Histone Methyltransferase Inhibitors Are Orally Bioavailable, Fast-Acting Molecules with Activity against Different Species Causing Malaria in Humans

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Current antimalarials are under continuous threat due to the development of drug resistance by malaria parasites. We previously reported promising in vitro parasite-killing activity with the histone methyltransferase inhibitor BIX-01294 and its analogue TM2-115. Here, we further characterize these diaminoquinazolines for in vitro and in vivo efficacy and pharmacokinetic properties to prioritize and direct compound development. BIX-01294 and TM2-115 displayed potent in vitro activity, with 50% inhibitory concentrations (IC50) of <50 nM against drug-sensitive laboratory strains and multirudr-resistant field isolates, including artemisinin-refractory Plasmodium falciparum isolates. Activities against ex vivo clinical isolates of both P. falciparum and Plasmodium vivax were similar, with potencies of 300 to 400 nM. Sexual-stage gametocyte inhibition occurs at micromolar levels; however, mature gametocyte progression to gamete formation is inhibited at submicromolar concentrations. Parasite reduction ratio analysis confirms a high asexual-stage rate of killing. Both compounds examined displayed oral efficacy in vivo mouse models of Plasmodium berghei and P. falciparum infection. The discovery of a rapid and broadly acting antimalarial compound class targeting blood stage infection, including transmission stage parasites, and effective against multiple malaria-causing species reveals the diaminoquinazoline scaffold to be a very promising lead for development into greatly needed novel therapies to control malaria.

The continuous evolution of antimalarial drug resistance by Plasmodium parasites is a major impediment to the elimination of this devastating disease. Artemisinin combination therapies (ACTs) are the current mainstay of malaria chemotherapy, but the development of artemisinin resistance in parasites was reported in 2008 and 2009 along the Thai-Cambodian border (1). This underscores the need to validate new antimalarial targets within the parasite and to develop new antimalarial treatments based on novel scaffolds with desirable characteristics, such as fast killing activity against multiple parasite life stages; efficacy against multidrug-resistant strains and multiple species of human malaria parasites, including Plasmodium vivax; and favorable pharmacokinetics to allow oral administration.

Epigenetic gene regulation mediated by histone-modifying enzymes has been shown to play an important role in malaria parasite transcriptional regulation, including the control of virulence genes involved in immune evasion (2, 3). Histone lysine methyltransferase (HKMT) enzymes present a novel potential target class for the development of antimalarials due to the association of histone methylation at distinct lysine positions with both overall transcriptional activation (H3K4me) and multicopy gene family transcriptional repression (H3K9me) (4, 5). Indeed, half of the identified Plasmodium falciparum HKMT enzymes were recently shown to be refractory to genetic disruption (6). The essential and important regulatory role of HKMT enzymes in malaria parasite biology, combined with information acquired through increased attention to them in the setting of cancer chemotherapy development (7), motivates the exploration of parasite HKMT enzymes as a novel target class for antimalarial treatment.

We previously demonstrated that BIX-01294, a histone methyltransferase inhibitor, and its analogue TM2-115 (Fig. 1A) cause...
rapid and irreversible parasite-killing activity in vitro throughout the intraerythrocytic asexual cycle (8). Importantly, the same class of molecule shows a “wake up” effect on dormant liver stage malaria parasites (hypnozoites), suggesting an important role of HKMTs in this yet ill-defined biological liver stage (9). These promising antimalarial characteristics led us to investigate these lead compounds against relevant multidrug-resistant (including artemisinin resistance) field isolates and clinical isolates of *P. falciparum* and *P. vivax*. Activity against sexual-stage gametocytes and their development into gametes, which are responsible for malaria disease transmission, were also evaluated. To better assess the potential of this novel class of molecules for preclinical development as antimalarials, we employed a number of relevant tests, such as oral bioavailability and efficacy in disease-relevant animal models. BIX-01294 and TM2-115 were subjected to a Peters test (see below) in mice infected with *Plasmodium berghei* and to a 4-day test in SCID mice infected with *P. falciparum* parasites. Pharmacokinetic analyses were undertaken to aid the interpretation of our in vivo results and to inform further series development.

**MATERIALS AND METHODS**

**Materials.** Antimalarials, including chloroquine (CQ) and atovaquone (ATQ), were obtained from Sigma-Aldrich; artesunate (AS) was obtained from Sigma-Aldrich and Holly Pharmaceuticals Co. Ltd. DSM1 ([5-methyl-[1,2,4]triazolo-[1,5-a][pyrimidin-7-yl]naphthalen-2-ylamine] (10) was a gift from Akhil Vaidya (Drexel University College of Medicine). BIX-01294 and TM2-115 were synthesized as previously described (11). The molecular masses of BIX-01294 and TM2-115 free-base compounds are both 491 g/mol, and the molecular masses of their trihydrochloride salts are both 600 g/mol.

**In vitro asexual-stage parasite assays.** Compound efficacy against drug-sensitive laboratory strain 3D7 parasites and Cambodian artemisinin-resistant isolates were performed using a previously described 3-day SYBR green I growth and proliferation assay in a 96-well format (12). *In vitro* parasite reduction ratio (PRR) studies were performed as previously described (3). Cambodian parasite isolates were collected in Palin province in 2010 and were culture adapted at Institut Pasteur in Cambodia, as previously described (13). All three strains harbor mutations (C580Y for KH10-PL3 and KH10-PL10; R539T for 3D7) in the propeller domain of the Kelch gene (PF3D7_1343700; K-13 propeller), recently associated with artemisinin resistance (14). Dose-response curves were fitted, and 50% inhibitory concentrations (IC50s) were calculated and compared using one-way analysis of variance (ANOVA) in GraphPad (San Diego, CA, USA) Prism Version 6.0e.

**Drug interaction studies.** Isobologram analysis was performed using the fixed-ratio method, as described previously (15), in combination with the SYBR green I assay to quantify *P. falciparum* strain 3D7 parasite growth and proliferation (12). BIX-01294 was analyzed in combination with the *P. falciparum* dihydroorotase dehydrogenase inhibitor DSM1 (10) or the antimalarials CQ, AS, and ATQ. The fractional inhibitory concentration (FIC) of each drug was calculated by dividing the IC50 for the drug in combination by the IC50 of the drug alone. Mean sum FIC values were computed to classify any interactions as synergistic (≤0.5), antagonistic (≥4), or indifferent (0.5 < mean sum FIC < 4) (16).

**Ex vivo clinical isolate parasite assays.** Assays were performed as described previously (17) with minor changes, as follows. Six *P. falcipa- rum* and four *P. vivax* isolates were collected from malaria patients with no prior antimalarial therapy attending the clinics of the Shoklo Malaria Research Unit (SMRU) in the Mae Sot region of Tak Province in northwestern Thailand under the ethical guidelines in the approved protocol OXTREC 027-025 (University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, Oxford, United Kingdom). After written consent, blood samples were obtained by venipuncture in 5-ml-volume lithium-heparinized tubes. Only samples with >90% early ring stages were chosen for drug sensitivity testing after the removal of leukocytes using a CF-11 column, as described previously (18). Parasite susceptibility was tested in parallel against chloroquine diphosphate (Sigma-Aldrich) and artesunate (Holly Pharmaceuticals Co. Ltd.), as previously described (17). Dose-response curves and IC50s using duplicate-weigh data for each drug concentration were determined using ICESTIMATOR (http://www.antimalarial-icestimator.net/MethodIntro.htm) (19, 20).

**Gametocyte and dual gamete formation assays.** Inhibition of early-stage (stages I to III) and late-stage (stages IV and V) *P. falciparum* strain NF54 gametocyte viability was performed as previously described (21). Male and female *P. falciparum* gametocyte formation assays were performed essentially as described previously (22) for the “carryover” format, in which test compounds were present for 24 h with stage V gametocytes and throughout gamete formation. For the “washout” format, after 24 h of compound incubation, gametocytes were washed three times over 6 h by total medium replacement, and gamete formation was then triggered in the absence of test compounds.

**Ookinete formation assays.** All work involving laboratory animals was performed in accordance with the EU regulations (EU directive 86/609/EEC) and within the regulations of the United Kingdom Animals (Scientific Procedures) Act 1986. The *P. berghei* ookinete development assay was set up exactly as described previously (23). Compounds were tested in quadruplicate independent biological replicates, and dose-response curves were fitted and IC50s were calculated using GraphPad (San Diego, CA, USA) Prism Version 6.0e.

**In vitro cytotoxicity assays.** Host cell cytotoxicity was determined in a 96-well format with a starting HEP2 cell density of 10,000 cells/well grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies). The cells were incubated with serial dilutions of test compounds for 3 days, and the resulting cell viability was quantified using Promega CellTiterBlue. Cell viability as a function of the test compound concentration was fitted to survival curves to estimate compound IC50s using GraphPad (San Diego, CA, USA) Prism Version 6.0e.

**Four-day Peters test.** *In vivo* efficacy against *P. berghei* was tested as previously described (24), with the modification that mice (n = 3 to 5) were infected with green fluorescent protein (GFP)-transfected *P. berghei* strain ANKA parasites (a gift from A. P. Waters and C. J. Janse, Leiden...
University, Leiden, The Netherlands), and parasitemia was determined using standard flow cytometry techniques. Compounds were dissolved or suspended in Tween 80-ethanol (70/30 [vol/vol]) and diluted 10-fold in water before dosing. The experimental mice were treated at 4, 24, 48, and 72 h postinfection with a 50-mg/kg of body weight oral dose of the compound (a 4-day Peters test) and were compared to an infected control group for reduction in parasitemia on day 4 (96 h postinfection) and for mean survival (monitored up to 30 days postinfection). Five control mice and three treated mice per treatment group were used. To support the interpretation of the in vivo efficacy results, 20-μl plasma samples were collected from two of the three mice from each treatment group for mouse snapshot exposure studies. All animal studies were approved by the Veterinary Office of Canton Basel-Stadt, and the animals were treated in accordance with institutional guidelines.

Four-day SCID mouse test. A cohort of age-matched female immunodeficient NOD-scid interleukin-2 receptor γcnull (IL-2Rγcnull) mice (Jackson Laboratory, Bar Harbor, ME) were engrafted with human erythrocytes (generously provided by the Red Cross Transfusion Blood Bank, Madrid, Spain) by daily injection with 1 ml of a 50% hematocrit erythrocyte suspension (RPMM 1640 medium, 25% [vol/vol] decomplemented human serum, 3.1 mM hypoxanthine) by the intraperitoneal route throughout the experiment, as described previously (25). After 7 days of injections, the mice reached 40% human erythrocytes in peripheral blood and were intravenously infected with 2 × 10⁷ P. falciparum PI3D70087/ N9-infected erythrocytes (day 0). On day 3 after infection, the mice were randomly allocated to treatments that were administered once a day for 4 consecutive days by oral gavage at 10 ml/kg. Both BIX-01294 and TM2-115 were dissolved in 70% Tween 80-30% ethanol and further diluted 1/10 in distilled water before administration.

Parasitemia was measured by flow cytometry in samples of peripheral blood stained with the fluorescent nucleic acid dye Syto-16 and anti-murine erythrocyte TER119 monoclonal antibody (Pharmingen, San Diego, CA, USA) in serial 2-μl blood samples taken every 24 h until assay completion (26).

In the efficacy experiment, the blood BIX-01294 and TM2-115 levels in the mice were measured in serial samples of peripheral blood (25 μl) taken by tail puncture at 0.25, 0.5, 1, 2, 4, 6, 8, and 23 h after the first administration. The blood samples were immediately lysed by mixing with 25 μl of water containing 0.1% saponin, frozen on dry ice, and stored at −80°C until analysis. The compounds were extracted from 10 μl of each lysate by liquid-liquid extraction in the MultiScreen Solvinit 0.45-μm Hydrophobic PTFE 96-well plate system (Millipore) and stored frozen at −80°C until analysis by liquid chromatography-tandem mass spectrometry (LC–MS-MS) in an AB Sciex API4000 (AB Sciex, Framingham, MA). The compound concentration-versus-time data were analyzed by noncompartmental analysis (NCA) using WinNonlin Professional Version 5.2 (Pharsight Corporation, Mountain View, CA, USA). Additional statistical analysis was performed with GraphPad (San Diego, CA, USA) Prism Version 5.01.

Efficacy was expressed as the daily exposure (area under the concentration-time curve [AUC] [μg · h/ml/day]) of BIX-01294 and TM2-115 in whole blood necessary to reduce parasitemia at day 7 by 90% with respect to vehicle-treated mice (AUCD90). The AUCD90 was estimated by fitting a four-parameter logistic equation for the log10 parasitemia at day 7 for individual i versus the AUC from 0 to 23 h (AUCD90i) of BIX-01294 and TM2-115 in blood for individual i using GraphPad (San Diego, CA, USA) Prism 6.0e.

All the experiments were approved by the Diseases of the Developing World, GlaxoSmithKline (GSK), Tres Cantos, Madrid, Spain (DDW) Ethical Committee on Animal Research and were carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals. The animal studies were performed at DDW Laboratory Animal Science facilities accredited by AAALAC. The human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents.

Functional hERG analysis. Functional human cardiac voltage-gated potassium channel ether-a-go-go-related gene (hERG) analysis was performed by Cyprotex (Macclesfield, United Kingdom). Briefly, mammalian cells expressing the hERG potassium channel were dispensed into 384-well planar arrays, and hERG tail currents were measured by whole-cell voltage clamping. A range of concentrations (8 nM to 25 μM) of the test compound were then added to the cells, and a second recording of the hERG current was made. The percent change in the hERG current was then calculated.

RESULTS

In vitro and ex vivo erythrocytic-stage antimalarial activities. We previously reported that BIX-01294 and TM2-115 (Fig. 1A) possess similar antimalarial efficacies against both drug-sensitive and drug-resistant laboratory strains of P. falciparum (8). The strains tested were resistant to longstanding antimalarials for which resistance has developed in the field, namely, chloroquine, mefloquine, and pyrimethamine. Reports of parasite resistance to the artemisinin derivatives, which represent the cornerstone of modern antimalarial combination therapies, developing along the Thai-Cambodian border prompted us to assess the efficacy of the compound series against clinically relevant artemisinin-resistant field isolates (13, 27). Parasite growth and proliferation in the presence of BIX-01294 and TM2-115 were assayed using three laboratory-adapted artemisinin-resistant field isolates from Pailin, Cambodia. The three isolates (3601 PC, KH10-PL03, and KH10-PL10) harbor mutations associated with artemisinin resistance and display a resistant phenotype in a ring stage survival assay (14.9%, 19.2%, and 27.3% survival, respectively, compared to 0.04% for sensitive strain 3D7 parasites) (14). The IC₅₀ of either BIX-01294 or TM2-115 were not significantly different between artemisinin-resistant field isolates and laboratory strain parasites (Table 1). These data suggest the mechanism of artemisinin resistance (K13 propeller mutations) does not significantly impact the efficacy of BIX-01294 and TM2-115 and suggests compounds developed from the series would be equally effective against both artemisinin-sensitive and emerging artemisinin-refractory parasites. Importantly, the IC₅₀ of BIX-01294 and TM2-115 are >100-fold higher against HepG2 human hepatic carcinoma cells (Table 1), as determined in a similar assay, indicating selectivity for parasites over host cells (Fig. 1B).

Of the Plasmodium species that infect humans, P. falciparum is the major contributor to worldwide malaria mortality. Though responsible for fewer malaria fatalities, P. vivax is the main cause of global morbidity and is responsible for instances of malaria relapse due to the ability of the species to remain as dormant hypnozoites for years within the liver of a previously infected individual (28). To identify whether BIX-01294 and TM2-115 have cross-species antimalarial activity, we assayed the compounds against clinical isolates of P. falciparum and P. vivax using an ex vivo red blood cell stage parasite progression assay (17). The data revealed similar IC₅₀ against either P. falciparum or P. vivax clinical isolates for both BIX-01294 and TM2-115 (Table 2). The overall IC₅₀ in the ex vivo assay are higher than those observed in the in vitro assay, including for chloroquine, likely due to experimental differences between the ex vivo and in vitro assays. Notably, the ex vivo assay reports on the progression from early- to late-stage parasites and thus reveals the effect of compound exposure during a single cycle of the 48-hour erythrocytic stage. These data suggest compounds developed from this series would be equally effective against the erythrocytic stages of the two most clinically relevant
species of human malaria parasites and are able to exert their killing effects on the time scale of a single 48-hour erythrocytic replication cycle.

**PRR analysis.** Currently used antimalarials display a wide range of parasite clearance times (PCTs) (3). The inherent killing rates of individual antimalarials not only impact the therapeutic onset and thereby the efficacy of a given treatment, but are also a major consideration in choosing combination therapy partners for novel antimalarial formulations. To best determine the rates of parasite killing by BIX-01294 and TM2-115, we performed a PRR analysis that quantifies the viable parasites after exposure to 10 times the IC₅₀ of test antimalarials for various lengths of time (3). P. falciparum parasites (strain 3D7A) were exposed to BIX-01294 or TM2-115 for periods ranging from 24 to 120 h or 24 to 48 h, respectively, after which time the compounds were washed out. The treated parasites were serially diluted into 96-well culture plates to determine the number of viable parasites remaining after 21 to 28 days of culture with fresh erythrocytes. The results from this analysis show that both BIX-01294 and TM2-115 display a rapid killing profile without any lag phase, essentially eliminating all viable parasites after a 48-hour exposure to either compound (Fig. 2). For BIX-01294, the log PRR value, representing the log-eliminate 99.9% of the starting parasitemia, was 29 h. TM2-115 cycle, was 4.7, and the 99.9% PCT value, representing the time to strain parasites or means and 95% confidence intervals (CI) for a single IC₅₀ determination for the artemisinin-resistant (Artr) strains.

**In vitro drug interaction studies.** We performed fixed-ratio isobologram analyses to determine whether any apparent interaction exists in vitro between BIX-01294 and existing compounds with known antimalarial mechanisms of action (15, 29). Mean sum FIC values of 1.1 to 1.4 for each drug combination reveal little if any interaction between BIX-01294 and the other parasite-killing compounds tested (Fig. 3). These data suggest that compounds developed from this series would be effective if employed in combination with existing antimalarials without a concern for drug antagonism.

**In vitro transmission stage activity.** Sexual-stage gametocytes are responsible for the transmission of malaria parasites from the infected host to the mosquito vector and then to subsequent, additional human hosts. Of the antimalarials currently in worldwide use, only primaquine has been proven effective at reducing the infectivity of the generally drug-insensitive mature gametocyte (30). Unfortunately, contraindications for primaquine use, including glucose 6-phosphate 1 dehydrogenase (G6PD) deficiency, which is prevalent in areas where malaria is endemic, prompts the search for new compounds with efficacy against gametocytes. To examine whether BIX-01294 or TM2-115 possesses potential transmission-blocking activity, we determined the IC₅₀ for each compound against P. falciparum gametocytes in either the early (stages I to III) or late (stages IV and V) phases of the 10- to 14-day gametocyte developmental period. The data show IC₅₀s of 1 to

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**TABLE 1** In vitro compound efficacies against a drug-sensitive (3D7) laboratory strain and multidrug-resistant, including artemisinin-resistant, field isolates from Pailin, Cambodia

<table>
<thead>
<tr>
<th>Compound treatment</th>
<th>P. falciparum strain 3D7</th>
<th>P. falciparum Artr² isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3601 PC</td>
<td>KH10-PL03</td>
</tr>
<tr>
<td>BIX-01294</td>
<td>19 ± 3</td>
<td>35 ± 14</td>
</tr>
<tr>
<td>TM2-115</td>
<td>32 ± 5</td>
<td>48 ± 18</td>
</tr>
<tr>
<td>Dihydroartemisinin</td>
<td>0.63 ± 0.41</td>
<td>1.3 (1.0–1.6)</td>
</tr>
<tr>
<td>Artesunate</td>
<td>0.97 ± 0.94</td>
<td>1.5 (1.1–2.1)</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>8.4 ± 1.2</td>
<td>68 ± 25</td>
</tr>
</tbody>
</table>

*IC₅₀ were determined in a 3-day SYBR green I-based growth and proliferation assay. HepG2 cell viability was determined in a 3-day CellTiterBlue assay. Values are means ± SD of two or three experiments for BIX-01294, TM2-115, and chloroquine. For dihydroartemisinin and artemesunate, the values are means ± SD from 20 or 21 experiments for 3D7 strain parasites or means and 95% confidence intervals (CI) for a single IC₅₀ determination for the artemisinin-resistant (Artr²) strains.

**TABLE 2** Ex vivo compound efficacies against clinical isolates of P. falciparum and P. vivax

<table>
<thead>
<tr>
<th>Compound treatment</th>
<th>IC₅₀ (nM)ᵃ</th>
<th>P. falciparum</th>
<th>P. vivax</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIX-01294</td>
<td>280 ± 90</td>
<td>390 ± 90</td>
<td></td>
</tr>
<tr>
<td>TM2-115</td>
<td>340 ± 160</td>
<td>240 ± 70</td>
<td></td>
</tr>
<tr>
<td>Chloroquine</td>
<td>66 ± 37</td>
<td>51 ± 21</td>
<td></td>
</tr>
<tr>
<td>Artesunate</td>
<td>1.1 ± 0.5</td>
<td>0.9 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

*IC₅₀ were determined in a parasite maturation assay based on microscopic evaluation of parasite progression from young ring stage to mature schizont stage parasites. The values are means ± SD of duplicate measurements of four to seven isolates per parasite species.

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**FIG 2** Parasite reduction ratio analysis. P. falciparum strain 3D7A parasites were exposed to 10 times the IC₅₀ of BIX-01294 or TM2-115, chloroquine, or artemisinin for the durations indicated; then, the compound was washed out, and the parasites were diluted to determine the number of viable parasites remaining. The values represent means ± SD of quadruplicate parasite measurements.
We also investigated the effect of BIX-01294 and TM2-115 on mature stage V gametocyte development into male and female gametes (22). The assay we employed reports on the ability of a test compound not necessarily to kill mature stage V gametocytes directly, but rather, to block their functional viability and prevent the onward development necessary for parasite transmission to the mosquito. Assays were performed to identify effects on gametocytes prior to gamete formation in a washout format and on gametocytes during gamete formation in a carryover format. The results from these dual gamete formation assays show BIX-01294 and TM2-115 to have inhibitory effects on the formation of both male and female gametes in both assay formats. Male gametes are particularly affected, with IC50s of 20 to 140 nM for either compound in both assay formats. These results suggest that treatment with either compound irreversibly compromises the capacity of stage V gametocytes to develop into gametes and strongly suggest transmission-blocking activity via decreasing the number of viable male gametes.

Additionally, we examined the effects of BIX-01294 and TM2-115 on P. berghei ookinete development, the parasite stage resulting from the zygote formed after gamete fertilization. This assay reports on the ability of a drug to inhibit the transformation of ex vivo P. berghei mature gametocytes into ookinetes, encompassing gamete formation, fertilization, and zygote development into the mature ookinete. Data from these experiments show both test compounds inhibit ookinete development with IC50s of 6 to 7 μM (Table 3).

**In vivo erythrocytic-stage activity against P. berghei in a mouse model.** We previously demonstrated that our diaminoquinazoline compounds possess activity against malaria parasites in an animal model of infection after intraperitoneal administration (8). To explore the oral efficacy of BIX-01294 and TM2-115, we performed a 4-day Peters test, which examines the abilities of compounds to reduce or clear blood stage infection (31). Mice were infected with P. berghei parasites and then treated via oral delivery at 4, 24, 48, and 72 h postinfection with 50 mg/kg of free-base or salt formulations of BIX-01294 or TM2-115 (Fig. 4A). Treatment with BIX-01294 free-base or salt formulations yielded

<table>
<thead>
<tr>
<th>Compound</th>
<th>P. falciparum gametocyte viability</th>
<th>P. falciparum gamete formation</th>
<th>P. berghei ookinete viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early stage</td>
<td>Late stage</td>
<td>Male</td>
</tr>
<tr>
<td>BIX-01294</td>
<td>1.07 ± 0.08</td>
<td>3.84 ± 0.36</td>
<td>0.120 ± 0.050</td>
</tr>
<tr>
<td>TM2-115</td>
<td>1.30 ± 0.08</td>
<td>2.63 ± 0.25</td>
<td>0.022 ± 0.001</td>
</tr>
<tr>
<td>Artesunate</td>
<td>0.0047 ± 0.0003</td>
<td>0.0076 ± 0.0004</td>
<td>0.022 ± 0.004</td>
</tr>
</tbody>
</table>

The values represent the means ± standard errors of the mean (SEM) of the fitted data for two to six independent IC50 determinations.
a 99.9% reduction in parasitemia versus untreated controls on day 4 postinfection (Fig. 4B). Treatment with TM2-115 free-base or salt formulations produced a 95% or 87% reduction in parasitemia versus untreated controls, respectively, on day 4 postinfection (Fig. 4B). These reductions in parasitemia were reflected in the overall survival rates of treated mice, where BIX-01294-treated mice survived for 12 to 16 days postinfection while TM2-115-treated mice survived for 6 to 10 days postinfection (Fig. 4C). Pharmacokinetic analysis revealed a long serum half-life ($t_{1/2}$) for both free-base and salt formulations of BIX-01294, though the salt form yields higher levels in serum than the base form but a much shorter $t_{1/2}$ for either form of TM2-115 (Fig. 4D). Thus, increased exposure to BIX-01294 relative to TM2-115 may account for the difference in efficacy between the two compounds. Though neither compound treatment resulted in complete parasite clearance, these data show that this diaminoquinazoline series exhibits oral efficacy against P. berghei rodent malaria parasites in a mouse model of infection.

**In vivo erythrocytic-stage activity against P. falciparum in a humanized mouse model.** To obtain a proof of concept of the therapeutic efficacies of BIX-01294 and TM2-115 against P. falciparum human malaria parasites in vivo, we employed NOD−scid IL2Rγcnull mice engrafted with human erythrocytes. Mice infected with P. falciparum were treated by oral delivery at days 3, 4, 5, and 6 postinfection with 75 or 100 mg/kg, and parasite levels were measured on days 3 to 7 (Fig. 5A). The levels of BIX-01294 and TM2-115 in blood were also measured for 23 h after the first dose in the same animals (Fig. 5B). In mice treated with BIX-01294, parasitemia fell below the limit of detection at day 5 or 6 of the experiment, and consistently, most parasites in the blood were pyknotic after 48 h of exposure (Fig. 5C). In infected mice treated with TM2-115, the parasite load remained essentially at the same level as when the infected mice were first treated, and parasites in the blood were a mixture of pyknotic and replicating parasites after 48 h of exposure (Fig. 5C). The difference in therapeutic responses between BIX-01294 and TM2-115 is consistent with the 5-fold-higher exposure of the former mice (BIX-01294 dose-normalized AUCs were 0.25 and 0.31 µg · h/ml per mg/kg versus TM2-115 dose-normalized AUCs of 0.07 and 0.06 µg · h/ml per mg/kg at 75 and 100 mg/kg, respectively) rather than to any intrinsic difference in potency or activity in vivo. Together, these data show that our diaminoquinazoline series are orally efficacious and kill P. falciparum rapidly in vivo.

**Functional hERG analysis.** Quinazoline-containing molecules have been known to inhibit hERG, presenting a potential liability for further series development (32). To assess whether our two diaminoquinazoline compounds possess any inhibitory activity against hERG, we obtained electrophysiology-based functional inhibition data for the two compounds (Fig. 6). BIX-01294 began inhibiting the hERG channel signal at concentrations above 10µM, producing 65% inhibition at 25µM, the highest concentration investigated. TM2-115 displayed very little hERG channel inhibition across the concentration range tested, inhibiting 17% at 25µM. These results indicate BIX-01294 inhibits hERG at con-
Subsequent demonstration of antimalarial characteristics against blood stage parasites (8). We initially screened several known histone methyltransferase inhibitors against parasites in culture and discovered that the diaminoquinazoline BIX-01294 and related analogues reduced parasite histone methylation levels and showed promising preliminary antimalarial activity during the entire asexual blood stage development (9). Further increased interest in these molecules for their potential in treating different life cycle stages of human malaria parasites. The current studies were designed to further investigate this series and to provide data to guide the development of diaminoquinazolines as potential novel antimalarials.

BIX-01294 and TM2-115 show comparable in vitro efficacies against drug-sensitive laboratory parasites and artemisinin-resistant clinical isolates. ACTs represent the front-line therapy in many areas where malaria is endemic. Emerging resistance to artemisinin and partner drugs in Southeast Asia threatens the effectiveness of all ACTs (1, 35–38). Artemisinin resistance manifests as reduced effectiveness of artemisinins against ring stage parasites (14). Compounds with efficacy against parasites resistant to this integral component of antimalarial combination therapy will be important when developing next-generation therapies with worldwide efficacy. Importantly, we have demonstrated BIX-01294 and TM2-115 to be equally effective throughout the erythrocytic cycle, including against ring stage parasites (8). This indicates that our diaminoquinazolines represent a promising compound class with a high likelihood of activity against existing and emerging multidrug-resistant parasite strains.

Activity against both P. falciparum and P. vivax clinical isolates demonstrates cross-species activity against the two most relevant human malaria pathogens for the compound series. This cross-species activity may be due to the high conservation of HKMT proteins in P. falciparum and P. vivax and their assumed conserved role in transcriptional regulation in all Plasmodium species. Activity against several Plasmodium species that affect humans is a very desirable characteristic of any potential new antimalarial therapy.

We previously showed a rapid parasite-killing effect in treatment and washout experiments (8). A more robust analysis of the parasite-killing rate has since been developed, and this improved method was employed to better characterize the rate and extent of parasite killing by BIX-01294 and TM2-115 (3). These studies revealed the parasite-killing rates of diaminoquinazoline compounds to be slightly higher than that of chloroquine, the fastest parasite killer currently in clinical use, after artemisinin derivatives. An advantage of this compound series is its demonstrated killing activity during the entire asexual blood stage development (8). This is probably due to the need for continual activation of genes throughout the 48-hour blood stage development, a process linked to epigenetic transcriptional regulation via histone methylation. Rapidly killing antimalarials are important for reducing initial parasite loads, especially in severe malaria cases, leading to the potential for development of resistance to both the drug itself and any partner drug of a combination chemotherapy. Importantly, the speed of action of an antimalarial is directly related to its mechanism of action. Thus, future compounds from this series that maintain activity against the same target will retain this rapid-killing effect.
Drug interaction studies in vitro showed BIX-01294 to have no significant interaction when tested in combination with chloroquine, artesunate, atovaquone, or the parasite dihydroorotate dehydrogenase inhibitor DSM1. The overall parasite-killing profile, together with a novel chemical structure and activity against characterized resistant strains, may indicate that BIX-01294 targets a parasite mechanism distinct from these other molecules with known mechanisms of action. The lack of any antagonism indicates BIX-01294 would in theory be a suitable partner drug in any combination antimalarial therapy comprised of compounds tested in these studies. As we develop this compound series further, interactions with potential partner drugs could be similarly investigated.

Populations of gametocytes, the parasite form responsible for malaria transmission, are female dominated, with a natural female-to-male ratio of approximately 4:1 (22). Compound activity against developing mixed-sex gametocytes appeared to be low relative to asexual blood stage parasites. Mean inhibitory concentrations against both early- and late-stage gametocyte viability were above 1 micromolar, comparable to that against human HepG2 cells, which we use as a measure of potential host cell toxicity. Inhibitory activity against subsequent mosquito stage ookinete development of P. berghei occurred at similarly high compound concentrations. However, in P. falciparum, dual gamete formation experiments demonstrated inhibition of male gamete formation in treated and washed mature stage V gametocytes at 20 to 140 nM, revealing potent and irreversible inhibition of the functional ability of gametocytes to progress to gametes. Overall, male gametocytes/gametes were 10-fold more susceptible to inhibition than the more abundant female gametes, and TM2-115 was roughly 5-fold more potent than BIX-01294. As such, while these diaminoquinazoline compounds appear less effective at inhibiting gametocyte and ookinete development and maturation, they are indeed effective at inhibiting gametocyte onward functional ability to develop into gametes, an essential first step in establishing an infection in the insect vector. The current understanding of transcriptional regulation in sexual-stage parasites is very limited. Differential expression profiles of the 10 annotated methyltransferase enzymes in asexual-stage parasites compared to sexual-stage gametocytes is apparent (http://plasmodb.org) and may account for decreased P. berghei levels and survival outcome. In a mouse model of P. falciparum infection, BIX-01294 and TM2-115 performed similarly to the P. berghei models. Four oral doses of TM2-115 beginning on day 3 postinfection decreased parasite levels relative to vehicle controls, though overall parasite levels remained essentially steady from the onset of treatment. Similar treatment of patent P. falciparum infection with BIX-01294 in the SCID mouse model produced a much greater decrease in parasite numbers, to below the limit of detection on day 5 or 6 postinfection, i.e., after three or four doses of 100 or 75 mg/kg, respectively. Together, these in vivo efficacy studies show this compound series to be orally bioavailable and active against both P. berghei, the parasite species in standard mouse models of malaria infection, and P. falciparum, the most relevant human malaria parasite. These studies establish the oral efficacy of the compound series and might support the use of P. berghei models, which are readily available relative to the P. falciparum model, to facilitate future compound progression. Importantly, the humanized SCID mouse model indicates that diaminoquinazoline compounds kill P. falciparum rapidly in vivo at a range of concentrations in blood that might be achievable in humans.

In summary, our in vitro and in vivo antimalarial activity studies of this diaminoquinazoline compound series indicate very promising activity against sexual- and multiple asexual-stage Plasmodium species with various genetic backgrounds, including multidrug-resistant profiles. These results support further development of the compound series as a novel antimalarial class, which is under way with regard to red cell stage efficacy and specificity and compound stability (39). Through iterative medicinal chemistry efforts, we aim to improve the pharmacokinetic characteristics of next-generation molecules while retaining the rapid-killing profile and improving efficacy and specificity against P. falciparum and P. vivax blood stage parasites and gametocyte functional viability. Ongoing target identification and subsequent target characterization efforts will greatly aid the progression of this compound series.

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