Here, we show that nitisinone can achieve a concentration profile in human blood that is overall superior to ivermectin's mosquitocidal properties. We present a proof-of-concept PK/PD modeling study of nitisinone, providing evidence for its potent mosquitocidal effect and potential for reducing residual malaria transmission in endemic settings.

RESULTS

Mosquito survival after ingesting blood meals containing different doses of nitisinone or ivermectin

Three related experiments were performed to determine mosquitocidal concentrations of nitisinone and ivermectin that could be used in subsequent experiments and for PK/PD modeling. To first establish an appropriate drug concentration starting point for screening the efficacy of nitisinone, we performed a pilot experiment (Experiment 1) using a broad concentration range of nitisinone (50-10,000 ng/mL) and ivermectin (15-5,000 ng/mL) (**table S1**, **fig. S1**). We then tested mosquitoes (Kisumu strain) against a range of physiologically relevant drug concentrations of nitisinone (3.8-3500 ng/mL; Experiment 2) and ivermectin (1.5-125 ng/mL) and replicated this study three times (**Table 1, Fig. 1**). The selected physiologically relevant concentrations were based on simulated drug PK profiles (i.e., the time it takes for a drug to be absorbed by and then excreted from the body), which corresponds to predicted drug concentrations in human blood at days 7, 14, 21 and 28 after the drug was ingested. The simulated oral dose of nitisinone was 1 mg/kg (the therapeutic dose for treating tyrosinemia type 1) for three days and the simulated dose for ivermectin was 0.6 mg/kg (the maximum safe dose for human ingestion) for three days (*37*). The maximal predicted ivermectin concentration (C_{max} 125 ng/mL) was also included as a positive control for mosquito mortality. A final, follow-up experiment (Experiment 3) was performed with a narrowed range of nitisinone

doses to better establish the sublethality of drug concentrations (100-250 ng/mL) (**table S2**, **fig. S2**). This third nitisinone dosing experiment also provided crucial data to enable improved accuracy in assessing the mosquito killing effect of nitisinone as this drug has a very steep concentration-effect curve.

Two treatment groups of mosquitoes were fed either nitisinone or ivermectin in bloodmeals and mortality was recorded daily over a 14-day period. Fourteen days is the extrinsic incubation period for *Plasmodium* development in the mosquito midgut and for its dissemination to the mosquito salivary glands from where the parasite is transmitted to its next human host. The goal of tracking mosquito survival for 14 days was to expand the data points that many systemic modeling experiments have included within their PK/PD calculations. Mosquito survival at each drug concentration (**Fig. 1**) was analyzed in a Cox regression to generate a hazard ratio comparing the likelihood of mortality to that of a drug free control blood meal (**Table 1**). In survival analyses, a hazard ratio is a measure, over time, of the frequency of an event occurring in one group compared to another group. In our study, the hazard ratio represents the likelihood of mosquito mortality; a bloodmeal with a hazard ratio of 4 means a mosquito is four times more likely to die after feeding, compared to if it had fed on the drug-free control blood meal.

The average median survival time of mosquitoes that had ingested nitisinone concentrations predicted at 7 days and 14 days post-administration to be 3500 ng/mL and 350 ng/mL, respectively, was 0.86 days (**Table 1**). More than 50% of mosquitoes were still alive at the end of the follow-up period for the nitisinone concentrations predicted at 21 days and 28 days post-administration (37 ng/mL and 3.8 ng/mL, respectively). The maximal predicted concentration (125 ng/mL) of ivermectin resulted in an average median survival time of 1.86 days, which increased to 3.5 days for the concentration predicted at 7 days (20 ng/mL) and 8.04 days for the concentration

predicted at 14 days (8.5 ng/mL) (**Table 1**). These results showed there was notable mosquitocidal activity at concentrations as low as 50 ng/mL for nitisinone (**table S1**) and 8.5 ng/mL for ivermectin (**Table 1**). Furthermore, nitisinone concentrations in the blood fed to mosquitoes had a significant killing effect ($p<0.0001$) at or above the concentration expected 14 days (350 ng/mL) after dosing , whereas this effect was lost by day 21 (37 ng/mL) (**Fig. 1A, C, E** and **Table 1**). In contrast, ivermectin (**Fig. 1B, D, F** and **Table 1**) demonstrated a significant mosquito killing effect ($p<0.0001$) only at the concentrations expected at or after 7 days post administration (20 ng/mL). Survival curves for the pilot (Experiment 1) and follow-up experiment (Experiment 3) are displayed in **fig S1 and fig S2**, respectively.

The concentration-effect relationship (potency over time) for nitisinone and ivermectin

The mosquitocidal activity of both drugs was analyzed in parallel using a non-linear predictive model to estimate the half maximal effective concentration (EC_{50}) and the Hill slope of the concentration-effect relationship of each drug. The EC_{50} describes the drug concentration that induces a mosquitocidal response that is halfway between the maximum and minimum hazard ratios at day 14. Hazard ratios from initial experiments one to three (**table S1, S2,** and **S3)** were combined for this analysis (**Fig. 2A**). Both drugs were mosquitocidal at their highest concentrations with hazard ratio values exceeding 20, which was the cut-off selected as the maximal hazard ratio in agreement with previous ivermectin PK/PD modeling (*38*). The estimated EC_{50} of ivermectin was 13.4 ng/mL, which is comparable to its reported EC_{50} in published clinical trial data of 26.1 ng/mL (*38*), whereas nitisinone gave an EC_{50} of 205.3 ng/mL (**table S4**). The Hill slope describes the steepness of a concentration-effect curve; having a steep Hill slope (>10) indicates a concentration-effect curve that is closer to an "all-or-nothing" relationship. A steeper

concentration-effect relationship was observed for nitisinone, with a Hill slope (h) of 12.85, compared to a shallower relationship for ivermectin (h = 2.3) (**table S4**).

Predicting the mosquitocidal effect of nitisinone and ivermectin in human blood using PK/PD relationships

It is important to translate the concentration-effect relationship of ivermectin and nitisinone to physiologically meaningful mosquitocidal activity. To do this, the exposure of both drugs was simulated in a human population model to demonstrate the PK profile (concentration of drug in the body) of each dosing regimen over time. The predicted concentration effect (hazard ratio) curve was then applied to the corresponding predicted concentration to demonstrate the mosquito killing effect over time associated with each dosing regimen (PD profile is the lethal effect of the drug on the mosquito). The PK/PD relationship showed superior mosquitocidal activity for nitisinone compared to ivermectin (**Fig. 2B, C**). This is defined by the length of time a drug remains above its EC_{50} (measured in days). Nitisinone remained above its EC_{50} for longer (15.7 days) than ivermectin did (10.4 days) (**Fig. 2B, table S5**). More importantly, nitisinone could achieve concentrations in the blood that remained above a hazard ratio threshold of 4.0 for longer than ivermectin (**Fig 2C**, **table S5**). This threshold has been defined in previous modeling as a critical point at which an impact on malaria incidence is predicted (*24*). Interestingly, the reported EC_{50} and the blood concentrations exceeding a hazard ratio of 4.0 for ivermectin were lower in a published clinical trial (*38*) compared to our *in vitro* results (**table S4**, **table S5**).

Efficacy of nitisinone and ivermectin for killing older mosquitoes

The mosquitocidal efficacy of nitisinone was compared to that of ivermectin for older (9-13 days) female *An. gambiae* (Kisumu strain) mosquitoes reared in an insectary. The average lifespan of *An. gambiae* females was predicted to be a month in captivity or two to three weeks in the wild (*39*). Testing older mosquitoes provided a realistic comparison

to what might be observed in field mosquito populations, as these mosquitoes are more likely to carry human infective *Plasmodium* sporozoites. Despite reports that older mosquitoes are more resistant to insecticides (*40*), there is also evidence of increased insecticide susceptibility with age (*41, 42*). It is thus important to determine if the efficacy of nitisinone and ivermectin would be compromised by the fitness of older mosquitoes. Nitisinone's mosquitocidal activity against older mosquitoes was observed as previously described using a Cox regression analysis (**Fig. 3, table S6**). Three replicate experiments were performed, repeating the same experimental design adopted for the experiment on younger mosquitoes (**table S7**). The experiment showed that nitisinone achieved mosquitocidal activity that was superior to ivermectin for at least 14 days (**Fig. 3C**) after drug administration. In contrast, ivermectin demonstrated reduced mosquitocidal activity at day 7 post drug administration **(Fig. 3B, table S7).**

Predicted drug concentrations in blood at 7 days post drug administration resulted in a median mosquito survival time of 0.83 days for nitisinone and 5.72 days (**table S6**) for ivermectin; that is, nitisinone-treated mosquitoes died before they could oviposit eggs. Concentrations predicted at 14 days for both drugs resulted in a median survival time of 0.83 days and 8.88 days for nitisinone and ivermectin, respectively (**table S6**). After 21 days there was no difference in the mosquito killing effect between nitisinone and ivermectin (**Fig 3D**).

Similar to the experiment on young (3-5 day old) mosquitoes (**Table 1**), nitisinone remained lethal to older mosquitoes ($p<0.0001$) for concentrations predicted up to two weeks after administering a nitisinone dose equivalent to 1 mg/kg (**table S6, table S7**). This demonstrated that older mosquitoes were not more resistant to nitisinone, and in fact there was no difference in mortality observed between old and young mosquitoes in terms of median survival time (0.83 days vs 0.86 days, respectively). However, the predicted killing effect of ivermectin (i.e., 3 day dose at 0.6 mg/kg) after 7 days was less

in older mosquitoes compared to young mosquitoes ($p<0.05$ (**table S6)** versus $p<0.0001$ (**Table 1**), respectively). This suggested that older mosquitoes are less sensitive to ivermectin (**Fig 3F**).

Nitisinone kills insecticide-resistant mosquitoes

In addition to testing the efficacy of nitisinone against an insecticide-susceptible strain of *An. gambiae* (Kisumu) in the previous experiments, we also tested whether an insecticide-resistant female *An. gambiae* strain (Tiassalé) (*43*) would die upon ingesting a nitisinone-containing bloodmeal. The Tiassalé mosquito strain is highly resistant to several insecticide classes including permethrin, deltamethrin, DDT, bendiocarb, dieldrin and propoxur (*44*). The mosquitocidal activity of nitisinone was simultaneously compared in age-matched Kisumu and Tiassalé female mosquitoes in three replicate experiments. We showed that the survival of the Tiassalé and the reference Kisumu mosquito strains was comparable and that both strains were susceptible to nitisinone at physiologically relevant drug concentrations (**Fig. 4, table S8, table S9**). The average mosquito median survival time for concentrations predicted at day 7 and 14 of the PK curve of nitisinone (1 mg/kg x 3) was 0.76 days for Tiassalé and 0.67 days for Kisumu (**Fig. 4, table S8**). Although there was no significant difference in the mortality of Kisumu and Tiassalé strains compared to nitisinone-free control bloodmeals, the higher 14-day hazard ratio (**table S8**) and lower median survival time indicated that the nitisinone-induced killing effect was more lethal to the Kisumu (insecticide-susceptible) strain compared to the Tiassalé (insecticide-resistant) strain. The higher mortality observed in untreated Tiassalé controls indicated that rearing this insecticide-resistant strain is often more difficult as this strain carries a higher fitness cost compared to Kisumu strain (*43*). This compromised fitness may have artificially contributed to the lower hazard ratio killing effect of nitisinone (**table S8**).

A single lower dose of nitisinone retains its mosquitocidal activity in human blood

To explore the full potential of nitisinone as a mosquitocidal compound for use in humans, the impact of lower (and consequently safer) dosing regimens were simulated (**fig. S3)**. Reports of side effects from long-term nitisinone treatment at therapeutic doses used to treat tyrosinemia type I, were previously identified in clinical trials (*45-47*). The PK/PD profiles of specific dosing regimens were generated to compare outcome parameters for patient doses below 1 mg/kg, reaching as low as 0.01 mg/kg (**fig. S3, fig. S4**, and **table S10**). A single dose of nitisinone (rather than therapy for 3 days) was also simulated, because of the operational preference for single administration and minimal drug exposure. A single simulated dose at 0.1 mg/kg is displayed in **fig. S3**. PK/PD simulations showed that nitisinone doses, lower than the tyrosinemia therapeutic dosing regimen, could attain concentrations above the EC_{50} for substantial periods of time. A dose half of that previously simulated (0.5 mg/kg) maintained blood concentrations above the EC_{50} for ten days, compared to 12 days for 1 mg/kg (**fig S4A**). **Fig S4B** shows that the same dose maintained the hazard ratio above 4 for comparable periods of time. Moreover, a single dose as low as 0.1 mg/kg maintained mosquitocidal activity for over 5 days (**table S10**).

Blood from individuals on nitisinone therapy kills mosquitoes

To determine the potential mosquito killing effect of circulating nitisinone in human blood, a small pilot experiment was performed using blood samples obtained from four alkaptonuria patients at the National Alkaptonuria Centre, Royal Liverpool University Hospital. Simulating what the nitisinone concentrations in human blood would reach with the alkaptonuria dosing regimen of 2 mg/day (regardless of body mass), we predicted that two days after starting nitisinone treatment, patient blood would become mosquitocidal (**Fig. 5A**). A predicted PD profile at this low dose also showed a favourable

hazard ratio profile that reached a maximal hazard ratio of 20 in only two days (**Fig. 5B**). Furthermore, this therapeutic dose was only 3% of the dose we previously predicted from modeling studies (70 mg/day), the recommended dose used to treat tyrosinemia type I (in a person weighing 70 kg), and is likely to have a more favourable safety profile.

We next tested whether blood samples from the three alkaptonuria patients taking 2 mg/day nitisinone would have mosquitocidal properties. Insectary-reared *An. gambiae* (Kisumu strain) were membrane-fed on neat or diluted alkaptonuria patient blood to obtain mosquito survival data from a range of *in vivo* nitisinone concentrations predicted to be above and below the EC_{50}. Blood from the three individuals on nitisinone therapy (at a 2 mg/day dose) killed mosquitoes within 12 hours of feeding (**Fig. 5C, D, E**). When blood samples were diluted to 1:2, 1:5 and 1:10, mosquito survival increased in a concentration-dependent manner. Notably, the blood sample from alkaptonuria patient 4, who had not yet started nitisinone treatment, was nitisinone-free and was not toxic to mosquitoes (**Fig. 5F**). **Table 2** shows the length of time the three patients had been on nitisinone therapy and the nitisinone concentrations in their blood 24 hours prior to mosquito feeding. The quantitated nitisinone blood concentrations and corresponding mosquito mortality support a concentration-dependent mosquitocidal effect. Patient 2 (**Fig 5D**) had the highest blood concentration of nitisinone, which correlated with this patient's blood sample showing the highest mosquito killing effect even at a 1:5 dilution (**Fig. 5, Table 3, table S11**).

Excess tyrosine in alkaptonuria donor blood due to nitisinone therapy does not contribute to nitisinone's mosquitocidal effects

To discern the potential impact of excess tyrosine on the nitisinone-induced mosquito killing effect (**Table 4**), we repeated the mosquito blood-feeding experiment on *An. gambiae* (Kisumu strain) using tyrosine alone or in combination with a sublethal dose of

nitisinone. The two physiological concentrations of tyrosine selected [high = 0.109 mg/mL (600 μM) and low = 0.036 mg/mL (200 μM)] are considered to be relevant according to recent clinical trial data obtained from nitisinone dosing regimens (*32*). The mosquitocidal effect of three doses of nitisinone, including nonlethal (3.8 ng/mL), sublethal (37 ng/mL) and lethal (250 ng/mL), established the killing baseline control (**Fig. 6A**). If tyrosine enhanced the killing effect of nitisinone, then mosquito killing profiles would show higher mortality than the baseline. We found that the addition of tyrosine alone to bloodmeals had no impact on mosquito survival when compared to the control bloodmeal **(Fig. 6B**). Bloodmeals containing tyrosine plus nonlethal nitisinone (**Fig. 6C**) or sublethal nitisinone doses (**Fig. 6D**) resulted in no significant difference in mosquito mortality, regardless of the concentration of tyrosine used (**Table 4, table S12**). These data show evidence that tyrosine did not act alone or in synergy with nitisinone to kill *An. gambiae* mosquitoes.

Metabolomics shows that nitisinone inhibits the Anopheline tyrosine pathway

To indirectly corroborate that nitisinone blocks tyrosine degradation in *An. gambiae*, we used mass spectrometry-based metabolomics. Mosquitoes were fed a sublethal dose of nitisinone in phosphate buffered saline (PBS) containing 50 mg/ml bovine serum albumin (BSA) (*35*). After feeding, mosquitoes were collected for analysis and grouped according to their degree of knockdown: Group A - complete knockdown (completely paralysed mosquitoes lying on their backs with twitching legs), Group B - partial knockdown (mosquitoes could still weakly move and were collected while clinging to cage walls and ceiling) and Group C - control (nitisinone-free mosquitoes were collected during flight). Control mosquitoes were fed on a nitisinone-free PBS/BSA meal. As previously observed in tsetse fly hemolymph (*35*), tyrosine and other metabolites related to the tyrosine degradation pathway accumulated in nitisinone-treated mosquitoes (**table S13** and **fig. S5**). Mosquitoes that reached a state of full paralysis (complete knockdown) showed a

higher accumulation of L-tyrosine, 3-hydroxyphenylpyruvate, 3-hydroxyphenyllactate, and 3-hydroxyphenylacetate. A higher abundance of L-phenylalanine was observed in mosquitoes able to crawl but not fly (partial knockdown) and abundance decreased as mosquitoes entered full paralysis. In addition, we discovered that nitisinone also affected abundance of citric acid cycle intermediates (**fig. S6**) and lipid metabolism **fig. S7**, **table S13**). These findings agree with the systemic paralysis and metabolite accumulation observed in other hematophagous arthropods (*34-36*) and shows that nitisinone also inhibits tyrosine degradation in Anopheline mosquitoes.

DISCUSSION

Parasite transmission-blocking compounds, such as endectocides, have the potential to complement existing disease control efforts to tackle residual transmission and accelerate a decline in global malaria. Here, we present a PK/PD modeling comparison between two drugs with the potential to block malaria transmission, nitisinone and ivermectin, and assess their mosquitocidal capabilities.

The inhibition of insect 4-hydroxyphenylpyruvate dioxygenase (HPPD), an Fe(II)-containing non-heme oxygenase catalyzing the catabolism of tyrosine, is lethal to mosquitoes in a dose-dependent manner as tyrosine detoxification is essential for the survival of blood-feeding arthropods (*34-36*). Feeding mosquitoes nitisinone killed them within 24 hours compared to taking four days for ivermectin. HPPD inhibition by nitisinone causes the accumulation of tyrosine and other intermediates in the tyrosine degradation pathway (*35, 48*) as confirmed by our metabolomics analysis. Although nitisinone was 12-fold less potent against *An. gambiae* than ivermectin (EC_{50} = 205.31 ng/mL vs EC_{50} = 13.43 ng/mL; **table S4**), its superior PK profile achieved concentrations 100 times that of ivermectin at relevant dosing regimens, providing a longer lasting mosquitocidal effect. We predicted that mosquitocidal concentrations in human blood

384 after three daily doses of 1 mg/kg nitisinone or 0.6 mg/kg ivermectin would last for 16 or
385 10 days, respectively (**Fig. 2B**).

386 We aimed to demonstrate a mosquitocidal effect in older female mosquitoes since
387 these are potential malaria vectors. The lethality observed matched that of the younger
388 mosquitoes; the predicted nitisinone concentration at two-weeks post-administration
389 showed 100% mortality in less than 24 hours post bloodmeal ingestion. No mortality with
390 older mosquitoes was observed for ivermectin concentrations even at one-week post
391 drug administration. Compared to the lethality this concentration had on younger
392 mosquitoes at two-weeks post administration, this poses the important question of how
393 effectively could ivermectin eliminate older mosquitoes? When *An. gambiae* females take
394 a second bloodmeal, it shortens the malaria parasite's extrinsic incubation period by more
395 than two days, which increases the basic reproduction number (R_0) by 12.2% (*49*). We thus
396 predict nitisinone at these dosing regimens could reduce the R_0 as all mosquitoes,
397 regardless of age, die rapidly post-blood feeding.

398 We then confirmed the killing effect of nitisinone against an insecticide-resistant
399 *An. gambiae* strain (Tiassalé), which is representative of wild mosquito populations. We
400 determined that the metabolic resistance mechanisms in the Tiassalé strain did not
401 compromise the nitisinone-induced mosquitocidal effect. It remains unknown how
402 ivermectin would perform against other *An. gambiae* strains, but evidence with other
403 vector species suggests that strain or species-specific differences could exist. For example,
404 with *Glossina palpalis gambiensis*, ivermectin's lethality differed from a closely related
405 tsetse fly subspecies, *G. palpalis palpalis* (*50*). In contrast, ivermectin was equally effective
406 at killing two other tsetse fly species, as previously discussed (*35*).

407 At currently recommended dosing regimens, ivermectin does not meet several
408 attributes for a new human endectocidal drug (*24*). Among the 'minimum essential'
409 criteria, a sustained hazard ratio of 4 or above, for 28 days following administration, is
410 specified. In our current analyses, nitisinone (three daily doses at 1 mg/kg) was predicted

411 to achieve a hazard ratio above 4 for 16.2 days post-administration which, despite being
412 below the essential described criteria, was higher than our 14.7-day prediction for the
413 highest dose of ivermectin (three daily doses at 0.6 mg/kg) (**table S5**).

414 We showed, by PK/PD modeling, that the concentration of nitisinone in human
415 blood, at recommended dosing regimens, remained above its EC_{50} for longer than
416 ivermectin. This sustained activity is due to the low volume of distribution and slow
417 clearance from the bloodstream, as the half-life of nitisinone is 54 hours (*51*). To compare,
418 although the half-life of ivermectin in human plasma averages 18 hours, the terminal half-
419 life of a 0.6 mg/kg ivermectin dose is approximately 100 hours (*22*). Previous PK/PD
420 modeling has shown that high, repeated or sustained doses of ivermectin are necessary
421 to be mosquitocidal (*52*).

422 Alongside efficacy and duration, the impact of an endectocide also depends on the
423 proportion of people eligible for drug administration (*53, 54*). Several groups are ineligible
424 to receive ivermectin, including young children and newborns; licensing is indicated for
425 individuals above 15 kg in weight only (*37*). Ivermectin treatment is also not
426 recommended for pregnant women as limited data exists to determine safety to the
427 unborn fetus (*55*). Furthermore, in areas where malaria and the filarial worm *Loa loa* co-
428 exist, life-threatening adverse reactions (i.e. encephalopathy) in patients infected with a
429 high burden of *Loa loa* microfilariae can result from ivermectin treatment (*56*). However,
430 in general, ivermectin is successfully administered to millions of people throughout its
431 mass drug administration for neglected tropical diseases and has a well-established safety
432 profile.

433 The safety information on nitisinone differs due to the rarity of disorders it is
434 indicated for and the resulting smaller patient cohorts. Nitisinone is licensed for use in
435 newborns and young children (*57*) and is prescribed for chronic use in patients with
436 alkaptonuria or tyrosinemia type 1 (*31, 58*). No harmful events during pregnancy have yet
437 been documented (*59-61*). The adverse events associated with long-term nitisinone use

are likely caused by dose-dependent increases in tyrosine metabolites accumulating in both serum and tissues (*62, 63*). Increasing serum tyrosine concentrations can cause a reversible ocular tyrosine keratopathy (*64*) and cognitive impairment in some patients (*65*). The wider effects of long-term tyrosine accumulation in other tissues and major organs remain unknown. Beyond drug safety concerns, the monitoring of tyrosine concentrations, as well as platelet and white blood cell counts, present substantial challenges. Furthermore, the necessary restricted dietary protein intake accompanying nitisinone therapy also complicate nitisinone administration in malaria-endemic regions.

There are limitations to our study. We emphasise the limitation of safety profiles for higher nitisinone doses and lack of safety data from healthy populations. However, an encouraging six-year retrospective study of 63 patients undergoing treatment for alkaptonuria demonstrated that nitisinone (at 2 mg/day) did not affect cognitive function despite the prevalence of nitisinone-induced hypertyrosinemia (*66*). Forthcoming safety data is expected from other studies repurposing nitisinone to treat phenylketonuria (*67*), oculocutaneous albinism (*68*), Hawkinsinuria (*69, 70*), metastatic neuroblastoma (*71*), osteoarthritis (*72*), colon cancer (*73*), and Parkinson's disease (*74*). These safety concerns further justified simulating the mosquitocidal potency of lower nitisinone doses and validating predictions using blood drawn from alkaptonuria patients undergoing low-dose nitisinone therapy. Further limitations to our work include the concentration-effect curves generated from pooled data and different generations of mosquitoes. Multiple experiments also resulted in variation that increased the uncertainty of the PD parameters. Furthermore, we used plasma concentration as a surrogate for blood concentration and assumed that the blood to plasma ratio for both drugs was close to 1.

The recommended nitisinone dose to treat chronic conditions ranges from 1 to 2 mg/kg per day (*57*). All clinical trials involve long term daily administration, including some trials using up to 10 mg/kg per day (*31*). With safety issues associated with higher doses in mind, and FDA reports of thrombocytopenia and leucopenia in some patients

using nitisinone, we assessed the feasibility of using lower dosing regimens. We predicted that nitisinone at lower doses remained mosquitocidal; a single dose of 0.1 mg/kg killed mosquitoes for approximately 5 days, compared to no mortality observed with any single dose of ivermectin (**table S10**).

Unlike tyrosinemia patients, individuals with alkaptonuria are prescribed a daily dose of only 2 mg nitisinone, regardless of body mass. This dose, equivalent to 0.029 mg/kg (for a 70 kg person), is substantially lower than the highest prescribed dose of 10 mg/kg for treating severe tyrosinemia type 1. By obtaining freshly donated blood from alkaptonuria patients, coupled with full blood analysis to confirm drug and metabolite blood concentrations, we confirmed that ingesting 2 mg nitisinone daily produced a mosquitocidal effect in human blood samples (**Fig. 5**). Blood from patient 2 (with a nitisinone concentration of 593 ng/mL) retained mosquitocidal activity even when diluted 1:5 (estimated concentration 119 ng/mL). It is important to compare this with the non-mosquitocidal blood from patient 4 that did not contain nitisinone. This indicated that alkaptonuria-related metabolites alone were not toxic to mosquitoes. However, as with all nitisinone therapies, tyrosine accumulates in human blood (**Table 2**) and in mosquitoes taking a human blood meal that contains nitisinone (**Fig. S5**). Interestingly, when mosquitoes were fed human blood dosed with tyrosine, their survival was not compromised (**Fig. 6B**). Hypothesising that increased tyrosine may enhance the efficacy of nitisinone, tyrosine was fed alongside nitisinone to mosquitoes. Again, mosquito mortality was not increased by the presence of tyrosine, suggesting that tyrosine itself did not enhance the lethality of nitisinone.

Despite initial safety concerns with nitisinone, a sustained mosquitocidal effect after a single dose warrants further investigation. As our modeling studies suggest, a 10-fold lower dose of 0.1 mg/kg of nitisinone could remain mosquitocidal for five days (**table S10**). Furthermore, the insecticidal activity of blood samples from patients receiving nitisinone provides an *in vivo* proof of concept.

With the annual high cost of nitisinone therapy, ~$11,000 to treat alkaptonuria and ~$380,000 to treat tyrosinemia type I *(75, 76)*, the advantages of repurposing nitisinone for mosquito control could contribute to reducing the drug's cost and increasing its availability for rare diseases. As such, nitisinone-based vector control tools may provide a complementary approach to treating malaria in malaria endemic areas.

MATERIALS AND METHODS

Study design

The overall objective of this study was to demonstrate the mosquitocidal effect of nitisinone against female *An. gambiae* mosquitoes, compared to ivermectin. There were a number of experimental variations including testing mosquitoes of different ages and insecticide-resistant strains. The selected treatments and doses of nitisinone and ivermectin included therapeutically relevant concentrations of both drugs when fed to mosquitoes in blood-feeding experiments. The mosquito survival data were combined with pharmacokinetic/pharmacodynamic modeling data that simulated the mosquitocidal profile for the concentration curve for each drug.

The study was based on calculations from the original mosquito blood feeding experiments for ivermectin (*21, 23, 24*). The experimental study design was guided by a survival power analysis using pilot data from previous experiments performed in our laboratory with mosquito survival data at a nitisinone dose of 37 ng/mL. The power analysis (powerCT function in R) showed that to obtain a power of 80% to observe a hazard ratio of 1.5 or more between the negative control and any treatment group with $p<0.05$, would require 101 mosquitoes in each arm and each experiment needed to be run for a minimum of 14 days. Data collection either ended when all fed mosquitoes had died in an experimental arm, or the recording period exceeded 14 days. The inclusion criteria for data were the fed status of female mosquitoes. Mosquitoes had to be fully

519 engorged for inclusion in the mosquito survival analysis counting, which is why they
520 were separated within an hour of feeding. The exclusion criteria were any non-blood fed
521 or partially blood-fed mosquitoes. Any survival data outliers were not eliminated from
522 survival data analysis and are visible in some survival curves. The endpoint of mortality
523 was selected as the outcome of interest for an endectocidal drug.
524
525 **Randomization**
526 Mosquitoes of varying age (from day of emergence) were evenly distributed into
527 experimental sample cages and randomly assigned to each treatment group.
528 The initial experiments were not blinded, partially due to the large difference in drug
529 concentrations selected for each experiment (10-fold difference). The data from blood
530 samples of alkaptonuria patients were blinded as the blood samples were collected
531 independently at the clinic, and the serum analysis data by mass spectrometry was not
532 received until weeks after the blood-feeding experiment was complete.
533
534 **Mosquito feeding experiments**
535 *An. gambiae* mosquitoes (Kisumu strain) were reared at the Liverpool Insect Testing
536 Establishment (LITE). This strain originates from Kenya (*77*) and it is susceptible to all
537 insecticides. The Tiassalé strain originates from Côte d'Ivoire and is resistant to several
538 insecticide classes including pyrethroids (*43*). Experiments 1, 2 and 3 were performed
539 using female mosquitoes (Kisumu) aged 3-5 days old. Experiment 1, as a pilot, tested a
540 wide range of concentrations of each drug (nitisinone: 50-10,000 ng/mL, ivermectin: 15-
541 5000 ng/mL), whereas experiment 2 (replicated three times) focused on concentrations
542 that corresponded to biologically relevant concentrations for each drug (nitisinone: 3.8-
543 3500 ng/mL, ivermectin: 1.5-125 ng/mL). Experiment 3 tested a narrow range of
544 nitisinone concentrations based within the steep slope of the concentration-effect curve
545 of nitisinone (100-250 ng/mL) to better resolve mosquito killing dynamics (Hill slope).

546 The same biological concentrations from experiment 2 were also tested in an
547 experiment on older female mosquitoes (9-13 days old), to explore if age-related
548 changes to mosquito immunity (senescence) would impact susceptibility to ivermectin
549 and nitisinone. These same concentrations were also used to test Kisumu and Tiassalé
550 strain female mosquitoes aged 3-5 days. The experiment using alkaptonuria blood
551 samples was performed with 3-5 day old female *An. gambiae* (Kisumu strain)
552 mosquitoes. To discern any additional effect of excess tyrosine on the nitisinone-
553 induced killing effect, three replicate experiments were performed on 3-5 day old
554 female *An. gambiae* (Kisumu strain) mosquitoes. The experiment included a nonlethal
555 (3.8 ng/mL), sublethal (37 ng/mL) and lethal dose of nitisinone (250 ng/mL). Two
556 bloodmeals were supplemented with concentrations of tyrosine alone that were
557 considered to be physiologically low and high (0.036 mg/mL, 200 μM and 0.109 mg/mL,
558 600 μM, respectively) and mosquito survival was compared to a control bloodmeal
559 without any tyrosine or nitisinone. The low and high tyrosine concentrations were
560 selected according to previous clinical blood serum tyrosine measurements (*31*).
561 Bloodmeals with nonlethal or sublethal doses of nitisinone were supplemented with
562 both low and high concentrations of tyrosine, and mosquito survival was compared to
563 that for bloodmeals containing nitisinone alone.
564 For each experiment, test mosquitoes were allowed to acclimatise to the testing
565 insectary for 48 hours prior to membrane feeding experiments using the Hemotek-
566 cellulose membrane feeding system. The mosquitoes were fed on sterile human
567 reconstituted plasma with red blood cells (National Blood Authority NC15 and NC05)
568 supplemented with known concentrations of either nitisinone, ivermectin or PBS (vehicle
569 control). Mosquitoes were fed for 30 minutes, and unfed mosquitoes were removed and
570 discarded. Mosquito counts were performed each day for two weeks. During this period,
571 the mosquitoes were maintained at 27°C, 80% humidity with a fixed light-dark cycle

(12h/12h) and were provided daily with fresh sugar (10% sucrose). All experiments were conducted at the Liverpool School of Tropical Medicine, UK.

Drug preparation

Five milligrams of nitisinone (2-[2-nitro-4-(trifluoromethyl) benzoyl] cyclohexane-1,3-dione; PESTANAL, analytical standard; Sigma-Aldrich) was dissolved in 600 µL of 1 M NaOH, then pH balanced with 100 µL of 6M HCl and brought up to 1 mL with 300 µL sterile PBS. The final stock concentration of nitisinone was 5 mg/mL. Aliquots of 50 µL were stored at -20˚C for up to one year. Ivermectin (Pharmaceutical Secondary Standard; Merck) was freshly solubilised in 95% ethanol (w/v) to make a stock concentration of 5 mg/mL.

Alkaptonuria patient blood sample preparation

Blood samples were donated by four anonymized participants from the National AKU Centre at the Royal Liverpool Hospital following informed consent and ethical committee approval (21/PR/1622, 23rd December 2021, IRAS project ID: 305562). Each enrolled participant had been receiving daily doses of 2 mg nitisinone for at least three years. Donor blood was collected into 9 mL VACUETTE CPDA tubes (Greiner Bio-One, Item No.: 455056) containing the anticoagulant citrate phosphate dextrose to match the anticoagulant in the diluent control blood from the National Blood Authority. Samples were diluted 1:2, 1:5 and 1:10 using control blood as diluent.

Mosquito Survival Analysis

Survival time (days) for female *An. gambiae* mosquitoes was defined as the time from blood feeding (time zero) to mosquito death during a 14-day period. Survival rates were plotted using the Kaplan-Meier method and compared using log-rank test. Survival time was analyzed using a Cox regression model, from which the hazard ratio (HR) between

each dose and control group was calculated for all experiments separately. The calculated hazard ratios of mortality for dose groups for both nitisinone and ivermectin from the experiments were combined to construct a non-linear squares concentration-effect curve unique to each drug. All statistical analyses were performed in R 3.5.3 (*78*).

PD Modeling

A concentration-effect relationship was created for each drug by using an E_{max} sigmoidal equation where drug concentration was related to its corresponding HR compared to control. The concentration-HR observed values were fitted according to Equation 1.

$$HR = \frac{HR_{max} \cdot C^h}{EC_{50}{}^h + C^h} + HR_{min} \qquad \textbf{Equation 1}$$

$\boldsymbol{HR_{max}}$ is the maximal HR value possible, $\boldsymbol{EC_{50}}$ is the concentration required to achieve half the maximal HR and ***h*** is the Hill slope, which describes the steepness of the concentration – effect curve. The $\boldsymbol{HR_{max}}$ was fixed to 20 to exclude exaggerated HRs achieved due to observations of 100% mortality in under 24 hours for higher drug concentrations. $\boldsymbol{HR_{min}}$ is the background natural death of mosquitoes and was fixed to 1 (i.e., the model assumes that the minimum possible drug effect causes mosquito mortality identical to that observed in the control). Data fitting was performed in R 3.5.3 (*78*) using non-linear regression through the R base nls function.

PK Simulations

PK and PK/PD simulations were performed using R through the IQRtools (v. 1.4.0) package (*79*), according to the protocol previously described (*38*). PK parameters were derived from the literature for nitisinone (*51*) and ivermectin (*38*) and are displayed in **table S4**. The simulations were performed using the differential equations:

$$\frac{dX_1}{dt} = -k_a \cdot X_1 \qquad \textbf{Equation 2}$$

$$\frac{dX_2}{dt} = k_a \cdot X_1 - \left(\frac{CL + Q}{V_c}\right) \cdot X_2 + \frac{Q}{V_p} \cdot X_3 \qquad \textbf{Equation 3}$$

$$\frac{dX_3}{dt} = \frac{Q}{V_c} \cdot X_2 - \frac{Q}{V_p} \cdot X_3 \qquad \textbf{Equation 4}$$

$$C_{PK} = \frac{X_2}{V_c} \qquad \textbf{Equation 5}$$

X_1, **X_2** and **X_3** are the drug concentrations in the human gut, blood and peripheral compartments, respectively. **k_a** is the rate of drug absorption from the gut to the blood in hours, **CL** is the overall drug clearance from the systemic compartment, **Q** is the intercompartmental clearance between the central and peripheral compartments and **V_p** is the volume of the peripheral compartment. **C_{PK}** is the predicted drug concentration in the blood at any given point of time.

The PK/PD simulations convert the changing predicted PK concentration over time (C_{PK}) (equations 2 to 5) to a predicted HR over time by substituting C with C_{PK} in Equation 1 at every time point in the simulation. A population simulation was generated for specified dosing regimens to provide comparative outcomes, as a result of administration with the specified drug and dosing regimen. Monte-Carlo population simulations were generated for a population of 1000 people each weighing 70 kg under a log normal distribution.

Metabolomics analysis

One cage of mated *An. gambiae* Kisumu females was allowed to feed for one hour on a solution containing 50 mg/mL bovine serum albumin fraction V (BSA), nitisinone (100 ng/mL) diluted in PBS (treated). Another age-matched cage was only fed 50 mg/mL BSA/PBS (control). As protein alone can activate nitisinone killing in blood-feeding insects (35), BSA was selected to reduce the complexity of blood-derived metabolites that could obscure the accuracy of the metabolomic assessment for these experiments. A fully engorged female *An. gambiae* mosquito visibly swells with approximately 4 µL of blood (80), thus unfed mosquitoes, even feeding on serum, are easy to identify and were immediately removed from the experimental cages.

Nitisinone-treated mosquitoes first lose the ability to fly and then rapidly progress to full paralysis and death. After 24 hours post feeding, whole mosquitoes were collected in pools of ten (four technical replicates per group) and snap frozen in liquid nitrogen. The three groups of mosquitoes collected were sorted according to treatment phenotypes: **Group A:** complete knockdown (fully paralyzed mosquitoes lying on their backs with legs twitching); **Group B:** partial knockdown (mosquitoes could still weakly move and were collected while clinging to cage walls and ceiling); **Group C:** from control cage (nitisinone-free mosquitoes). Dead mosquitoes were not analyzed.

Frozen samples were shipped to the Glasgow Polyomics Facility on dry ice and processed in house. Briefly, samples were ground on dry ice using a handheld homogeniser (VWR, Cordless Motor - Pestle Motor, Model 47747-370) and the homogenate was re-suspended in 200 µL of extraction solvent (chloroform/methanol/water, 1:3:1, V/V/V). All samples, including blanks, were left on a shaker at 4°C for one hour, centrifuged at 16,000 x *g* for 10 min at 4°C and the supernatant was transferred into a fresh tube. Quality control samples were generated by pooling 10 µL of each sample. Samples were stored at -80°C until further LC-MS analysis. Metabolomics samples were separated using Q-Exactive Orbitrap mass spectrometer (ThermoFisher) in both positive and negative modes

(switching mode) and coupled to a high-performance liquid chromatography (HPLC) system (Dionex) with a ZIC-pHILIC column (Merck SeQuant) as described before (81). Raw data were acquired as previously described (82) and further processed using XCMs and mzMatch (peak picking and peak matching, respectively). Metabolite identification and relative quantitation was performed using the IDEOM interface (83). Fragmentation patterns were integrated in the analysis using the polyomics integrated metabolomics pipeline (84). Data were deposited into the MetaboLights repository (https://www.ebi.ac.uk/metabolights/) under accession number MTBLS11617.

Statistics

The statistical test used for determining significance was a Cox regression analysis that used the control sample as the comparator. P values were obtained from the Cox regression analysis in R. In addition, p values for Cox-rank tests were applied to the mosquito survival data in the survival curves. The 5% and 95% confidence intervals were generated during the Cox regression analysis. Additionally, the PK/PD modeling applied to simulate the PK/PD curves were the appropriate methods to use, based on previously published modeling on ivermectin (22). The confidence intervals were generated during the Cox regression hazard ratio analysis. The generation of survival data was consistent with proportional hazards in a Cox regression. R code is held in stored in Zenodo (10.5281/zenodo.14219339, https://zenodo.org/records/14872460).

SUPPLEMENTARY MATERIALS

Materials and Methods

Figs. S1 to S7

Tables S1 to S13

Reproducibility checklist

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FIGURE CAPTIONS

Fig. 1. Mosquito survival after a bloodmeal containing nitisinone or ivermectin. Mosquito survival after consuming a bloodmeal containing either nitisinone or ivermectin at a range of concentrations based on predicted clinical human blood concentrations at

7, 14, 21 and 28 days of drug treatment. Kaplan-Meier survival curves show three replicate experiments measuring mosquito survival over 14 days after feeding on a bloodmeal containing four concentrations of nitisinone **(A, C, E)** or five concentrations of ivermectin **(B, D, F)**. Nitisinone doses (ng/mL): 3.8 (orange), 37 (teal), 350 (yellow) and 3500 (dashed dark blue); ivermectin doses (ng/mL): 1.5 (orange), 3.5 (light blue), 8.5 (teal), 20 (yellow) and 125 (dashed dark blue). The effect of drug-containing bloodmeals for both nitisinone and ivermectin were compared to control mosquitoes fed a drug-free blood meal (black). P values were calculated from log-rank tests to determine the significance of the difference in survival between the control group and the drug group (n=3485 mosquitoes analyzed; N=3 experiment replicates).

Fig. 2. Concentration-effect relationship of nitisinone and ivermectin and their predicted clinical exposure and effect. (A) Concentration-effect relationship for nitisinone (brown) and (ivermectin (blue). Y-axis represents hazard ratio values from Cox regression data **(tables S1, S2 and S3)**. Hazard ratio values were capped at a value of 20 for comparison. Vertical dashed lines represent the estimated EC_{50} concentration for each drug as predicted by the non-linear model and according to the E_{max} equation. (n=4979 mosquitoes analyzed; N=5 experiment replicates). **(B)** Simulated PK profile for ivermectin (blue) and nitisinone (brown) according to published PK parameters. The horizontal dashed lines show the EC_{50} for each drug. **(C)** Predicted Hazard Ratio (HR) value over time for nitisinone and ivermectin after drug administration. The dashed black line represents the HR cut-off value of 4. The lines in **(C)** and **(D)** are the medians of 500 simulations of a population of 500 people based on three-day dosing regimens for nitisinone (1 mg/kg/day) and ivermectin (0.6 mg/kg/day).

Fig. 3. Mosquitocidal efficacy of nitisinone versus ivermectin. (A) Shown is the predicted PK profiles for nitisinone (brown) and ivermectin (blue) in human blood when

the drug is administered for three consecutive days at doses of 1.0 mg/kg (nitisinone) or 0.6 mg/kg (ivermectin) to human patients. Predicted PK profiles were used to select drug concentrations expected to be observed in human blood after discrete time durations following drug treatment. The predicted concentrations, shown as points in the figure, were then used to compare mosquitocidal activity of the two drugs in older (9-13 days) female *An. gambiae* mosquitoes fed a bloodmeal containing either drug. (**B-E)** Comparison of the mosquitocidal activity of nitisinone and ivermectin in bloodmeals at concentrations that are predicted in human blood at days 3 (ivermectin only) and days 7, 14, 21 and 28 post-administration (ivermectin and nitisinone). **(F)** Overall prediction of drug activity (Hazard ratio) over time and the difference in mosquitocidal activity between nitisinone (brown) and ivermectin (blue). (n=3395 mosquitoes analyzed; N=3 experimental replicates).

Fig. 4. Survival of insecticide-resistant mosquitoes in response to bloodmeals containing different nitisinone concentrations. Survival of insecticide-susceptible (Kisumu strain) (**A, C, E**) and insecticide-resistant (Tiassalé strain) (**B, D, F**) *An. gambiae* mosquito strains after ingestion of nitisinone in a bloodmeal. Kaplan-Meier survival curves show mosquito survival over 14 days following the ingestion of a bloodmeal containing different concentrations (ng/mL) of nitisinone. Each panel represents one of three replicates. In all experiments, the effect of nitisinone-containing bloodmeals was simultaneously compared to control bloodmeals that did not contain nitisinone. P values were calculated using a log-rank test to determine the significance of the difference in survival between the control group and the nitisinone group. (n=4390 mosquitoes analyzed; N=3 experimental replicates).

Fig. 5. Mosquito survival after feeding on blood samples from alkaptonuria patients treated with nitisinone. (A) Predicted PK profile of the concentration of nitisinone in human blood over a one-month period at the therapeutic dose used to treat patients with alkaptonuria (AKU). The simulation is based on a 2 mg daily dosing regimen for nitisinone. The dashed line represents the predicted EC_{50}. **(B)** Predicted PD profile with the same dose of 2 mg per day over one month. The dashed line represents the HR cut-off value of 4. **(C-F)** Kaplan-Meier curves show mosquito survival over 14 days after feeding on blood samples from three AKU patients (**C-E**) treated with 2 mg/ml nitisinone daily for at least one month prior to the experiment or blood samples from one patient with AKU (**F**) who had not yet started nitisinone treatment. In all experiments, the effect of nitisinone-containing bloodmeals on mosquito survival was compared to that for a drug-free bloodmeal control. P values were calculated from log-rank tests. In panels **C, D, E**, nitisinone-containing blood dilutions are neat (orange), 1:2 (light blue), 1:5 (teal) and 1:10 (pink). In all panels, nitisinone-free control blood (from a donor without AKU) is shown in black. **(F)** Mosquito survival after feeding on a blood sample from an AKU patient yet to commence nitisinone treatment (purple). Nitisinone concentrations in AKU patient blood 24 hours prior to donation were measured by mass spectrometry: 493.85 ng/mL (Patient 1), 592.61 ng/mL (Patient 2), 428 ng/mL (Patient 3), 0 ng/mL (Patient 4) (**Table 2**). (n=1843 mosquitoes analyzed; one experiment).

Fig. 6. Mosquito survival after taking bloodmeals containing nitisinone and tyrosine. Survival of insecticide-susceptible female *An. gambiae* (Kisumu strain) mosquitoes fed with bloodmeals containing nitisinone in the presence of increasing concentrations of tyrosine. **(A)** Survival of mosquitoes fed bloodmeals containing 3.7 ng/mL (orange), 37 ng/mL (light blue) or 250 ng/mL (teal) nitisinone compared to a control nitisinone-free bloodmeal (black). **(B)** Survival of mosquitoes fed bloodmeals containing a low (0.036 mg/ml, orange) or high (0.109 mg/ml, light blue) tyrosine concentration compared to a

tyrosine-free control bloodmeal (black). **(C)** Survival of mosquitoes fed a non-lethal dose of nitisinone (3.7 ng/mL) in combination with either a high (light blue) or low (orange) tyrosine concentration, compared to nitisinone alone (black). **(D)** Survival of mosquitoes fed a sublethal dose of nitisinone (37 ng/mL) in combination with either a high (light blue) or low (orange) tyrosine concentration, compared to nitisinone alone (black). Results are combined from three replicate experiments. P values were calculated using a log-rank test. (n=3059 mosquitoes analyzed; N=3 experimental replicates).

Tables

Table 1. Average of Hazard Ratios for nitisinone and ivermectin. Hazard ratios for female *An. gambiae* (Kisumu) mosquitoes fed a bloodmeal containing physiologically relevant concentrations of nitisinone or ivermectin were generated using a Cox regression analysis. Concentrations represent predicted concentrations in human blood at 7, 14, 21 and 28 days after oral ingestion of selected doses of either nitisinone (1 mg/kg/day) or ivermectin (0.6 mg/kg/day). Hazard ratios were averaged from data combined from three replicate experiments (**Fig. 1**) n = average number of mosquitoes tested. Hazard ratios and sample sizes are averages. Replicate-level Hazard ratios are displayed in **table S3**.

Nitisinone					**Ivermectin**				
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Median survival (days)**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**	**Median survival (days)**
Control	110	---	---	---	Control	110	---	---	---
3.8 (PK day 28)	117	0.71 [0.27-1.66]	0.285	---	1.5 (PK day 28)	120	0.77 [0.3-1.47]	0.467	---
37 (PK day 21)	114	1.30 [0.4-3.3]	0.26	---	3.5 (PK day 21)	139	1.49 [0.4-4.89]	0.206	---
350 (PK day 14)	107	86.24 [22.41-494.02]	**<0.0001**	0.86	8.5 (PK day 14)	99	6.44 [1.33-21.62]	<0.01	8.04
3500 (PK day 7)	111	93.49 [26.98-495.48]	**<0.0001**	0.86	20 (PK day 7)	118	17.34 [6.98-46.58]	**<0.0001**	3.53

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Table 2. Metabolic analysis of blood from patients with alkaptonuria. Blood donor information along with measured concentrations of nitisinone and metabolites were taken the day before patients donated blood that was used for feeding mosquitoes. At the time of blood procurement, Patients 1, 2 and 3 were on long-term treatment of 2 mg daily doses of nitisinone (confirmed by the expected nitisinone-induced hypertyrosinemia), whereas Patient 4 was about to commence nitisinone treatment (note elevated homogentisic acid, yet low tyrosine). BMI, body mass index; HGA, homogentisic acid, Tyr, tyrosine; PHE, phenylalanine; HPPA, hydroxyphenyl pyruvic acid; HPLA, hydroxyphenyl lactic acid.

Patient No.	Nitisinone (ng/mL)	Sex	BMI	Treatment length	HGA (µmol/L)	TYR (µmol/L)	PHE (µmol/L)	HPLA (µmol/L)	HPPA (µmol/L)
Patient 1	493.85	M	31.3	3 years	3.6	**883**	56	70	41
Patient 2	592.61	F	33.7	8 years	<3.1	**843**	39	25	57
Patient 3	428.00	M	31.4	9 years	8	**916**	52	46	31
Patient 4	***0***	*M*	*27.0*	***0***	***47.2***	*67*	*79*	*<5*	*<10*

Table 3. Average of Hazard Ratios for blood samples from alkaptonuria patients. 14-day Hazard Ratios for *An. gambiae* mosquitoes (Kisumu strain) fed blood obtained from three patients with alkaptonuria being treated with nitisinone (Patients 1-3) were generated using Cox regression analysis. Hazard ratios and sample sizes are averages. n = number of mosquitoes tested. Also see **Fig. 5** and **table S11**.

Alkaptonuria donor blood			
Nitisinone Conc. [ng/mL]	**n =**	**14-day HR [95 CI]**	**P value**

Control	161	---	---
Neat	136	**21.9** [4.68 – 54.8]	**<0.0001**
1:2	105	**16.2** [3.37 – 41.7]	**<0.0001**
1:5	122	1.84 [0.609 – 4.64]	**<0.318**
1:10	143	1.07 [0.57 – 1.92]	0.92

Table 4. Average Hazard Ratios for bloodmeals containing either tyrosine alone, nitisinone alone, or nitisinone in combination with tyrosine fed to mosquitoes in three replicate experiments. The 14-day Hazard Ratios were generated using Cox regression analysis for female *An. gambiae* (Kisumu strain) mosquitoes fed bloodmeals containing varying concentrations of either nitisinone, tyrosine or both (**Fig. 6**). Serum tyrosine concentrations considered to be high (0.109 mg/mL) or low (0.036 mg/mL) were determined from previous nitisinone clinical trial data (*31*). Both high and low doses of tyrosine were fed to mosquitoes alone or together with sublethal or nonlethal doses of nitisinone that were equivalent to predicted nitisinone concentrations in human blood 21 and 28 days after receiving three doses of nitisinone over three days (1 mg/kg x 3 days). A nitisinone concentration associated with the maximum killing effect was also included as a positive control (250 ng/mL)(**Fig 6A**). Replicate-level hazard ratios are displayed in **table S12.**

Nitisinone and/or tyrosine concentrations [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**
Control (no tyrosine or nitisinone)	108	---	---
Low tyrosine alone (0.036mg/mL)	93	1.38 (0.63-3.05)	0.495

High tyrosine alone (0.109mg/mL)	108	1.07 (0.47-2.42)	0.461
Nitisinone 3.8 ng/mL + low Tyr	91	1.21 (0.53-2.77)	0.412
Nitisinone 3.8 ng/mL + **high** Tyr	89	1.61 (0.76-3.46)	0.235
Nitisinone 37 ng/mL + low Tyr	90	1.24 (0.54-2.87)	0.589
Nitisinone 37 ng/mL + **high** Tyr	113	1.79 (0.85-3.74)	0.43
Nitisinone alone 250 ng/mL (C_{max})	119	**28.21** (15.38-51.9)	**<0.0001**
Nitisinone alone 37 ng/mL (PK day 21)	93	1.04 (0.46-2.42)	0.666
Nitisinone alone 3.8 ng/mL (PK day 28)	116	1.64 (0.78-3.46)	0.493

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Author contributions

LRH, MS, GA, and AA-S conceptualized this study. LRH, AT, MPB, LRR, GA, and AA-S developed the methodologies and designed the experiments. LRH, AT, CR, NG, DM, CR, MPB and GA performed all the experimental work. GA and AT performed the PK/PD modeling. LRH and NG ran the *Anopheles* metabolomics experiment. DM, CR and MPB conducted the in-depth metabolomics analysis. LRR provided access to alkaptonuria patients. LRH, MPB, LRR, GA, and AA-S supervised this study. AA-S, LRH, MPB, LRR, and AT acquired funding. GA, AT and LRH wrote the original drafts. LRH, AT, GA, MS, DL, JNB, GB, CR, MPB, LRR, and AA-S edited subsequent drafts. All authors proofread and approved the final manuscript.

Competing interests Authors declare that they have no competing interests,

Data and materials availability

All data needed to evaluate the conclusions of this study are in the main text or the supplementary materials. The R code is held in Zenodo (DOI 10.5281/zenodo.14219340) and other datafiles supporting the manuscript are available on Zenodo (A. Trett, 2024). Metabolomics raw data are deposited in the MetaboLights repository (https://www.ebi.ac.uk/metabolights/) under accession number MTBLS11617.

1194 Figure 1.

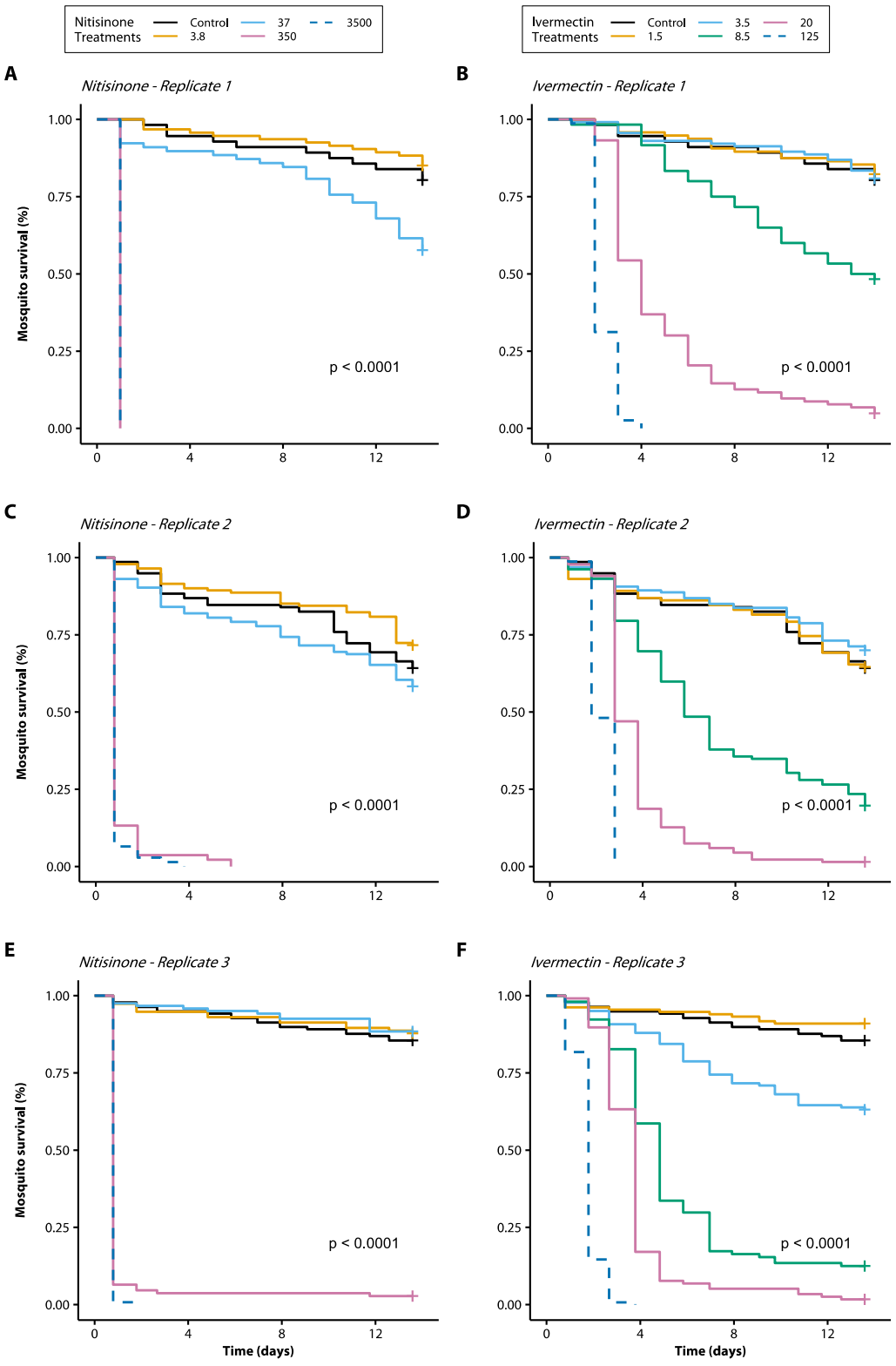

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Figure 2.

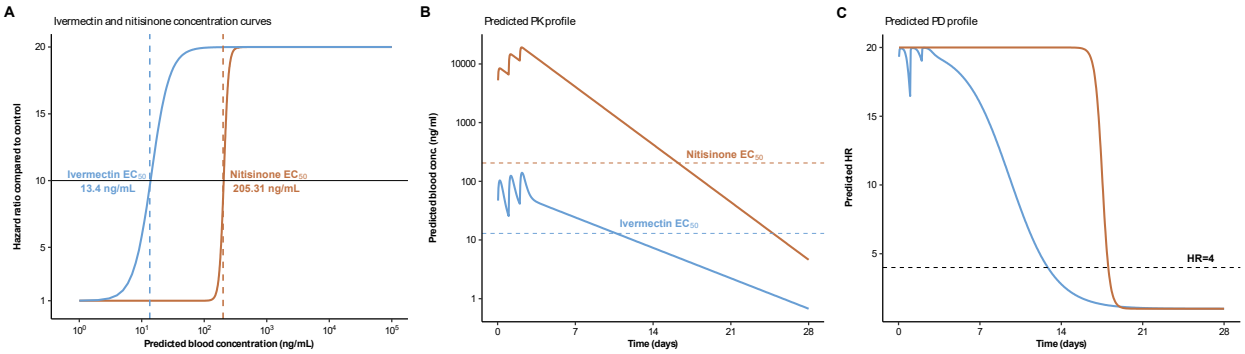
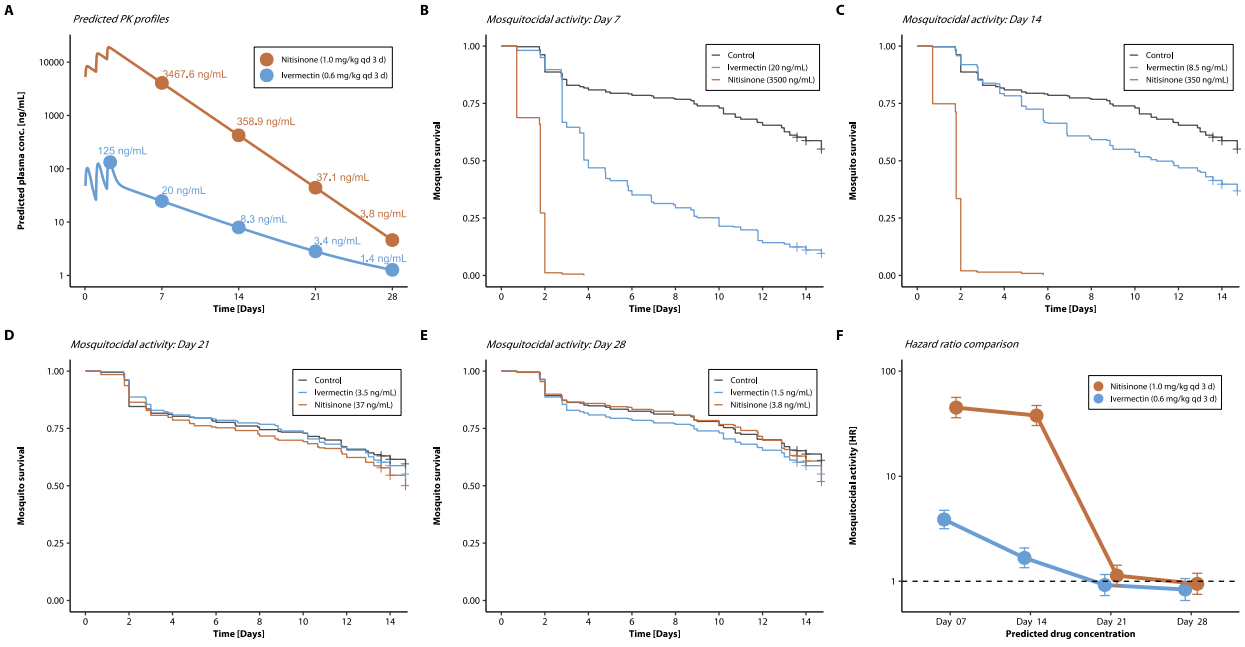
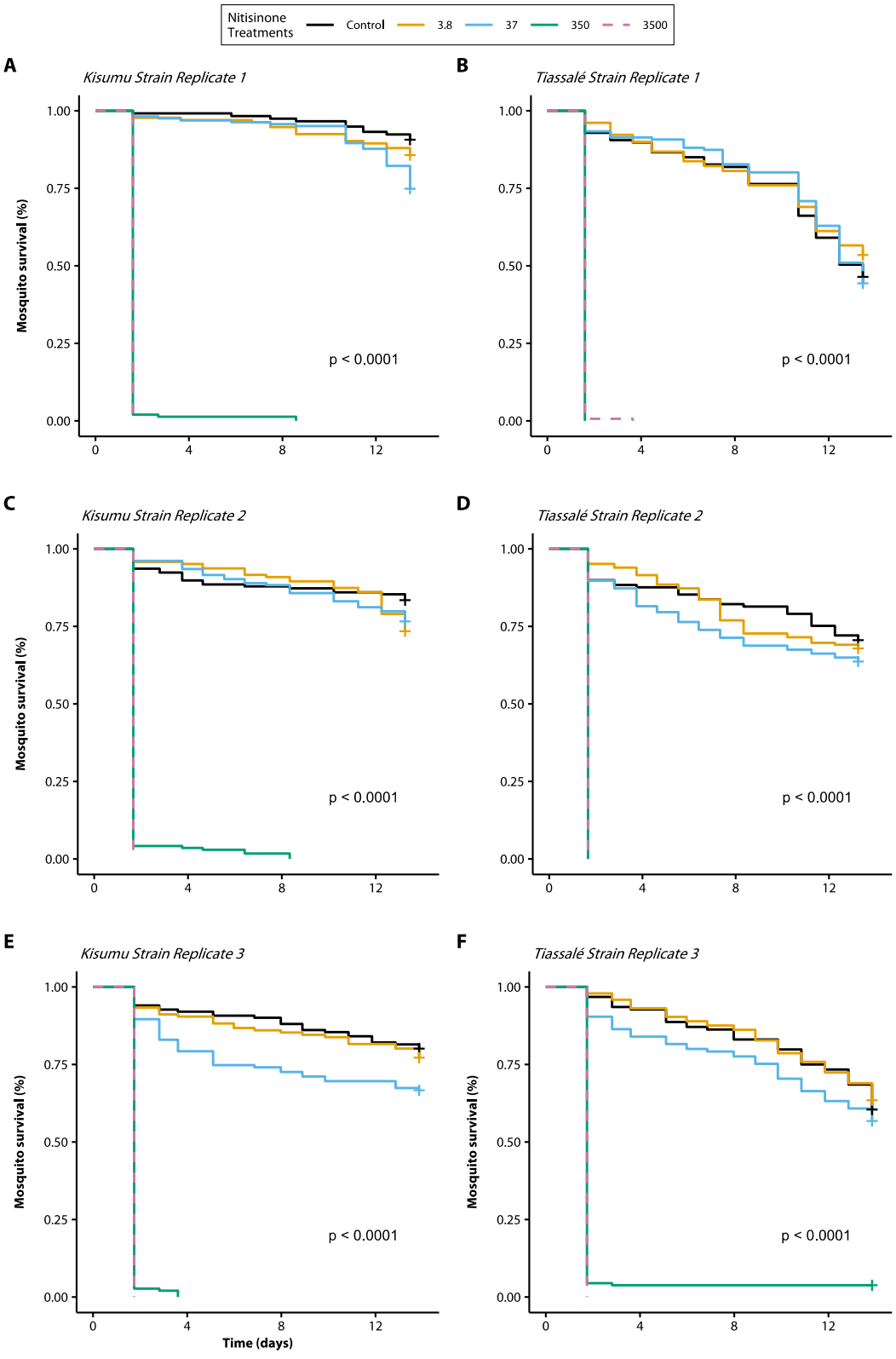


Figure 3.



1205 Figure 4.



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1207 Figure 5.

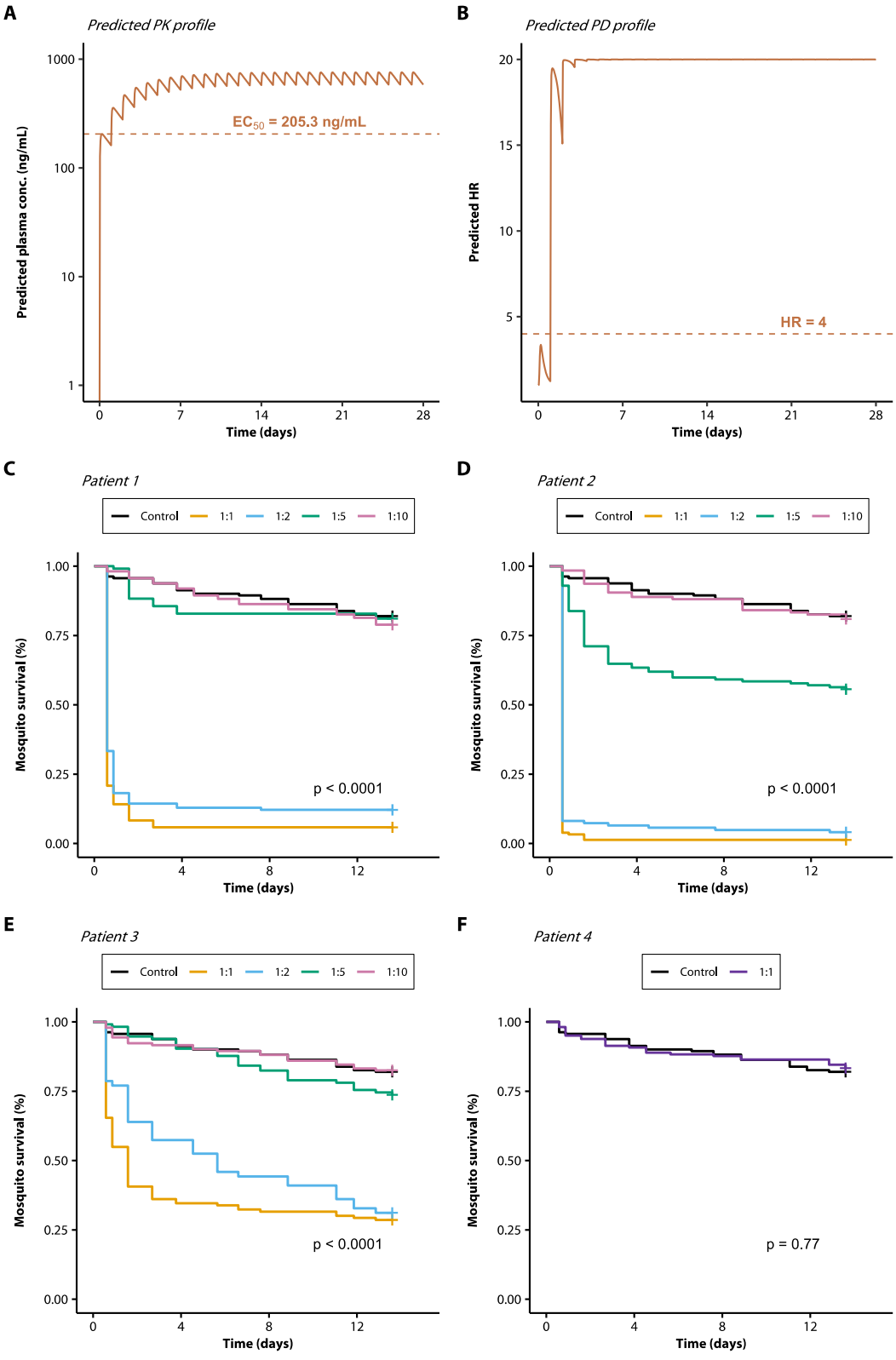

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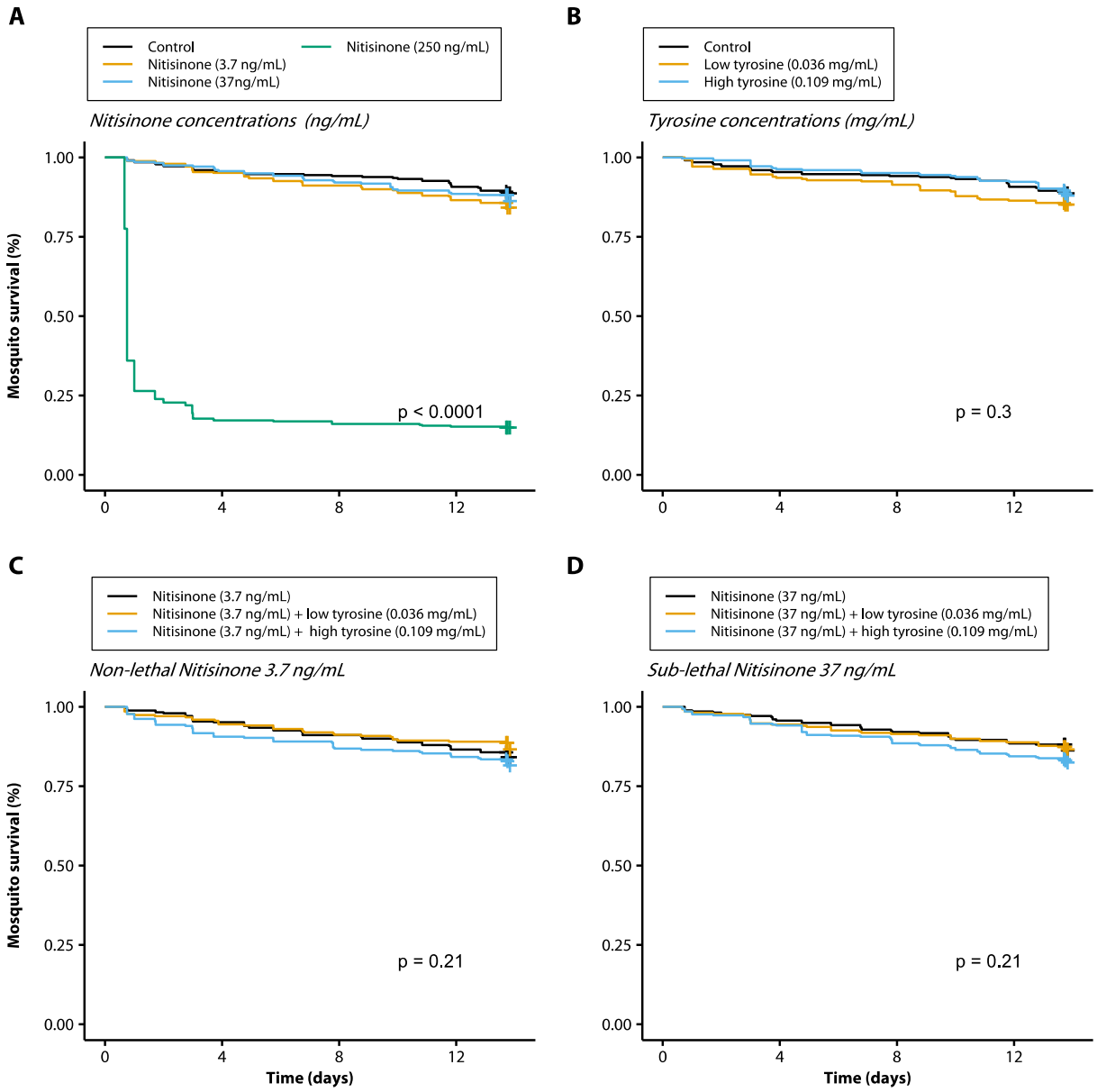
Figure 6.


A
Control
Nitisinone (3.7 ng/mL)
Nitisinone (37ng/mL)
Nitisinone (250 ng/mL)
Nitisinone concentrations (ng/mL)
Mosquito survival (%)
1.00
0.75
0.50
0.25
0.00
p < 0.0001
0
4
8
12
B
Control
Low tyrosine (0.036 mg/mL)
High tyrosine (0.109 mg/mL)
Tyrosine concentrations (mg/mL)
p = 0.3
C
Nitisinone (3.7 ng/mL)
Nitisinone (3.7 ng/mL) + low tyrosine (0.036 mg/mL)
Nitisinone (3.7 ng/mL) + high tyrosine (0.109 mg/mL)
Non-lethal Nitisinone 3.7 ng/mL
p = 0.21
Time (days)
D
Nitisinone (37 ng/mL)
Nitisinone (37 ng/mL) + low tyrosine (0.036 mg/mL)
Nitisinone (37 ng/mL) + high tyrosine (0.109 mg/mL)
Sub-lethal Nitisinone 37 ng/mL
p = 0.21
Time (days)

Anopheles mosquito survival and pharmacokinetic modeling show the mosquitocidal activity of nitisinone

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[†]These authors contributed equally to this work

This file includes:

Supplementary Methods

Figs. S1 to S7

Tables S1 to S13

Supplementary Methods

Chemical measurements

A freshly drawn aliquot of serum was acidified using perchloric acid (10% v/v 5.8 M) and frozen at -20°C. Metabolite concentrations in blood serum were measured by liquid chromatography tandem mass spectrometry (*63*). Analysis was performed using Agilent 6490 Triple Quadrupole mass spectrometer with Jet-Stream® electrospray ionisation (ESI-MS/MS) coupled with an Agilent 1290 infinity UHPLC pump and HTC autosampler. The method incorporates reverse-phase chromatographic separation on an Atlantis C18 column (100 mm x 3.0 mm, 3 µm) and initial conditions of 80:20 water:methanol with 0.1% formic acid (v/v) increased linearly to 10:90 over five minutes. Matrix-matched calibration standards and quality controls were utilized with appropriate isotopically labelled internal standards. Quantitation was achieved in multiple reaction mode (MRM) with two product ion transitions for both tyrosine (positive ionisation) and homogentisic acid (HGA) (negative ionisation). Samples were prepared by dilution in a combined internal standard solution (final concentrations of 0.4 µmol/L $_{13}C_6$-HGA and 2 µmol/L d_2-tyrosine in 0.1% formic acid 9 (v/v) in deionised water). All serum quantitation analysis was performed by the Department of Clinical Biochemistry and Metabolic Medicine at the Royal Liverpool University Hospital.

Metabolomics analysis

Mosquitoes were fed a sublethal dose (100 ng/mL) of nitisinone that was added to 50 mg/mL bovine serum albumin (BSA) reconstituted and filter sterilised in phosphate buffered saline (PBS). As protein alone can trigger nitisinone killing in blood-feeding insects (*35*), BSA was chosen to reduce the complexity of blood-derived metabolites that could obscure the accuracy of the metabolomic assessment for these experiments. In a preliminary experiment, we determined that mosquito mortality is comparable when 100 ng/mL nitisinone is added to either blood (34%) or 50 mg/ml BSA solubilised in sterile PBS (44%) within a 24 h period.

Two cages of mated, *An. gambiae* Kisumu females were allowed to feed for one hour on 50 mg/mL BSA/PBS, either with nitisinone (100 ng/mL) (treated) or without (control). A fully engorged female *An. gambiae* mosquito visibly swells with approximately 4 µL of blood or serum (*80*), thus unfed mosquitoes are easy to identify and were immediately discarded. Nitisinone-treated mosquitoes first lose the ability to fly and then rapidly progress to full paralysis and death. The three groups of mosquitoes collected for metabolomic analysis were grouped according to mosquito behaviour:

Group A- complete knockdown (completely paralysed mosquitoes lying on their backs with twitching legs);

Group B- partial knockdown (mosquitoes could still weakly move and were collected while clinging to cage walls and ceiling);

Group C- control (nitisinone-free mosquitoes were collected during flight).

Dead mosquitoes (blackened bloated abdomens) were not selected for analysis to avoid confounding death-associated data. After 24 hours, whole mosquitoes were collected in pools of ten (four technical replicates per group) and snap frozen in liquid nitrogen. Frozen samples were shipped to the Glasgow Polyomics Facility on dry ice and

processed in house according to well established protocols. Briefly, samples were ground on dry ice using a handheld homogeniser (VWR, Cordless Motor - Pestle Motor, Model 47747-370). Homogenate was re-suspended in 200 µL of extraction solvent (chloroform/methanol/water, 1:3:1, V/V/V). All samples, including blanks, were left on a shaker at 4°C for one hour, centrifuged at 16,000 *g* for 10 min at 4°C and the supernatant was transferred into a fresh tube. Quality control samples were generated by pooling 10 µL of each sample. Samples were stored at -80°C until further LC-MS analysis. Metabolomics samples were separated using Q-Exactive Orbitrap mass spectrometer (ThermoFisher) in both positive and negative modes (switching mode) and coupled to a high-performance liquid chromatography (HPLC) system (Dionex) with a ZIC-pHILIC column (Merck SeQuant) as described before (*81*). Raw data were acquired as previously described (*82*) and further processed using XCMs and mzMatch (peak picking and peak matching, respectively). Metabolite identification and relative quantitation was performed using the IDEOM interface (*83*). Fragmentation patterns were integrated in the analysis using the polyomics integrated metabolomics pipeline (*84*). Data were deposited into the MetaboLights repository (https://www.ebi.ac.uk/metabolights/) under accession number MTBLS11617.

Supplementary Figures

Cox regression analysis

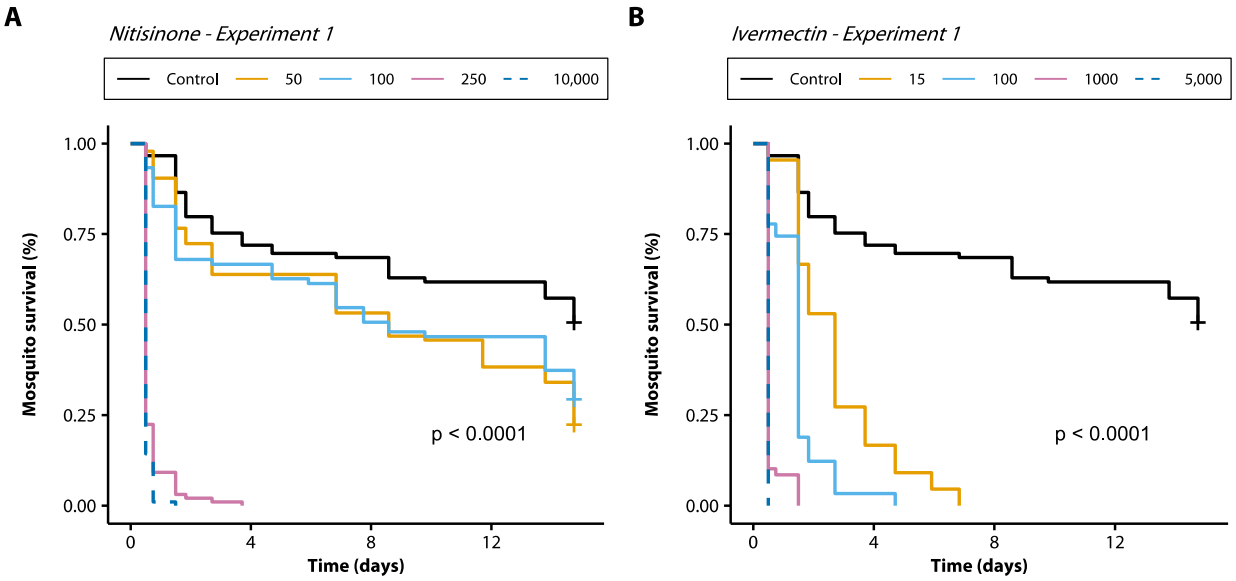


fig. S1. Mosquito survival after consuming a bloodmeal containing either a concentration series of nitisinone or ivermectin (Experiment 1). Kaplan-Meier curves show mosquito survival (y-axis) over 14 days (x-axis) after feeding on blood containing nitisinone **(A)** or ivermectin **(B)** at four initial concentrations (top panel legends show ng/mL). The effect of drugged bloodmeals were simultaneously compared to drug-free controls (black line). P values were calculated from log-rank tests.

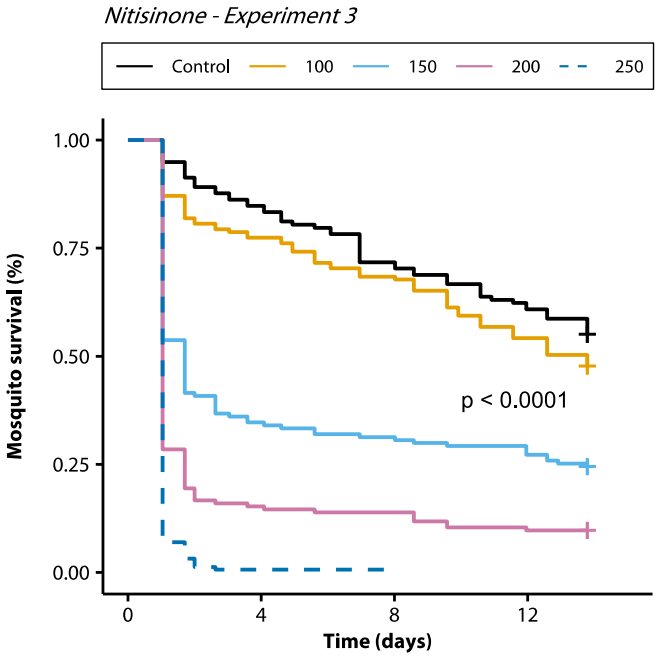


fig. S2. Mosquito survival after consuming a bloodmeal containing a narrow range of nitisinone concentrations. Kaplan-Meier curves show mosquito survival (y-axis) over 14 days (x-axis) after feeding on blood containing nitisinone at four concentrations (top panel legends show ng/mL). The effect of drugged bloodmeals were simultaneously compared to drug-free controls (black line). P values were calculated from log-rank tests.

PK modelling: Single dose simulations

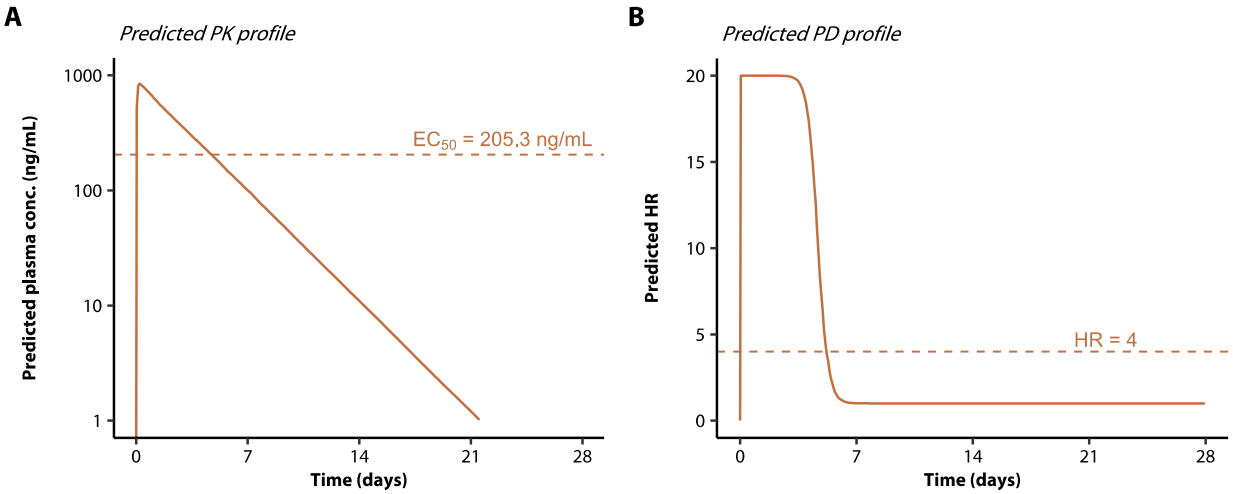


fig. S3. Predicted PK/PD profile for a single dose of 0.1 mg/kg nitisinone. Panel **(A)** shows the simulated PK profile according to its PK parameters. The horizontal dashed line shows the EC_{50} for nitisinone. Panel **(B)** shows the predicted Hazard ratio (HR) value for the dosing regimen over time. The dashed line represents the cut-off HR value of 4. The Monte Carlo population simulation was run for a population of 500 individuals weighing 70 kg.

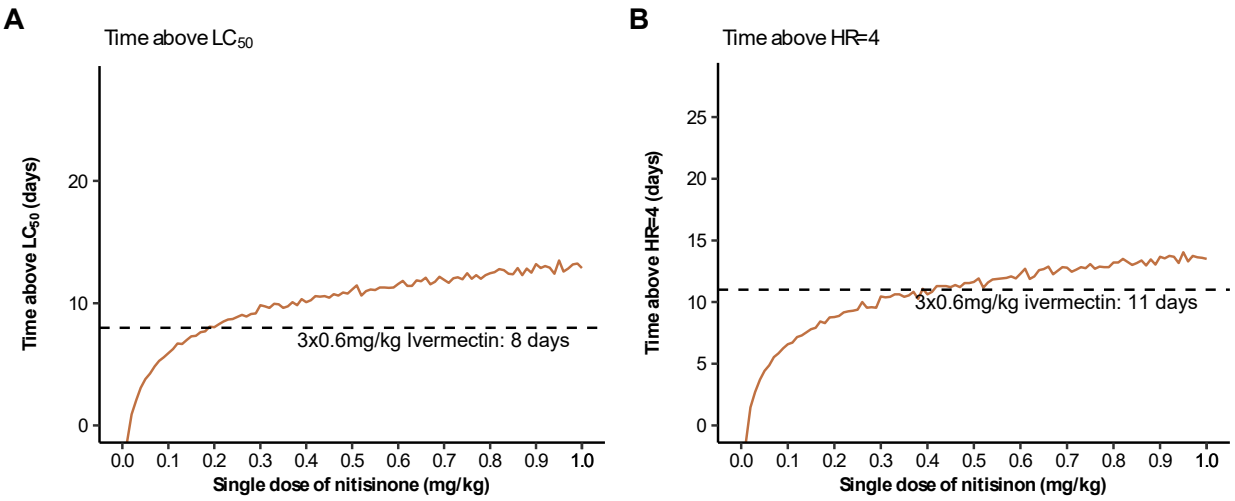
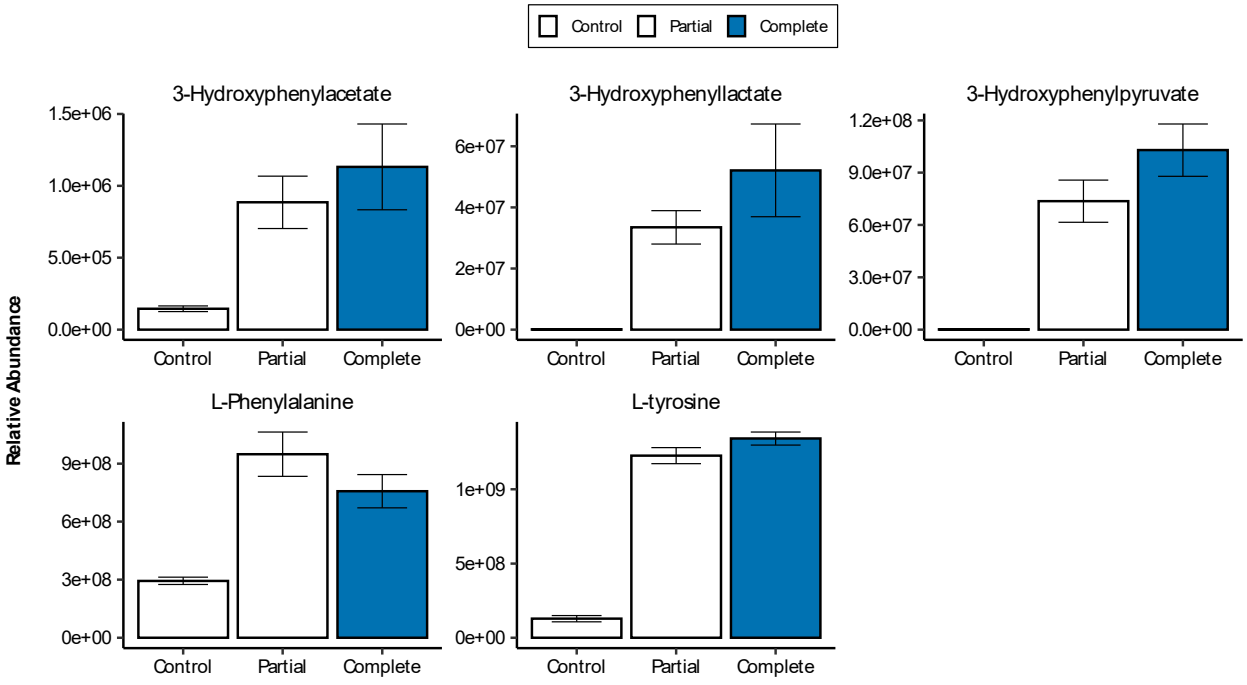


fig. S4. Predicted simulations of the PK and PD profiles for different doses of nitisinone in human blood. Simulations were run for a range of 100 single doses of nitisinone, ranging from 0.1 mg/kg to 1 mg/kg. Each Monte Carlo simulation was based on a population of 500 individuals each weighing 70 kg. **(A)** Predicted time nitisinone concentration lies above the LC_{50}. **(B)** Predicted time nitisinone concentration lies above a Hazard Ratio (HR) of 4**.** Darker shading represents the 25 - 75% confidence intervals and lighter shading represents the 5 - 95% confidence intervals. For comparison, the outcome of three doses of 0.6 mg/kg of ivermectin is indicated by the black dashed lines. PK/PD simulations are based on *in vitro* generated PD parameters and published PK parameters for both drugs (*38, 51*). The 25% and 75% confidence intervals were generated on the population average of simulations.



1354

1355 **fig. S5. Tyrosine catabolism pathway metabolites isolated from female *An. gambiae*, 24**
1356 **hours after ingesting a sublethal dose (100 ng/mL) of nitisinone.** The relative abundance of
1357 several key metabolites involved in tyrosine metabolism are compared between the groups:
1358 control mosquitoes (grey), partially paralyzed mosquitoes (yellow) and mosquitoes completely
1359 paralysed (blue). See **table S13** for statistical analysis.

1360

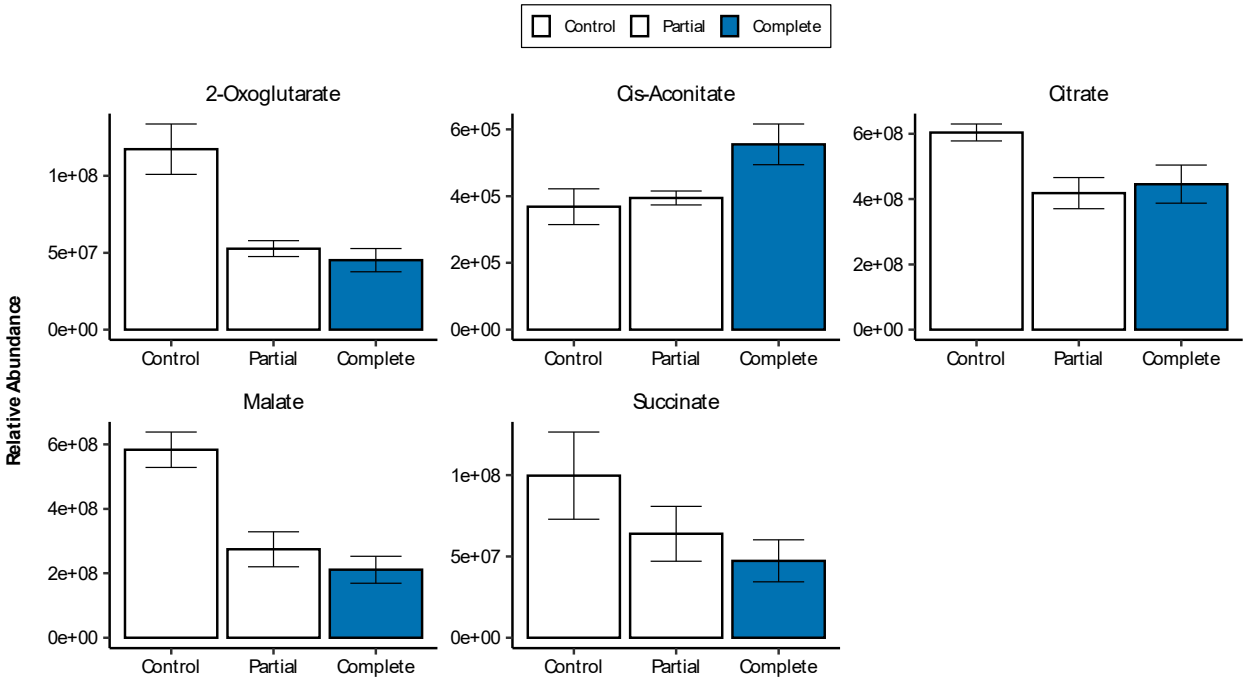


fig. S6. Citric acid cycle metabolites isolated from female mosquitoes 24 hours after ingesting nitisinone. The relative abundance of five metabolites involved in the citric acid cycle are compared between the groups: control mosquitoes (grey), partially paralysed mosquitoes (yellow) and mosquitoes completely paralysed (blue). See **table S13** for statistical analysis.

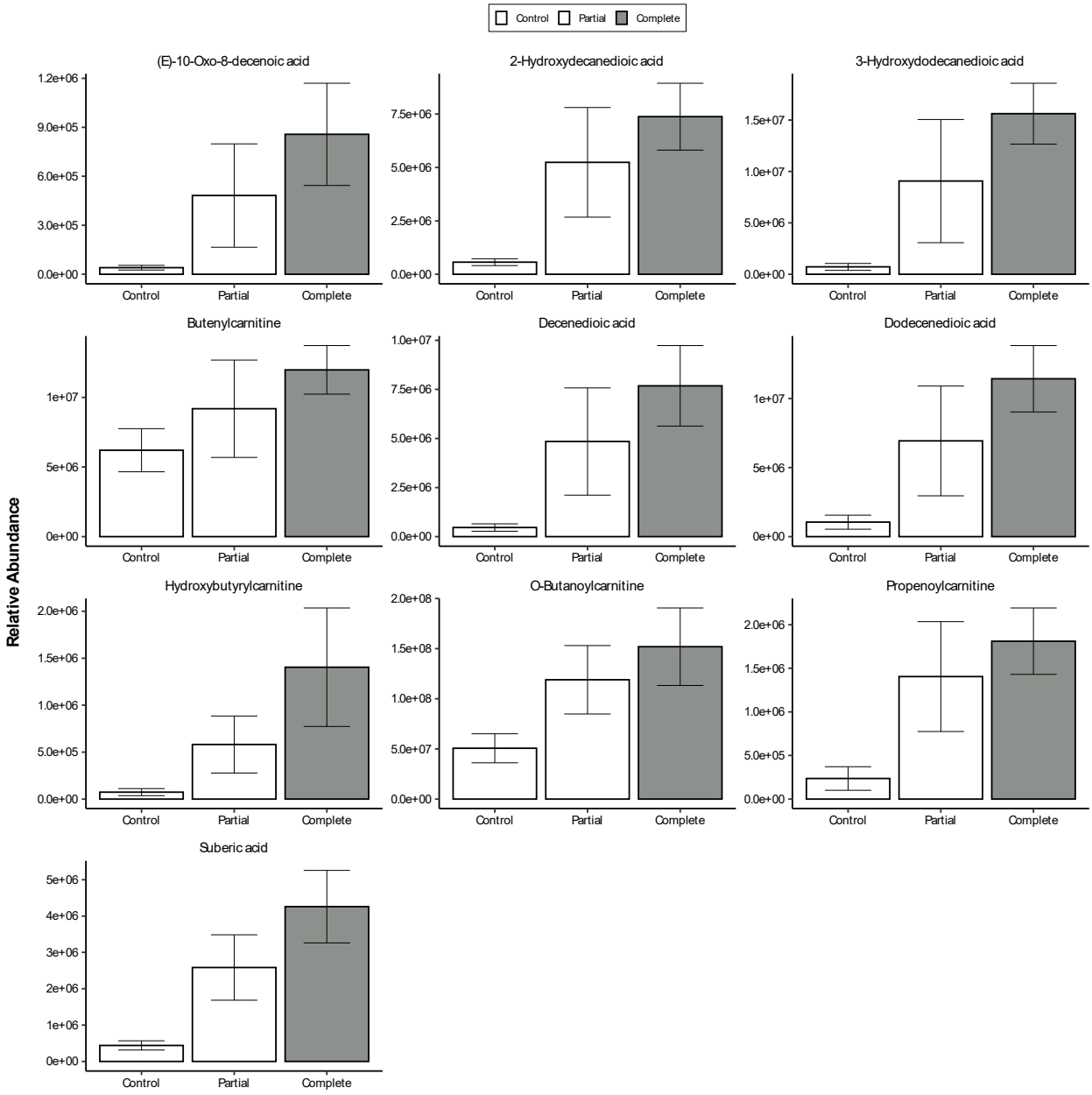


fig. S7. Lipid species detected in female *An. gambiae* mosquitoes, 24 hours after ingesting nitisinone. The relative abundance of nine metabolites linked to lipid metabolism are compared between the groups: control mosquitoes (lightest grey), partially paralysed mosquitoes (medium grey) and mosquitoes with full paralysis (darkest grey). See **table S13** for statistical analysis.

Supplementary Tables

table S1. Hazard ratios (HRs) for nitisinone and ivermectin, Experiment 1. 14-day HRs for *An. gambiae* mosquitoes, feeding on blood containing nitisinone or ivermectin at varying concentrations, were generated using Cox regression analysis. n = number of mosquitoes tested in each group.

Experiment 1 (Pilot experiment)							
Nitisinone				**Ivermectin**			
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	89	---	---	Control	89	---	---
50	94	1.99 [1.37 – 2.9]	<0.001	**15**	**66**	**6.76 [4.22 – 10.85]**	**<0.0001**
100	75	1.8 [1.21 – 2.69]	<0.01	**100**	**90**	**16.92 [10.43 – 27.46]**	**<0.0001**
250	**98**	**27.90 [18.17 – 42.9]**	**<0.0001**	**1000**	**59**	**99.77 [56.95 – 174.77]**	**<0.0001**
10000	**98**	**40.52 [25.69 – 63.92]**	**<0.0001**	**5000**	**84**	**158.85 [88.91 – 283.78]**	**<0.0001**

table S2. Hazard ratios for nitisinone, Experiment 3. 14-day HRs generated using Cox regression analysis for *An. gambiae* mosquitoes feeding on blood containing nitisinone at selected concentrations (100, 150, 200, 250 ng/mL). n = number of mosquitoes tested in each group.

Experiment 3 (Concentration-refined follow-up experiment)			
	Nitisinone		
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**
Control	138	---	---
100	155	1.89 [0.78 – 1.81]	0.421
150	**147**	**3.99 [2.76 – 5.78]**	**<0.0001**
200	**144**	**7.89 [5.48 – 11.35]**	**<0.0001**
250	**157**	**17.55 [12.12 – 25.41]**	**<0.0001**

table S3. Hazard ratios (HRs) for nitisinone and ivermectin, Experiment 2. 14-day HRs were generated using Cox regression analysis for *An. gambiae* mosquitoes that had fed on blood containing concentrations of nitisinone or ivermectin. Predicted human drug concentrations in blood at 7, 14, 21 and 28 days after receiving either a 1 mg/kg nitisinone or 0.6 mg/kg ivermectin for three days were determined. Results are generated from three replicate experiment and n = number of mosquitoes tested in each group.

Nitisinone				Ivermectin			
Replicate 1							
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	56	---	---	Control	56	---	---
3.8 (PK day 28)	94	0.54 [0.27 - 1.06]	0.072	1.5 (PK day 28)	96	0.71 [0.37 - 1.34]	0.285
37 (PK day 21)	78	1.86 [1.04 - 3.3]	<0.05	3.5 (PK day 21)	115	0.73 [0.4 - 1.35]	0.317
350 (PK day 14)	77	**185.30 [69.5 - 494.02]**	**<0.0001**	8.5 (PK day 14)	60	2.37 [1.33 – 4.25]	<0.0001
3500 (PK day 7)	69	**185.30 [69.3 - 495.48]**	**<0.0001**	20 (PK day 7)	103	**11.87 [6.98 – 20.2]**	**<0.0001**
---	---		---	125 (PK Cmax)	77	**76.75 [41.97 – 140.34]**	**<0.0001**
Replicate 2							
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	137	---	---	Control	137	---	---
3.8 (PK day 28)	141	0.75 [0.49 – 1.14]	0.162	1.5 (PK day 28)	130	0.98 [0.69 – 1.47]	0.936
37 (PK day 21)	144	1.25 [0.86 – 1.82]	0.009	3.5 (PK day 21)	160	0.81 [0.54 – 1.21]	0.299
350 (PK day 14)	136	**33.54 [22.41 – 50.21]**	**<0.0001**	8.5 (PK day 14)	132	3.72 [2.64 – 5.23]	0.022
3500 (PK day 7)	139	**40.79 [26.98 – 61.65]**	**<0.0001**	20 (PK day 7)	134	**11.69 [8.22 – 16.63]**	**<0.0001**
---	---		---	125 (PK Cmax)	158	**41.86 [28.49 – 61.51]**	**<0.0001**
Replicate 3							
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	138	---	---	Control	138	---	---
3.8 (PK day 28)	115	0.84 [0.42 – 1.66]	0.608	1.5 (PK day 28)	133	0.61 [0.3 – 1.25]	0.179
37 (PK day 21)	121	0.79 [0.4 – 1.56]	0.496	3.5 (PK day 21))	141	3.14 [1.87 – 5.26]	<0.0001
350 (PK day 14)	108	**39.87 [23.86 – 66.62]**	**<0.0001**	8.5 (PK day 14)	104	**13.26 [8.12 – 21.65]**	**<0.0001**
3500 (PK day 7)	126	**54.38 [31.87 – 92.79]**	**<0.0001**	20 (PK day 7)	117	**28.51 [17.42 – 46.67]**	**<0.0001**
---	---		--	125 (PK Cmax)	137	**205 [119.38 – 352.04]**	**<0.0001**

table S4. Parameters used for the PK/PD simulations of nitisinone and ivermectin in table S5. PK parameters were extracted from published research (*38, 51*). PD parameters were generated in-house from the mosquito feeding experiments for both nitisinone and ivermectin.

Parameters used for PK/PD simulation	Nitisinone	Ivermectin
K_a [h^{-1}]	0.89	0.226
CL [L/h]	0.105	10.1
VC [L]	7.8	164
Q [L/h]	0	21.4
V_p [L]	1	887
HR_{max}	20	20
EC_{50} [ng/mL]	205.31	13.43
h	12.85	2.3

table S5. Results of PK/PD simulation for nitisinone and ivermectin in human blood. Predicted time above the EC_{50}, EC_{95} and Hazard Ratio (HR) of 4 for nitisinone or ivermectin generated from PK/PD simulations based on in-house generated PD parameters and published PK parameters. The PK and PD parameters used are displayed in table S4. The fourth column (grey highlight) represents predicted observed times above the EC_{50}, EC_{95} and HR of 4 in humans receiving the same dose of ivermectin in previously published clinical trials.

Parameter	**Nitisinone (1 mg/kg, 3 d)**	**Ivermectin (0.6 mg/kg, 3 d) (*in vitro*, this study)**	**Ivermectin (0.6 mg/kg, 3 d) (*in vivo* Clinical trial)**
Time above EC_{50} [days]	15.7	10.4	6.8 (*38*)
Time above EC_{95} [days]	14.9	3.3	< 3 (*38*)
Time HR > 4 [days]	16.2	14.7	7.0 – 10.0 (*24*)

table S6. <u>Averaged</u> Hazard ratios for nitisinone and ivermectin in older mosquitoes. 14-day HRs generated using Cox regression analysis for older *An. gambiae* mosquitoes (9-13 days) fed on blood containing nitisinone or ivermectin. The predicted blood concentrations represent 7, 14, 21 and 28 days after receiving doses of each drug (1 mg/kg x 3 days nitisinone; 0.6 mg/kg x 3 days ivermectin). Results were generated from three replicated experiments. HRs and sample sizes are averages. Experiment-level HRs are displayed in **table S7**.

Nitisinone					**Ivermectin**				
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Median survival (days)**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**	**Median survival (days)**
Control	115	---	---	9.93	Control	115	---	---	9.93
3.8 (PK day 28)	116	0.78 [0.49-1.4]	0.287	12.61	1.5 (PK day 28)	112	0.93 [0.55-1.69]	0.709	10.93
37 (PK day 21)	111	1.05 [0.58-1.82]	0.448	11.5	3.5 (PK day 21)	125	1.09 [0.54-1.94]	0.399	6.43
350 (PK day 14)	115	**25.78** [10.49-50.21]	**<0.0001**	0.83	8.5 (PK day 14)	103	1.86 [0.57-5.23]	0.405	8.88
3500 (PK day 7)	116	**30.14** [12.41-61.65]	**<0.0001**	0.83	20 (PK day 7)	107	4.92 [0.97-16.63]	<0.05	5.72
---	---	---	---	---	125 (PK Cmax)	110	**19.23** [3.24-61.51]	**<0.0001**	2.51

table S7. Hazard ratios for replicates of nitisinone and ivermectin fed to older mosquitoes (9 – 13 days). 14-day HRs generated using Cox regression analysis for older *An. gambiae* Kisumu (aged 9-13 days) fed on blood containing nitisinone or ivermectin. The selected concentrations are predicted concentrations from humans after 7, 14, 21 and 28 days having received either drug (1 mg/kg x 3 days nitisinone or 0.6 mg/kg x 3 days ivermectin). Data shown for three replicates and n = number of mosquitoes tested in each group.

Nitisinone				**Ivermectin**			
Replicate 1							
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	104	---	---	Control	104	---	---
3.8 (PK day 28)	97	0.71 [0.5 – 1.02]	0.067	1.5 (PK day 28)	91	0.79 [0.55 – 1.14]	0.203
37 (PK day 21)	96	0.83 [0.58 – 1.19]	0.306	3.5 (PK day 21)	104	1.40 [1.01 – 1.94]	<0.05
350 (PK day 14)	109	**25.52 [16.49 – 39.51]**	**<0.0001**	8.5 (PK day 14)	74	0.83 [0.57 – 1.21]	0.332
3500 (PK day 7)	91	**27.87 [17.62 – 44.07]**	**<0.0001**	20 (PK day 7)	93	1.35 [0.97 – 1.88]	0.077
---	---		---	125 (PK Cmax)	78	**4.60 [3.24 – 6.52]**	**<0.0001**
Replicate 2							
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	137	---	---	Control	137	---	---

3.8 (PK day 28)	141	0.75 [0.49 – 1.14]	0.162	1.5 (PK day 28)	130	0.98 [0.66 – 1.47]	0.936
37 (PK day 21)	144	1.25 [0.86 – 1.82]	0.009	3.5 (PK day 21)	160	0.81 [0.54 – 1.21]	0.299
350 (PK day 14)	136	**33.54 [22.41 – 50.21]**	**<0.0001**	8.5 (PK day 14)	132	3.72 [2.64 – 5.23]	<0.0001
3500 (PK day 7)	139	**40.79 [26.98 – 61.65]**	**<0.0001**	20 (PK day 7)	134	**11.69 [8.22 – 16.63]**	**<0.0001**
---	---		---	125 (PK Cmax)	158	**41.86 [28.49 – 61.51]**	**<0.0001**
Replicate 3							
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	104	---	---	Control	104	---	---
3.8 (PK day 28)	110	0.89 [0.57 – 1.4]	0.619	1.5 (PK day 28)	116	1.00 [0.6 – 1.69]	0.989
37 (PK day 21)	92	1.07 [0.66 – 1.74]	0.792	3.5 (PK day 21)	112	1.06 [0.6 – 1.85]	0.851
350 (PK day 14)	101	**18.27 [10.49 – 31.84]**	**<0.0001**	8.5 (PK day 14)	103	1.04[0.64 – 1.67]	0.883
3500 (PK day 7)	119	**21.77 [12.41 – 38.17]**	**<0.0001**	20 (PK day 7)	95	1.72 [1.12 – 2.66]	<0.05
---	---		---	125 (PK Cmax)	**95**	**11.23 [6.78 – 18.59]**	**<0.0001**

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table S8. Averaged Hazard ratios (HRs) for nitisinone fed to insecticide-susceptible and -resistant mosquitoes). The 14-day HRs for *An. gambiae* Kisumu (susceptible) and Tiassalé (resistant) mosquitoes feeding on blood containing nitisinone were generated using Cox regression analysis. The predicted blood concentrations represent 7, 14, 21 and 28 days after receiving 1 mg/kg x 3 days nitisinone. HRs were combined from the three replicate experiments. HRs and sample sizes are averages. Replicate-level HRs are displayed in **table S9**.

Kisumu strain (insecticide-susceptible)					**Tiassalé strain (insecticide-resistant)**				
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Median survival (days)**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**	**Median survival (days)**
Control	142	---	---	---	Control	127	---	---	13.46
3.8 (PK day 28)	137	1.46 [0.7 – 3.34]	0.326	---	3.8 (PK day 28)	146	0.95 [0.59 – 1.67]	0.55	13.46
37 (PK day 21)	151	2.07 [0.87 – 5.61]	0.055	---	37 (PK day 21)	144	1.18 [0.73 – 2.02]	0.503	13.46
350 (PK day 14)	155	**148.16** [36.91 – 664.35]	**<0.0001**	0.67	350 (PK day 14)	151	**52.62** [11.67 – 120.05]	**<0.0001**	0.76
3500 (PK day 7)	157	**190.09** [53.48 – 839.45]	**<0.0001**	0.67	3500 (PK day 7)	153	**61.43** [18.73 – 143.61]	**<0.0001**	0.76

table S9. Hazard ratios for nitisinone bloodmeal replicates fed to insecticide-susceptible and -resistant mosquitoes. 14-day HRs generated using Cox regression analysis for *An. gambiae* Kisumu (insecticide-susceptible) and Tiassalé (insecticide-resistant) mosquitoes feeding on blood containing nitisinone at varying concentrations. Concentrations represent predicted drug blood concentrations in humans at 7, 14, 21 and 28 days after receiving a dose of nitisinone (1 mg/kg x 3 days). n = number of mosquitoes tested in each group.

Kisumu strain (insecticide-susceptible)				**Tiassalé strain (insecticide-resistant)**			
Replicate 1							
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	118	---	---	Control	127	---	---
3.8 (PK day 28)	133	1.59 [0.76 – 3.34]	0.221	3.8 (PK day 28)	129	0.84 [0.59 – 1.19]	0.325
37 (PK day 21)	163	2.88 [1.48 – 5.61]	<0.01	37 (PK day 21)	151	1.01 [0.73 – 1.38]	0.973
350 (PK day 14)	150	**309.5 [144.2 – 664.5]**	**<0.0001**	350 (PK day 14)	145	**68.60 [42.39 – 111.01]**	**<0.0001**
3500 (PK day 7)	174	**385.8 [177.2 – 839.5]**	**<0.0001**	3500 (PK day 7)	145	**70.99 [44.08 – 114.35]**	**<0.0001**
Replicate 2							
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	157	---	---	Control	129	---	---
3.8 (PK day 28)	143	1.44 [0.87 – 2.39]	0.156	3.8 (PK day 28)	165	1.1 [0.73 – 1.67]	0.655
37 (PK day 21)	154	1.61 [0.97 – 2.66]	0.06	37 (PK day 21)	157	1.34 [0.89 – 2.02]	0.162
350 (PK day 14)	168	**75.30 [46.37 – 122.3]**	**<0.0001**	350 (PK day 14)	152	**72.76 [44.1 – 120.1]**	**<0.0001**
3500 (PK day 7)	165	**95.84 [57.8 – 158.85]**	**<0.0001**	3500 (PK day 7)	160	**85.97 [51.46 – 143.6]**	**<0.0001**

Replicate 3							
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**	**Conc. [ng/mL]**	**n**	**14-day HR [95 CI]**	**P value**
Control	151	---	---	Control	124	---	---
3.8 (PK day 28)	136	1.16 [0.7 – 1.92]	0.554	3.8 (PK day 28)	145	0.92 [0.62 – 1.36]	0.671
37 (PK day 21)	135	1.88 [1.18 – 2.98]	<0.01	37 (PK day 21)	125	1.19 [0.81 – 1.75]	0.374
350 (PK day 14)	146	**59.64 [36.91 –96.38]**	**<0.0001**	350 (PK day 14)	157	**16.50 [11.67 – 23.35]**	**<0.0001**
3500 (PK day 7)	133	**88.67 [53.5 – 146.99]**	**<0.0001**	3500 (PK day 7)	153	**27.32 [18.73 – 39.86]**	**<0.0001**

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Population simulation modeling

table S10. Monte Carlo population simulations of the PD profile for a single dose of nitisinone. Predicted time above HR=4 and EC_{50} for ten different doses of nitisinone in a population of 500 people weighing 70 kg.

Predicted nitisinone dose (mg/kg)	Total mg for 70 kg individual	Time above HR = 4 (Days)	Time above EC_{50} (Days)
0.1	7	5.21	4.58
0.2	14	7.33	6.79
0.3	21	9.08	8.46
0.4	28	9.42	8.83
0.5	35	10.42	9.79
0.6	42	10.83	10.25
0.7	49	11.17	10.58
0.8	56	11.71	11.08
0.9	63	12.46	11.83
1	70	12.51	11.91

table S11. Hazard ratios for diluted and neat alkaptonuria patient blood samples. 14-day HRs generated using Cox regression analysis for donor blood fed *An. gambiae* Kisumu (insecticide susceptible strain). Four blood samples were donated by individuals with alkaptonuria. Patients 1, 2 and 3 had received 2 mg nitisinone daily for years, whilst patient 4 had not yet begun nitisinone treatment. Undiluted (neat) control blood was the human blood (nitisinone-naïve) used to maintain mosquito colonies. Data show n = number of mosquitoes tested in each group.

Patient 1			
Blood dilutions	**n =**	**14-day HR [95 CI]**	**P value**
Control (neat)	161	---	---
Neat	120	**22.13 [14.65 – 33.42]**	**<0.0001**
1:2	132	**15.67 [10.41 – 23.6]**	**<0.0001**
1:5	111	1.07 [0.61 – 1.87]	0.818
1:10	161	1.17 [0.71 – 1.92]	0.536

Patient 2			
Blood dilutions	**n =**	**14-day HR [95 CI]**	**P value**
Control (neat)	161	---	---
Neat	154	**36.48 [24.28 – 54.8]**	**<0.0001**
1:2	123	**27.62 [18.3 – 41.69]**	**<0.0001**
1:5	142	2.99 [1.92 – 4.64]	<0.0001
1:10	126	1.06 [0.62 – 1.82]	0.84

Patient 3			
Blood dilutions	n =	**14-day HR [95 CI]**	**P value**
Control (neat)	161	---	---
Neat	133	**7.09 [4.68 – 10.76]**	**<0.0001**
1:2	61	**5.42 [3.37 – 8.69]**	**<0.0001**
1:5	114	1.47 [0.88 – 2.45]	0.138
1:10	143	0.97 [0.57 – 1.66]	0.92

Patient 4			
Blood dilutions	n =	**14-day HR [95 CI]**	**P value**
Control (neat)	161	---	---
Neat	162	0.92 [0.55 – 1.56]	0.767

table S12. Hazard ratios for bloodmeals containing either nitisinone or tyrosine or both, fed to mosquitoes in three replicate experiments. 14-day HRs were generated using a Cox regression analysis for *An. gambiae* Kisumu mosquitoes that had fed on blood containing varying concentrations of either nitisinone, tyrosine or both. Tyrosine serum concentrations considered to be physiologically high (high Tyr = 0.109 mg/mL) and low (low Tyr = 0.036 mg/mL) were extracted from the previous nitisinone clinical trial data (*32*). Both high and low doses of tyrosine were alone given to mosquitoes via a bloodmeal or alongside lower doses of nitisinone that were equivalent to the predicted human concentrations (PK) at day 21 (37 ng/mL) and day 28 (3.8 ng/mL) after receiving three doses of nitisinone over three days (1 mg/kg x 3 days). The C_{max} was a lethal dose of nitisinone at 250 ng/mL, which served as a mortality control. Data show n = number of mosquitoes tested in each group.

Replicate 1			
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**
Control	90	---	---
Low tyrosine	86	1.27 (0.569-2.835)	0.56
High tyrosine	92	0.69 (0.277-1.71)	0.421
3.8 + low Tyr	88	0.74 (0.296-1.829)	0.509
3.8 + high Tyr	91	1.61 (0.753-3.432)	0.22
37 + low Tyr	57	0.86 (0.32-2.337)	0.774
37 + high Tyr	110	1.45 (0.688-3.04)	0.33
250 (C_{max})	85	**8.96 (4.67-17.201)**	**<0.0001**
37 (PK day 21)	91	1.17 (0.524-2.61)	0.703
3.8 (PK day 28)	137	1.13 (0.538-2.377)	0.745
Replicate 2			
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**
Control	112	---	---
Low tyrosine	106	1.13 (0.559-2.287)	0.732
High tyrosine	142	0.88 (0.438-1.757)	0.712
3.8 + low Tyr	97	0.83 (0.383-1.817)	0.648
3.8 + high Tyr	81	1.77 (0.891-3.508)	0.103
37 + low Tyr	125	0.94 (0.467-1.909)	0.872
37 + high Tyr	139	1.02 (0.518-2.008)	0.954
250 (C_{max})	166	**37.45 (21.706-64.608)**	**<0.0001**
37 (PK day 21)	109	1.18 (0.589-2.361)	0.642
3.8 (PK day 28)	118	0.88 (0.425-1.822)	0.73

Replicate 3			
Conc. [ng/mL]	**n**	**14-day HR [95 CI]**	**P value**
Control	122	---	---
Low tyrosine	87	1.75 (0.754-4.039)	0.193
High tyrosine	91	1.64 (0.707-3.787)	0.25
3.8 + low Tyr	88	2.07 (0.919-4.66)	0.079
3.8 + high Tyr	94	1.47 (0.622-3.45)	0.382
37 + low Tyr	87	1.91 (0.84-4.367)	0.122
37 + high Tyr	91	2.89 (1.351-6.169)	0.006
250 (C_{max})	105	**38.22 (19.77-73.89)**	**<0.0001**
37 (PK day 21)	78	0.78 (0.267-2.285)	0.652
3.8 (PK day 28)	94	2.91 (1.37-6.18)	0.005

table S13. Raw data showing the relative abundance of metabolites isolated from female *An. gambiae*, 24 hours after ingesting nitisinone. Tyrosine metabolites (blue), citric acid metabolites (orange) and lipid species (grey).

*****homogentisate could not be confidently identified in the dataset.

Metabolite	Mass	Retention Time	Group A mean (complete knockdown)	Group B mean (partial knockdown	Group C mean (control)	Fold Change (Log2 A/C)	P value (A/C)	Adjusted P value (A/C)
L-tyrosine	182.081	661.19	1.3×10^{9}	1.2×10^{9}	1.3×10^{8}	3.4	<0.0001	1×10^{9}
3-Hydroxyphenylpyruvate	179.035	470.6	1×10^{8}	7.4×10^{7}	164220	9.29	<0.0001	6.56×10^{13}
3-Hydroxyphenyllactate	181.051	519.25	5.2×10^{7}	3.3×10^{7}	118824	8.79	<0.0001	1.16×10^{10}
3-Hydroxyphenylacetate	151.04	552.28	1132072	885502	145040	2.94	<0.0001	3.01×10^{7}
L-phenylalanine	166.086	532.1	7.6×10^{8}	9.5×10^{8}	2.9×10^{8}	1.36	<0.0001	5.74×10^{6}
***Homogentisate**	167.035	617.19	3841204	1895344	143426	4.74	<0.0001	9.65×10^{7}
Citrate	191.02	815.66	4.5×10^{8}	4.2×10^{8}	6×10^{8}	-0.45	<0.0001	1.76×10^{2}
2-Oxoglutarate	145.014	756.3	4.5×10^{7}	5.3×10^{7}	1.2×10^{8}	-1.38	<0.0001	2.25×10^{5}
Cis-Aconitate	173.009	856.13	555163	394582	368234	0.6	<0.0001	4×10^{3}
Malate	133.014	763.26	2.1×10^{8}	2.7×10^{8}	5.8×10^{8}	-1.48	<0.0001	5.25×10^{5}
Succinate	117.019	730.78	4.7×10^{7}	6.4×10^{7}	1×10^{8}	-1.07	<0.0001	8.4×10^{3}
Dodecenedioic acid	227.129	463.79	1.1×10^{7}	6929355	1047448	3.53	<0.0001	1.52×10^{5}
Decenedioic acid	199.098	552.69	7681470	4846710	464366	4.13	<0.0001	7.55×10^{6}
(E)-10-Oxo-8-decenoic acid	183.103	518	856843	482099	40473.6	4.43	<0.0001	7.55×10^{6}
3-Hydroxydodecanedioic acid	245.14	497.56	1.6×10^{7}	9066308	718300	4.56	<0.0001	4.92×10^{6}
2-Hydroxydecanedioic acid	217.108	594.19	7378568	5240015	565300	3.73	<0.0001	2.6×10^{6}
Suberic acid	173.082	623.28	4259504	2583870	439380	3.29	<0.0001	1.67×10^{6}
Propenoylcarnitine	216.123	405.45	1811107	1404745	235713	3.12	<0.0001	1.84×10^{4}
O-Butanoylcarnitine	232.154	458.87	1.5×10^{8}	1.2×10^{8}	5.1×10^{7}	1.6	<0.0001	3.47×10^{4}
Butenylcarnitine	228.125	244.4	1.2×10^{7}	9182612	6205794	0.97	<0.0001	1.1×10^{2}
Hydroxybutyrylcarnitine	248.149	246.88	1403537	580553	55123.4	4.29	<0.0001	4.57×10^{5}

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