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The Spiroindolone Drug Candidate NITD609 Potently Inhibits Gametocytogenesis and Blocks *Plasmodium falciparum* Transmission to *Anopheles* Mosquito Vector

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The global malaria agenda has undergone a reorientation from control of clinical cases to entirely eradicating malaria. For that purpose, a key objective is blocking transmission of malaria parasites from humans to mosquito vectors. The new antimalarial drug candidate NITD609 was evaluated for its transmission-reducing potential and compared to a few established antimalarials (lumefantrine, artemether, primaquine), using a suite of *in vitro* assays. By the use of a microscopic readout, NITD609 was found to inhibit the early and late development of *Plasmodium falciparum* gametocytes *in vitro* in a dose-dependent fashion over a range of 5 to 500 nM. In addition, using the standard membrane feeding assay, NITD609 was also found to be a very effective drug in reducing transmission to the *Anopheles stephensi* mosquito vector. Collectively, our data suggest a strong transmission-reducing effect of NITD609 acting against different *P. falciparum* transmission stages.

Plasmodium falciparum malaria is responsible for almost 800,000 deaths and 250 million clinical cases annually (35). The number of deaths associated with malaria gradually went down in several countries of sub-Saharan Africa during the previous decade, thanks to increased financial investments, implementation of long-lasting-insecticide-impregnated bed nets, and artemisinin-based combination therapy (35). As expressed by the MalEra initiative, the global malaria agenda has undergone a reorientation from control of clinical cases to malaria elimination and eventually eradication (5).

For decades, antimalarial drug development has been almost entirely focused on asexual blood stages, which are directly responsible for morbidity and mortality, while mostly ignoring the nonpathogenic life cycle stages responsible for malaria transmission and subsequent spread of parasites in the population. Transmission stages emerging from asexual blood stages follow a number of transformation steps and eventually appear in the blood circulation as mature stage V male and female gametocytes (6). Once these mature gametocytes are ingested by blood-feeding *Anopheles* mosquitoes, the sporogonic cycle is initiated by rapid transition of male and female gametocytes into gametes, instantly followed by fertilization. The formed oocysts finally release sporozoites that migrate to the mosquito salivary glands. Infected mosquitoes inject a small number of sporozoites into the human host with every blood meal. A drug that substantially reduces or preferably entirely blocks transmission of malaria from human to mosquito would be an important tool (5).

Here we describe a series of *in vitro* assays performed to test the effects of drugs on the early and late development of laboratory-adapted *P. falciparum* strain NF54 as well as *in vivo* oocyst development in mosquitoes. Data are shown for the new antimalarial drug candidate NITD609 as well as a few established antimalarial drugs (lumefantrine, artemether, primaquine) (24). Lumefantrine and artemether were chosen due to being the two compounds of the combination therapy drug Coartem, which was reported to have transmission-blocking activity in an earlier study (26). Primaquine, which acts as a transmission-blocking agent *in vivo*, consistently shows no such effect *in vitro* and was added to the experiments presented in this paper for further replication of earlier results (7, 8, 10, 19, 27).

MATERIALS AND METHODS

***In vitro* anti-asexual activity assay.** *P. falciparum* drug-sensitive strain NF54 was cultivated in a variation of a medium previously described (12, 28), consisting of RPMI 1640 supplemented with 0.5% Albumax II, 25 mM HEPES, 25 mM $NaHCO_3$ (pH 7.3), 0.36 mM hypoxanthine, and neomycin at 100 μg/ml. Human erythrocytes served as host cells. Cultures were maintained in an atmosphere of 3% O_2, 4% CO_2, and 93% N_2 in humidified modular chambers at 37°C. Compounds were dissolved in dimethyl sulfoxide (DMSO) (10 mM), diluted in hypoxanthine-free culture medium, and titrated in duplicate experiments over a 64-fold range in 96-well plates. Infected erythrocytes (1.25% final hematocrit and 0.3% final parasitemia) were added into the wells. After 48 h of incubation, 0.5 μCi of [^{3}H]hypoxanthine was added per well and plates were incubated for an additional 24 h. Parasites were harvested onto glass fiber filters, and radioactivity was counted using a Betaplate liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded and expressed as a percentage of the untreated control data. Fifty percent inhibitory concentrations (IC_{50}) were estimated by linear interpolation (13).

Early and late gametocyte development assays. Figure 1 shows microscopic images of Giemsa-stained blood films representing the development and maturation of gametocytes emerging from replicating asexual parasites. The distinct morphological stages (I to V) form the basis for measuring potential effects of compounds on early gametocyte develop-

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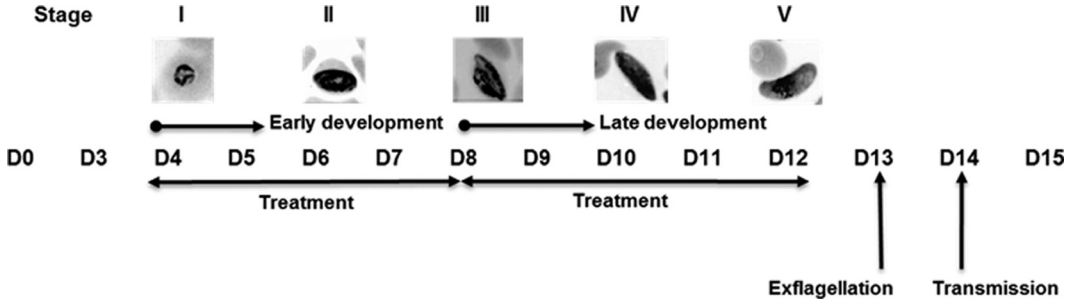

FIG 1 Timeline and check points for drug testing during *P. falciparum* gametocyte and sporogonic development. The timeline data are presented in days, showing the gametocyte culture grown from asexual parasites seen at day 0 (D0) to the beginning of the early development assay on D4, the end of the early development assay as well as the beginning of the late development assay on D8, and the end of the late gametocyte development assay on D12. Gametocytes were checked for exflagellation on D13. A standard membrane feeding assay (SMFA) was carried out on D14. The double-pointed horizontal arrows below the timeline represent the time periods during which test compounds were added in the culture (treatment). Every assay was started anew from a culture matured to the given day defined as the starting point of the assay. Light microscope images of gametocyte stages I to V are shown at ×100 magnification.

ment (gametocyte stages I and II) and late gametocyte development (stages III to V) (4, 6).

Cultures of *P. falciparum* strain NF54 were routinely grown for gametocyte production as described previously (20). Briefly, isolates of NF54 were suspended in 5% group O red blood cells (RBCs) in culture medium (RPMI 1640 with HEPES [5.94 g/liter], hypoxanthine [0.05 g/liter]), 10% human serum, 5% bicarbonate [42 ml/liter]), with a parasite density of 0.5%. Blood was obtained from selected healthy Dutch blood bank donors without any history of malaria.

For early gametocyte development, parasite cultures were started in an automated tipper system in RBCs (22). After 4 days, parasites were cultured in 24-well plates (Nunc flat bottom) up to day 8. Growth medium that included different concentrations of test compounds (volume, 1 ml) was replaced manually once a day. The numbers of healthy stage II gametocytes were counted on day 8 (number of parasites/5,000 RBCs) by the same person, using Giemsa-stained films prepared from culture samples, and morphology was documented. Healthy stage II gametocytes have a half-moon shape within RBCs, with pigment residing on the straight edge of the half-moon and in the middle of the RBC. The early development assay was repeated three times.

For late gametocyte development, gametocyte cultures were grown in a tipper system at 5% hematocrit as described previously (21, 22). Culture medium was replaced automatically twice a day (15). Test compounds were added to cultures from day 8 up to day 12. On day 13, healthy stage V male and female gametocytes were counted by the same person using Giemsa-stained films prepared from culture samples. Gametocyte maturation and quality were assessed according to morphology, pigmentation, and overall appearance; healthy stage V gametocytes are banana-shaped with rounded ends. Stage V females have a slightly blue color, with the pigment clustered in the middle, whereas males have a slight pink color and pigment evenly spread throughout the parasite.

SMFA. The standard membrane feeding assay (SMFA) was used to test potential effects of compounds or drugs on sporogonic development in the mosquito as described before (30). Briefly, 14-day-old cultures of strain NF54, showing 0.3% to 0.5% mature gametocytes, were first checked in a prefeed for their quality and potential to form oocysts. When ookinetes were seen 22 h after the feed, parasite culture material was used to test the potential effects of compounds on sporogony; 300 μl of culture material was added to 180 μl of washed packed cells and centrifuged for 20 s. After removal of the supernatant, 150 μl of human control serum with or without test compound was added to the pellet. Each suspension was immediately injected into an individual membrane-covered minifeeder, and 20 3-to-5-day-old *Anopheles stephensi* mosquitoes were allowed to feed for 10 to 15 min (30). Six days after feeding, 20 mosquitoes per feeder were dissected. Absolute numbers of oocysts were counted using a light microscope after staining the mosquito stomach with 2% merbromin (Mercurochrome) (15, 30).

Selected test compounds. Parasite cultures were treated with a dose range of NITD609, lumefantrine, artemether, or primaquine (5 mM stock) dissolved in DMSO. Stock dilutions were subsequently made in complete media that contained human control serum, RPMI 1640, and sodium bicarbonate (NaBic). Control cultures contained a final concentration of 0.1% DMSO. Compound concentrations were selected on the basis of their respective 50% effective concentrations (EC_{50}s) on asexual stages, covering a range of 5 to 500 nM. NITD609 was synthesized as described in reference 24. Lumefantrine and artemether were provided by Novartis Pharma. Primaquine (bisphosphate) was obtained from Sigma-Aldrich (catalog no. 160393) and used without further purification.

Data analyses. The results were gathered on datasheets using Microsoft Excel and SPSS Inc. PASW Statistics 18.0. Calculations of percentages of control results were done using formulas within the datasheets of Microsoft Excel. Figures were made using GraphPad Prism 5. Statistical analyses were performed using SPSS version 18.0. Differences in compound-dose responses compared to control results were analyzed with a multiple-comparison model using Dunnett's *post hoc t* test, as appropriate. The effect of drugs on oocyst counts was determined in two experiments that were analyzed by negative binomial regression models where incidence rate ratios (IRR) were presented with *P* values and estimates were adjusted for correlations between observations from the same experiment. To avoid multiple comparisons between drugs at different concentrations and control feeds, experiments were analyzed in two steps. First, oocyst counts in control feeds were compared to counts in feeds with the different drugs at any concentration (e.g., comparison of NITD609 at any concentration with control feed). Second, a dose-dependent effect of drugs was determined in a model where drug concentrations were added as categorical variables (e.g., for NITD609, 5 nM = 1, 50 nM = 2, 500 nM = 3) and compared with the lowest drug combination as a reference value. *P* values < 0.05 were considered statistically significant in all analyses.

RESULTS

Effect of compounds on asexual stages. To select a dose range of the individual compounds for their capacity to interrupt transmission stages, we first determined the *in vitro* activity on asexual stages of the chloroquine-sensitive NF54 and -resistant K1 strains (Table 1). NITD609 was the most potent compound and displayed subnanomolar potency, while both lumefantrine and artemether were slightly less potent, with single-digit nanomolar IC_{50} values in accordance with the literature (3, 24, 36, 37). It was decided to run the gametocytocidal activity assays at concentrations of 5, 50, and 500 nM for NITD609 and artemether (ranging from roughly 5 to 500 times and 2 to 200 times their respective IC_{50} values). It is worth noting that upon treatment with lumefan-

TABLE 1 *In vitro* antimalarial activity against asexual blood stages

Compound	IC_{50} (nM [mean ± SD])[a]	
	NF54	K1
NITD609	0.5 ± 0.1	0.6 ± 0.2
Lumefantrine	2.8 ± 0.7	1.1 ± 0.2
Primaquine	1,191 ± 276	557 ± 183
Artemether	3.5 ± 1.0	2.2 ± 0.3

[a] IC_{50} values of NITD609, lumefantrine, primaquine, and artemether against *Plasmodium falciparum* strains NF54 and K1 are expressed as means ± standard deviations of the results of at least three independent assays.

trine-artemether, the maximum plasma concentration of artemether in malaria patients reaches about 100 to 200 nM (11). Since the lumefantrine component of lumefantrine-artemether can achieve plasma concentrations of 5 to 10 μM, we tested slightly higher concentrations of lumefantrine, i.e., 50, 500, and 5,000 nM (roughly 20 to 2,000 times its IC_{50} value). At the recommended treatment regimen (0.25 to 0.5 mg/kg/day for 14 days), primaquine maximum plasma concentrations are in the micromolar range (14, 34). Primaquine showed poor activity against *P. falciparum* asexual stages that was in line with previous studies; thus, we tested concentrations of 500, 2,500, and 5,000 nM (about 0.5, 2.5, and 5 times the IC_{50} value) (3).

Effect of compounds on early gametocyte development. The effect of NITD609, lumefantrine, artemether, and primaquine on early *P. falciparum* gametocyte development was tested in a 24-well plate assay. Figure 2 shows that NITD609 was the most effective inhibitor of early gametocyte development. The activity was dose dependent, with significant inhibition seen for NITD609 at 50 and 500 nM ($P = 0.001$). In addition, as shown in Fig. 3, there was a clear damaging effect on gametocyte morphology, with the remaining stage II gametocytes showing swollen rounded forms. Similar but somewhat weaker inhibition was observed with lumefantrine, with significant dose-dependent suppression at the two highest concentrations of 500 and 5,000 nM ($P = 0.001$). Artemether was effective only at the highest concentration of 500 nM ($P = 0.001$), while primaquine showed no significant effect on early gametocyte development under the conditions tested.

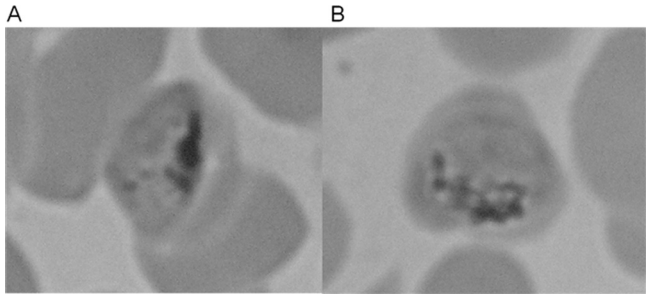

FIG 3 Photos of morphological effect of NITD609 on stage II gametocytes. (A) Normal stage II gametocyte in control culture. (B) Aberrant NITD609-affected stage II gametocyte with swollen rounded form.

Effect of compounds on late gametocyte development. Next, potential effects of these compounds on late gametocyte development were studied. The same four compounds were added into cultures in the automated tipper system during the period of *P. falciparum* gametocytogenesis from stage II up to fully mature stage V gametocytes. Results are presented in Fig. 4. In both experiments, NITD609 showed a dose-dependent inhibiting effect on late gametocyte development. Lumefantrine showed inhibitory effects only at the two highest test concentrations of 500 and 5,000 nM. Artemether showed an effect on late gametocyte development only at the highest concentration (500 nM), whereas the results for the cultures treated with lower concentrations were

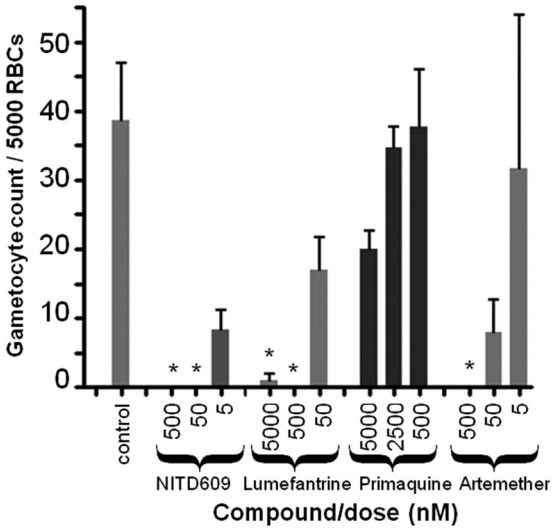

FIG 2 Effect of drugs on early gametocyte development. Bars represent means (with standard errors of the means [SEM]) of gametocyte stage II counts per 5,000 erythrocytes. Each compound/dose combination was quantified as a single measurement; assays were replicated three times. Statistical parametric tests were done with absolute numbers of gametocytes. Bars marked with an asterisk represent a statistically significant difference between compound/dose and control test results ($P < 0.01$).

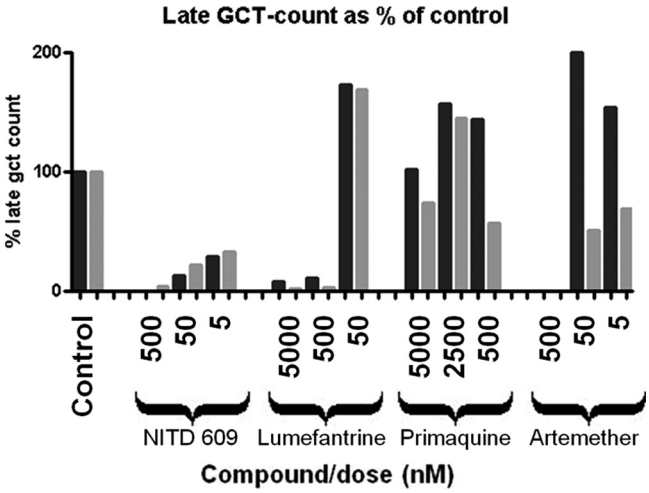

FIG 4 Late development of gametocytes. Two independent experiments were done under similar conditions, each with a medium control. Total gametocyte numbers per 5,000 erythrocytes per experiment were counted and are expressed as percentages of control results, with the mean of the control results set to 100%. Dark bars represent percentages of control results from experiment 1, and light bars represent percentages of control results from experiment 2, each shown for the different compound/dose concentrations tested. Controls for experiment 1 were done in duplicate (mean, 93 stage V gametocytes/5,000 RBCs; standard deviation [SD], 67.9), controls for experiment 2 were done in triplicate (mean, 254; SD, 95.7).

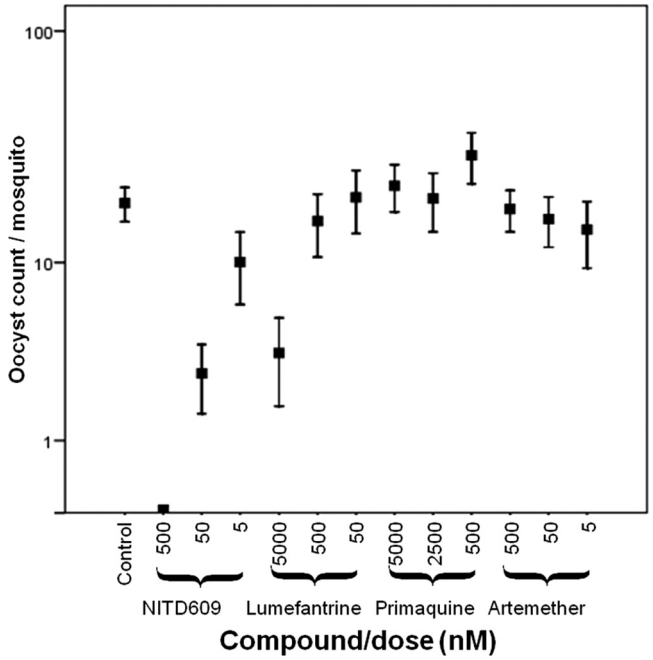

FIG 5 Effect of drugs on oocyst count. Dots represent arithmetic mean numbers of oocysts from 20 dissected mosquitoes. Standard deviation (SD) lines show the variations in the results of two separate experiments. The results for both experiments are combined in the figure; thus, 40 mosquitoes were dissected for each tested compound dilution. Eighty mosquitoes were used in the negative-control experiment.

inconsistent. The cultures grown in the presence of primaquine were not affected.

Effect of compounds on oocyst development. Finally, these compounds were added to the blood meal of blood-feeding mosquitoes in the SMFA to test potential effects on sporogonic development (Fig. 5). Compared to control feeds, adding NITD609 (IRR, 0.21; $P < 0.001$) and lumefantrine (IRR, 0.51; $P < 0.001$) at any concentration resulted in a reduced oocyst count. There was no statistically significant effect on oocyst counts of adding artemether (IRR, 0.86; $P = 0.23$) or primaquine (IRR, 1.20; $P = 0.12$) at any concentration. There was evidence for a dose-dependent effect of NITD609 on oocyst counts in mosquitoes; oocyst counts decreased with increasing NITD609 concentration (IRR, 0.20; $P < 0.001$). Similarly, a dose-dependent inhibition of oocyst formation was observed for lumefantrine where the number of oocysts decreased with increasing lumefantrine concentration (IRR, 0.57; $P < 0.001$). Increasing doses of artemether resulted in higher oocyst counts (IRR, 1.34; $P = 0.001$); there was no dose-dependent effect on oocyst counts seen with primaquine (IRR, 0.88; $P = 0.14$).

DISCUSSION

The *in vitro* assay platform presented here evaluates the effects of the drugs on different steps of the entire process of gametocytogenesis from early and late gametocyte development in *in vitro* culture to oocyst development in *Anopheles* mosquitoes. In these assays, the spiroindolone drug candidate NITD609 shows potent dose-dependent inhibition against all steps in gametocyte and sporogonic development of *P. falciparum in vitro*. Significant inhibitory activity was also found for lumefantrine, while such activity was present only at the highest tested concentration of 500 nM for artemether. Under our assay conditions, primaquine did not show any significant activity at concentrations as high as 5 μM.

The spiroindolones represent a novel class of antimalarial drug candidates with robust activity against *Plasmodium* symptomatic asexual stages (37). Consistent with its subnanomolar potency for asexual stages, NITD609 was found to be the most potent compound in our early and late gametocyte development assays, with very significant gametocytemia reduction at the lowest tested concentration of 5 nM, which is still 10 times the IC_{50} for asexual parasites. Remarkably, NITD609 also shows potent dose-dependent activity in the SMFA, which is unprecedented for (candidate) antimalarial drugs; NITD609 completely blocks transmission of the parasite at 500 nM, suggesting a further inhibiting effect on the (remaining) stage V mature gametocytes going into fertilization in the mosquito midgut and/or on the production of ookinetes or oocysts. The data underline the potency of NITD609 as a potential transmission-blocking drug with inhibiting activity in several critical steps of this part of the life cycle.

Artemisinin derivatives are known for the fastest parasite clearance time *in vivo* of all known antimalarial drugs (2, 9, 17, 33). Some *in vivo* studies conducted with artemisinin derivatives have also shown reducing effects on gametocyte prevalence (18, 32). This has been speculated to be the consequence of fast killing of asexual stages, although *in vitro* data suggest that dihydro-artemisinin may target early rather than late gametocyte development (7). Our results are consistent with this hypothesis but contrast with results reported for artesunate (9). We saw an inconsistent but, in both experiments, insignificant effect on late gametocyte development at the lower doses of artemether, likely due to variations between culture flasks. Furthermore, there was no inhibitory effect on mature gametocytes with artemether in the SMFA, even at the highest tested concentration of 500 nM, while Chotivanich et al. reported potent activity for artesunate in a similar assay (9). The explanation for this discrepancy may lie in methodological differences; in the study by Chotivanich et al., mosquito vectors were fed with mature gametocytes that had been cultured in a drug solution for 24 h prior to analysis using the SMFA, whereas the protocol used in our study entails adding drugs into a mature gametocyte culture immediately before the SMFA.

Although with reduced *in vitro* potency compared to NITD609, lumefantrine also inhibits both early and late gametocyte development and significantly reduces transmission at a high tested concentration (5 μM). As previously reported (26), the transmission-reducing activity of lumefantrine-artemether is speculated to be borne largely by artemether. However, our *in vitro* data, as well as data recently published by Adjalley et al. (1), suggest that lumefantrine may contribute significantly to this activity.

The only antimalarial drug reported to have a gametocytocidal effect on *P. falciparum in vivo* is the 8-aminoquinolone primaquine. While accelerating gametocyte clearance, primaquine did not affect gametocyte development (23). A dosing regimen of primaquine is currently being further tested as a potential transmission-reducing therapy (25). Although it has been previously shown that it effectively clears gametocytes in human patients (16), primaquine did not show significant activity in any of our assays. We observed only a small, statistically insignificant reduc-

tion of the formation of stages I and II gametocytes and only at a relatively high concentration (5,000 nM). The observation that primaquine did not affect *P. falciparum* gametocytes *in vitro* is in agreement with previous reports (7, 8, 10, 19, 27) and can be explained by the fact that primaquine needs to be bioactivated or metabolized to exploit its gametocytocidal properties (29).

In conclusion, our data support the notion that, if the drug is shown to be safe and efficacious at doses providing plasma levels in the range of 50 to 500 nM in humans, NITD609 could be used to exert transmission-blocking activity through direct and cidal activity against mature gametocytes. This is particularly important, because this suggests that spiroindolones could also be an important tool to treat asymptomatic carriers, which constitute a constant reservoir of mature gametocytes in areas of high transmission. Collectively, our data support the notion that if NITD609 is successful in the clinics as an treatment against acute malaria, this compound could also provide transmission-blocking activity to a novel combination therapy.

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REFERENCES

1. **Adjalley SH, et al.** 2011. Quantitative assessment of Plasmodium falciparum sexual development reveals potent transmission-blocking activity by methylene blue. Proc. Natl. Acad. Sci. U. S. A. **108**:E1214–E1223.
2. **Adjuik M, et al.** 2004. Artesunate combinations for treatment of malaria: meta-analysis. Lancet **363**:9–17.
3. **Akoachere M, et al.** 2005. In vitro assessment of methylene blue on chloroquine-sensitive and -resistant Plasmodium falciparum strains reveals synergistic action with artemisinins. Antimicrob. Agents Chemother. **49**:4592–4597.
4. **Alano P.** 2007. Plasmodium falciparum gametocytes: still many secrets of a hidden life. Mol. Microbiol. **66**:291–302.
5. **Alonso PL, et al.** 2011. A research agenda to underpin malaria eradication. PLoS Med. **8**:e1000406. doi:10.1371/journal.pmed.1000406.
6. **Baker DA.** 2010. Malaria gametocytogenesis. Mol. Biochem. Parasitol. **172**:57–65.
7. **Buchholz K, et al.** 2011. A high-throughput screen targeting malaria transmission stages opens new avenues for drug development. J. Infect. Dis. **203**:1445–1453.
8. **Chevalley S, Coste A, Lopez A, Pipy B, Valentin A.** 2010. Flow cytometry for the evaluation of anti-plasmodial activity of drugs on Plasmodium falciparum gametocytes. Malaria J. **9**:49. doi:10.1186/1475-2875-9-49.
9. **Chotivanich K, et al.** 2006. Transmission-blocking activities of quinine, primaquine, and artesunate. Antimicrob. Agents Chemother. **50**:1927–1930.
10. **Dixon MW, Peatey CL, Gardiner DL, Trenholme KR.** 2009. A green fluorescent protein-based assay for determining gametocyte production in Plasmodium falciparum. Mol. Biochem. Parasitol. **163**:123–126.
11. **Djimde A, Lefevre G.** 2009. Understanding the pharmacokinetics of Coartem. Malar. J. **8**(Suppl. 1):S4. doi:10.1186/1475-2875-8-S1-S4.
12. **Dorn A, Stoffel R, Matile H, Bubendorf A, Ridley RG.** 1995. Malarial haemozoin/beta-haematin supports haem polymerization in the absence of protein. Nature **374**:269–271.
13. **Huber W, Koella JC.** 1993. A comparison of three methods of estimating EC50 in studies of drug resistance of malaria parasites. Acta Trop. **55**:257–261.
14. **Kim YR, et al.** 2004. Pharmacokinetics of primaquine and carboxyprimaquine in Korean patients with vivax malaria. Arch. Pharm. Res. **27**:576–580.
15. **Kone A, et al.** 2010. Sulfadoxine-pyrimethamine impairs Plasmodium falciparum gametocyte infectivity and Anopheles mosquito survival. Int. J. Parasitol. **40**:1221–1228.
16. **Lederman ER, et al.** 2006. Combined chloroquine, sulfadoxine/pyrimethamine and primaquine against Plasmodium falciparum in Central Java, Indonesia. Malar. J. **5**:108. doi:10.1186/1475-2875-5-108.
17. **Nosten F, et al.** 2000. Effects of artesunate-mefloquine combination on incidence of Plasmodium falciparum malaria and mefloquine resistance in western Thailand: a prospective study. Lancet **356**:297–302.
18. **Okell LC, Drakeley CJ, Ghani AC, Bousema T, Sutherland CJ.** 2008. Reduction of transmission from malaria patients by artemisinin combination therapies: a pooled analysis of six randomized trials. Malar. J. **7**:125. doi:10.1186/1475h2875-7-125.
19. **Peatey CL, et al.** 2009. Effect of antimalarial drugs on Plasmodium falciparum gametocytes. J. Infect. Dis. **200**:1518–1521.
20. **Ponnudurai T, Leeuwenberg AD, Meuwissen JH.** 1981. Chloroquine sensitivity of isolates of Plasmodium falciparum adapted to in vitro culture. Trop. Geogr. Med. **33**:50–54.
21. **Ponnudurai T, et al.** 1989. Infectivity of cultured Plasmodium falciparum gametocytes to mosquitoes. Parasitology **98**(Pt. 2):165–173.
22. **Ponnudurai T, Meuwissen JH, Leeuwenberg AD, Verhave JP, Lensen AH.** 1982. The production of mature gametocytes of Plasmodium falciparum in continuous cultures of different isolates infective to mosquitoes. Trans. R. Soc. Trop. Med. Hyg. **76**:242–250.
23. **Pukrittayakamee S, et al.** 2004. Activities of artesunate and primaquine against asexual- and sexual-stage parasites in falciparum malaria. Antimicrob. Agents Chemother. **48**:1329–1334.
24. **Rottmann M, et al.** 2010. Spiroindolones, a potent compound class for the treatment of malaria. Science **329**:1175–1180.
25. **Shekalaghe SA, et al.** 2010. In Tanzania, hemolysis after a single dose of primaquine coadministered with an artemisinin is not restricted to glucose-6-phosphate dehydrogenase-deficient (G6PD A-) individuals. Antimicrob. Agents Chemother. **54**:1762–1768.
26. **Sutherland CJ, et al.** 2005. Reduction of malaria transmission to Anopheles mosquitoes with a six-dose regimen of co-artemether. PLoS Med. **2**:e92. doi:10.1371/journal.pmed.0020092.
27. **Tanaka TQ, Williamson KC.** 2011. A malaria gametocytocidal assay using oxidoreduction indicator, alamarBlue. Mol. Biochem. Parasitol. **177**:160–163.
28. **Trager W, Jensen JB.** 1976. Human malaria parasites in continuous culture. Science **193**:673–675.
29. **Vale N, Moreira R, Gomes P.** 2009. Primaquine revisited six decades after its discovery. Eur. J. Med. Chem. **44**:937–953.
30. **van der Kolk M, et al.** 2005. Evaluation of the standard membrane feeding assay (SMFA) for the determination of malaria transmission-reducing activity using empirical data. Parasitology **130**:13–22.
31. Reference deleted.
32. **Wells TN, Alonso PL, Gutteridge WE.** 2009. New medicines to improve control and contribute to the eradication of malaria. Nat. Rev. Drug Discov. **8**:879–891.
33. **White NJ.** 2008. The role of anti-malarial drugs in eliminating malaria. Malar. J. **7**(Suppl. 1):S8. doi:10.1186/1475-2875-7-S1-S8.
34. **WHO.** 2011. Guidelines for the treatment of malaria, 2nd ed. WHO, Geneva, Switzerland.
35. **WHO.** 2010. WHO Global Malaria Program: world malaria report 2010. WHO, Geneva, Switzerland.
36. **Wong RP, et al.** 2010. In vitro sensitivity of Plasmodium falciparum to conventional and novel antimalarial drugs in Papua New Guinea. Trop. Med. Int. Health **15**:342–349.
37. **Yeung BK, et al.** 2010. Spirotetrahydro beta-carbolines (spiroindolones): a new class of potent and orally efficacious compounds for the treatment of malaria. J. Med. Chem. **53**:5155–5164.