Small-Molecule Xenomycins Inhibit All Stages of the *Plasmodium* Life Cycle

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Widespread resistance to most antimalaria drugs in use has prompted the search for novel candidate compounds with activity against *Plasmodium* asexual blood stages to be developed for treatment. In addition, the current malaria eradication programs require the development of drugs that are effective against all stages of the parasite life cycle. We have analyzed the antimalarial properties of xenomycins, a novel subclass of small molecule compounds initially isolated for anticancer activity and similarity to quinacrine in biological effects on mammalian cells. *In vitro* studies show potent activity of Xenomycins against *Plasmodium falciparum*. Oral administration of xenomycins in mouse models result in effective clearance of liver and blood asexual and sexual stages, as well as effective inhibition of transmission to mosquitoes. These characteristics position xenomycins as antimalarial candidates with potential activity in prevention, treatment and elimination of this disease.

Malaria is still a major world health problem, causing more than 600,000 deaths annually, mainly among African children (1). The enormous progress in malaria control achieved in the past decade appears to have slowed down recently, in parallel with a plateau in international funding. Despite the availability of preventive measures, such as bed-nets, mosquito repellents, and insecticides, their efficacy and implementation is only partial, and malaria cases reached 207 million in 2012.

Small-molecule drugs remain the dominant clinical option for treating malaria, both prophylactically and curatively. Unfortunately, effective drugs such as chloroquine, fansidar (sulfadoxine and pyrimethamine), atovaquone, mefloquine, and now artemisinin suffer from resistance problems (2). Malaria treatment still relies mostly on chloroquine for *Plasmodium* species other than *falciparum*. However, the generalized spread of resistance in *Plasmodium falciparum* requires new drugs for effective patient treatments. Compounds with unrelated mechanisms of import/action to those currently in use are highly sought after to avoid development of resistance in the field.

Recent renewed emphasis on the eradication of malaria has highlighted the need for drugs with gametocidal activity that would block disease transmission. Most current antimalarials in use do not prevent transmission, either because they do not have gametocidal activity or because their short half-lives prevent effective clearance of gametocytes (3).

A novel preventive malaria treatment to target *Plasmodium* liver stage is also highly desirable, not only because the liver stage is well suited for prophylactic intervention but also because the liver can serve as a reservoir for *Plasmodium vivax* and *Plasmodium ovale* hypnozoites dormant parasite forms that may lead to relapses long after the initial blood infection has been eliminated (4). Primaquine, the current standard of care for liver-stage malaria, has significant disadvantages since it causes hemolytic anemia in patients with glucose-6-phosphate-dehydrogenase deficiency. Also, it cannot be prescribed for pregnant women.

It has become a consensus that, in addition to the standard requirements for any new drug in the field (safety and efficacy, oral delivery, stability, etc.), novel antimalarial compounds to be

used in areas of endemicity should also present low susceptibility to resistance development, as well as target multiple stages of the parasite (3, 4).

There is a significant coincidence of anticancer and antimalarial properties in several families of compounds that is possibly mediated by the coincidence on basic metabolic requirements associated with the high proliferation rate of both cell types (5). Here, we have analyzed the antimalarial properties of several small molecules from the recently introduced curaxin family of anticancer compounds (6). From a chemical standpoint, curaxins represent carbazole derivatives, typically with two strong electron-withdrawing groups linked to positions 3 and 6 of the carbazole ring and a secondary or tertiary amino group spaced from carbazole nitrogen by two to three carbon atoms N-linker. Proprietary chemical class of curaxins is protected by worldwide patent portfolio (J. Tucker, S. Sviridov, L. Brodsky, C. Burkhart, A. Purmal, K. Gurova, and A. Gudkov A, Carbazole compounds and therapeutic uses of the compounds [patent application 61/102,913], 6 October 2008). During the course of structure-activity relationship and hit to anticancer lead studies, more than 200 curaxins were synthesized and characterized *in vitro* for their p53 activation potential. For the 20 most promising curaxins, a more detailed assessment of pharmacological properties was performed. This eval-

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FIG 1 Chemical structures of quinacrine (A) and ten xenomycins (B).

uation included *in vitro* (solubility, metabolic stability in the presence of animal and human liver microsomes, hERG inhibition, and cytochrome 450 inhibition) and *in vivo* (toxicity, pharmacokinetics, and anticancer efficacy) studies. Some of the selected curaxins (Fig. 1) were evaluated as antiprotozoal agents in the present study. The idea of testing selected curaxins for antimalarial properties came from the close similarity of biological effects caused by curaxins in mammalian cancer cells to those of old antimalarial drug quinacrine; despite obvious differences in chemical structure curaxins and quinacrine simultaneously activate p53 and inhibit NF- κ B pathways acting as nongenotoxic DNA intercalators (6–8). Although the mechanism of activity of quinacrine against *Plasmodium* is not known, similarities of anticancer effects of curaxins and quinacrine suggested testing of curaxins against malaria. To differentiate a subclass of curaxins expressing antimicrobial properties, the proprietary name xenomycins was assigned to these compounds. Our studies show that xenomycins are effective against every stage of the *Plasmodium* life cycle, including liver, blood asexual, gametocytes, and transmission to mosquitoes. The development of xenomycins as antimalarial drugs has the potential to deliver multipurpose compounds that would be effective in preventing and treating infection, as well as blocking the transmission of malaria.

MATERIALS AND METHODS

Reagents. Xenomycin compounds were provided for the present study by Incuron, LLC (Buffalo, NY). CBL0100, CBL0159, CBL0174, CBL0175,

and CBL0212 were custom synthesized by Dalton Pharma (Toronto, Canada). CBL0176, CBL0207, and CBL0252 were custom synthesized by Jubilant Chemsys, Ltd. (New Delhi, India). CBL0137 was synthesized by Aptuit, Inc. (Kansas City, MO). All xenomycin compounds were >97% pure as determined by high-pressure liquid chromatography (HPLC) and used as monohydrochloride salts. Dimethyl sulfoxide (DMSO) was used for the solubilization of compounds used for testing *in vitro* and never exceeded 0.5%. A 0.2% concentration of hydroxypropyl methylcellulose was used for the solubilization of compounds used for *in vivo* testing.

Studies of metabolic stability in the presence of rat or human liver microsomes were conducted by Absorption Systems (Exton, PA). Typically, pooled rat or human liver microsomes prepared by Absorption Systems were thawed at room temperature and kept on ice prior to the experiments. The test compound was prepared in DMSO and diluted with acetonitrile. Briefly, an incubation mixture containing 1 mg protein/ml of liver microsomes, 0.1 M phosphate buffer (pH 7.4), 5 mM MgCl₂, and test compound (5 μ M in the final incubation) was preincubated at 37°C in a shaking water bath for 3 min. CYP-mediated reaction was initiated by the addition of 1 mM NADPH, and the reaction mixture was incubated at 37°C in a shaking water bath. Aliquots of the incubation solutions were withdrawn in duplicate ($n = 2$) at 0 and 60 min for the test compound and at 0 and 5 min for the positive control (testosterone). The reaction was immediately terminated by adding ice-cold acetonitrile containing 0.1% formic acid, and the samples were stored at -80°C until analysis. The positive control (20 μ M testosterone) was performed in parallel to confirm the enzyme activities of the liver microsomes used. After centrifugation at 3,000 rpm for 10 min, the supernatants were transferred into HPLC vials, and the concentrations of the test compound or positive

control (testosterone) were determined by liquid chromatography-tandem mass spectrometry.

Measurements of hERG inhibition were contracted at IPS Therapeutique, Inc. (Sherbrooke, Quebec, Canada). This screening study was aimed at quantifying the *in vitro* effects of test articles on the rapid component of the delayed rectifier potassium current (IKr) generated under normoxic conditions in stably transfected human embryonic kidney cells (HEK 293). The hERG assay was used to assess the potential of a compound to interfere with the rapidly activating delayed-rectifier potassium channel. Typically, an hERG inhibition assay was performed with 0.1, 1, and 3 μ M concentrations of test compound. The hERG-expressing HEK293 cell line was propagated in minimum essential medium (MEM) complemented with 10% fetal bovine serum, 1% MEM sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, 1% penicillin-streptomycin, and 400 μ g/ml G-418 (Geneticin) as the selection agent (all ingredients from Multicell/Wisent, St-Bruno, Quebec, Canada). The line was maintained in 75-cm² flasks (Falcon, canted neck with angled blue vented seal cap). The cells were passed using 0.25% trypsin (Gibco/Invitrogen, Burlington, Ontario, Canada) when they reached a confluence of ca. 80%. Appropriate amounts of the cell suspension were added to culture dishes for patch clamping or transferred to a culture flask after trypsinization. The assay involved whole-cell current acquisition by manual patch clamp and semiautomated current density analysis. Statistical comparisons were made by using repeated paired Student *t* tests; the currents recorded after exposure to the different test articles concentrations were statistically compared to the currents recorded in baseline conditions. Differences were considered significant when the *P* value was <0.05.

Aqueous solubility experiments were conducted in classical "shake flask" format. Briefly, a suspension of test sample in water, phosphate-buffered saline, and 300 mg/ml Captisol or saline was incubated with rocking for at least 24 h at room temperature. The supernatant was separated from undissolved pellet by centrifugation and filtered through 0.2- μ m-pore-size filter to eliminate undissolved microparticles. The concentration of test article in filtrate was determined by HPLC.

Maximum tolerated doses of xenomycin compounds. For each xenomycin compound, the oral maximum tolerated doses (MTD) in mg/kg were determined for single and repetitive administrative regimens using 6- to 8-week-old National Institutes of Health (NIH) Swiss male and female mice (Harlan Laboratories, Indianapolis, IN). Typically, four mice per group (two males and two females) were used in single oral gavage administration studies. Six mice per group (three males and three females) were used in repetitive dosing (five daily oral administrations) study. Three to four dose levels were used in each study, along with a vehicle control group. Normally, the single MTD of xenomycins was the highest test dose in the subsequent repetitive MTD study. For accurate dose delivery before each administration, the mice were weighed, and the dose volume (10 ml/kg) was adjusted for each animal was based on individual body weight. After administration (on day 1), the mice were monitored twice daily (a.m. and p.m.) for mortality and morbidity. In particular, mice were observed for changes in skin, fur, eyes, and mucous membranes, in respiratory, circulatory, autonomic and central nervous system functions, in somatomotor activity, and in behavior patterns. Compound toxicity was assessed based on animal survival, visual signs of toxicity, and body weight changes that were monitored during the entire treatment period, followed by a 7-day recovery. The highest dose level causing no mortalities/morbidities or $\geq$ 15% body weight loss was considered the maximum tolerated.

Mice. The present study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol (IACUC 140112) was approved by the Institutional Animal Care and Use Committee of New York University School of Medicine, which is fully accredited by the Association For Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Groups of five female, 6- to 8-weeks old Swiss Webster mice were used for each condition, as specified.

Cell lines. NIH 3T3 mouse embryonic fibroblasts were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin at 37°C in 5% CO₂.

Toxicity of xenomycins against 3T3 mouse fibroblasts *in vitro*. Using 96-well plates, 5×10^5 3T3 mouse fibroblasts were added to each well in 100 μ l of supplemented DMEM culture medium. The cells were incubated at 37°C for at least 2 h to allow for fibroblast adherence. Xenomycins were then serially diluted in duplicate wells. Negative and positive controls of cells alone and ionomycin (Life Technologies, USA), respectively, were added to each plate. Plates were incubated for 96 h at 37°C in 5% CO₂. After incubation, cell viability was determined by adding 10 μ l of PrestoBlue (Life Technologies) to each well, followed by incubation for 4 h at 37°C in the cell culture incubator. Plates were analyzed on a fluorescence plate reader (Victor; Perkin-Elmer, USA) measuring excitation and emission wavelengths of 531 and 590 nm, respectively. The results are the averages of triplicate determinations.

Activity of xenomycins against *P. falciparum* blood stage *in vitro* and synchronization of *P. falciparum* cultures. *P. falciparum* 3D7 and the multidrug-resistant strains W2 and V1S (both resistant to chloroquine, pyrimethamine, quinine, and sulfadoxine) were kindly provided by David Fidock (Columbia University) and Kirk Deitsch (Cornell University), respectively. Using 96-well plates, 100 μ l of *P. falciparum* growth medium (RPMI 1640, 25 mM HEPES, 0.1 mg/ml gentamicin, 0.05 mg/liter hypoxanthine [pH 6.75]), supplemented with 0.25% sodium bicarbonate and 0.5% Albumax II (Invitrogen), were added to each well. Xenomycins from a DMSO stock at 10 mM were then serially diluted in triplicate. DMSO at the highest concentration used (0.5%) was tested in parallel, showing no differences from the control samples. Asynchronous *P. falciparum* in a 100- μ l volume was then added to each well at 0.25% late-stage parasitemia with 5% hematocrit, as well as controls (medium, red blood cells, *P. falciparum*, and *P. falciparum* with 2 μ M chloroquine). The plates were placed in a sealed chamber and maintained under atmospheric conditions of 5% oxygen, 5% carbon dioxide, and 90% nitrogen at 37°C for 96 h.

After incubation, plates were placed at -80°C for 24 h. The plates were then thawed at 37°C for 4 h. Once thawed, 100 μ l of from each resuspended well was transferred into a black, 96-well plate. To each well, 100 μ l of a solution containing 0.2 μ l of SYBR green I nucleic acid dye (Molecular Probes) in 1 ml of lysis buffer (20 mM Tris [pH 7.5], 5 mM EDTA, 0.008% saponin, 0.08% Triton X-100) was added. The plates were covered in aluminum foil, followed by incubation on a plate shaker for 1 h at room temperature. Plates were analyzed on a fluorescence plate reader (Victor) at excitation and emission wavelengths of 485 and 530 nm, respectively. The results are the averages of triplicate determinations.

To synchronize *P. falciparum* for the analysis of the different intra-erythrocytic stages, cultures at 5% hematocrit were passed through LD magnetic cell sorting columns (Miltenyi Biotec) to obtain a highly purified fraction of trophozoites. A Giemsa-stained smear of the collected fraction was performed to confirm efficacy. Selected infected red blood cells (iRBC) (mostly trophozoites) were put back in culture for an additional 18 h to obtain ring stages or for 44 h to obtain schizonts. Cultures were incubated with the indicated compounds at 10 μ M from stocks in vehicle (0.2% hydroxypropyl methylcellulose).

Activity against *Plasmodium berghei* asexual blood stages *in vivo*. Groups of five mice were infected with 10^3 blood-stage *P. berghei* ANKA expressing luciferase (*P. berghei*-luc) (9) by intraperitoneal injection. On day 3 postinfection, mice were anesthetized by isoflurane inhalation, injected with 150 mg of D-luciferin potassium salt substrate/kg, and imaged using an IVIS imager (Lumina II In Vivo Imaging System; Perkin-Elmer). Accumulated light intensity was measured in each mouse to determine the baseline infection levels before treatment. Mice were then started on a 5-day course of treatment (administered once per day by oral gavage) with the xenomycin compounds at the doses indicated in Table 2 in 0.2% hydroxypropyl methylcellulose, as a vehicle (days 3 to 7 postinfection). A

TABLE 1 Activity of xenomycins against *P. falciparum* 3D7 blood stage *in vitro*^a

Compound	Mean ± SEM		
	IC ₅₀ (nM)	TC ₅₀ (nM)	TC ₅₀ /IC ₅₀
CBL0100	9.2 ± 1.8	83.4 ± 5.4	9.1
CBL0137	166.0 ± 41.3	495.4 ± 79.8	3.0
CBL0159	39.1 ± 3.1	334.6 ± 81.7	8.6
CBL0174	215.5 ± 15.1	174.3 ± 33.1	0.8
CBL0175	61.9 ± 3.6	506.8 ± 28.5	8.2
CBL0176	77.3 ± 7.9	342.6 ± 52.3	4.4
CBL0207	30.2 ± 3.3	112.9 ± 25.7	3.7
CBL0212	31.2 ± 4.5	150.3 ± ND	4.8
CBL0252	27.3 ± 1.7	638.2 ± 63.7	23.4
CBL0253	14.4 ± 2.2	298.5 ± 20.4	20.7
Quinacrine	15.8 ± 1.7	8,743.2 ± 72.3	553.4
Chloroquine	19.6 ± 8.2	8,452.1 ± 826.4	4312.3

^a IC₅₀, half-maximal inhibitory concentration versus *P. falciparum* 3D7 growth; TC₅₀, half-maximal inhibitory concentration versus 3T3 fibroblasts after 4 days of contact.

group of negative-control mice were treated with vehicle alone, and a group of positive-control mice were treated with chloroquine (20 mg/kg/day). On day 8 postinfection, 24 h after 5 days of treatment, the mice were imaged a second time.

Activity against *P. berghei* liver stages *in vivo*. Groups of five mice were exposed for 15 to 20 min to the bites of 10 to 50 mosquitoes infected with *P. berghei* ANKA expressing luciferase (9). Mice were anesthetized by the intraperitoneal injection of xylazine (300 mg/kg) and ketamine (3,500 mg/kg) before exposure to mosquitoes. Mice were treated once per day with vehicle or test compounds at the doses indicated in Table 3 via oral gavage on day -1, day 0, and day 1 after infection. The negative-control group received 0.2% hydroxypropyl methylcellulose (vehicle). The positive-control group received primaquine (30 or 40 mg/kg/day) and different doses of xenomycins solubilized in vehicle. All groups were imaged 40 to 42 h after infection.

Activity of xenomycins against *P. berghei* gametocytes *in vivo* and transmission to mosquitoes. Groups of five mice were infected with 10⁷ *P. berghei* ANKA-infected erythrocytes. At 3 days postinfection (on day 0), initial gametocytemia was determined by blood smear. Mice were then treated via oral gavage with 0.2% hydroxypropyl methylcellulose (vehicle control), primaquine (positive control), or test compounds (xenomycins) for 2 days (on days 0 and 1). Gametocytemia was counted again on day 2 posttreatment (day 5 postinfection). Analysis of variance with repeated measures and a Greenhouse-Geisser correction was used to determine statistical significance. Difference between groups were analyzed with Bonferroni post hoc test. Mice were anesthetized with ketamine/xylazine and groups of 50 *Anopheles stephensi* mosquitoes were allowed to feed on each group of 5 mice from for 20 min with feeding disruption once per minute. *A. stephensi* mosquitoes were obtained from the NYU School of Medicine Insectary Core Facility.

The infected mosquitoes were incubated at 18°C for 11 days to allow oocyte formation. For each group, 50 mosquitoes were dissected and their midguts removed. Ten groups of five midguts selected made randomly. Single data points were collected by homogenizing each group of five midguts into one well of a 96-well plate. D-Luciferin potassium salt (200 µl at 200 µg/ml) was added. Luminescence was measured by using a Victor plate reader.

RESULTS

Xenomycins have strong activity against *P. falciparum* blood stages *in vitro*. Initial screening was done with a 10 compound subset of xenomycins (Fig. 1), with adequate chemical and metabolic stability, solubility, and suitability for oral administration, as previously established during hit-to-lead optimization of curaxins

TABLE 2 Activity against *P. berghei* asexual blood stages in mice

Compound	Dose (mg/kg/day)	% reduction(s) over control (vehicle) ^a
CBL0100	3.2	29
CBL0137	25	61, 96
CBL0159	16	92, 98
CBL0174	25	59
CBL0175	20	73
CBL0176	16	0, 54
CBL0207	16	97, 94
CBL0211	9	22
CBL0212	9	68, 91
CBL0252	16	85, 91
CBL0253	3.2	84
Quinacrine	100	100
Chloroquine	40	100, 100

^a Multiple values indicate the results for more than one independent experiment.

(6). In the present study, the antimalaria properties of 10 xenomycins were tested against *P. falciparum* (3D7) blood stage *in vitro* according to a standard protocol based on a DNA-fluorescent dye for detecting parasite growth in asynchronous cultures during 96 h (10). The *in vitro* activity (IC₅₀) of several xenomycins was in the nanomolar range, which is comparable to the one observed for chloroquine (IC₅₀ ~19 nM, Table 1). The cytotoxicity of xenomycins against 3T3 mouse fibroblast cells after 4 days of contact was substantially lower compared to their effect against *P. falciparum* (Table 1). However, the best ratio between TC₅₀ and IC₅₀ was observed for chloroquine. Therefore, cytotoxicity reduction against normal cell remains an important issue to be addressed during further lead compound structure optimization (Table 1).

Xenomycins have strong activity against the *P. berghei* blood stage *in vivo*. Prior to antimalarial evaluation of xenomycins *in vivo*, dose-range finding studies had been conducted with these compounds using NIH Swiss mice. The doses used in antimalarial efficacy experiments (Tables 2 and 3) corresponded to 75 to 80% of the MTD (five daily oral administrations) level for each xenomycin compound tested. Preclinical toxicology testing of oral CBL0137 has included studies in mice and monkeys with drug administration through 28 days of daily dosing followed by a 14-day recovery period; the principal adverse effects were reversible

TABLE 3 Activity of xenomycins against liver-stage *P. berghei* *in vivo*

Compound	Dose (mg/kg/day)	% reduction(s) over control (vehicle) ^a
CBL0100	3.2	100
CBL0137	25	97, 97, 100
CBL0159	16	0
CBL0174	25	93, 46
CBL0175	20	84
CBL0176	16	85, 43
CBL0207	16	100, 100
CBL0211	9	98, 97
CBL0212	9	99, 100
CBL0252	16	100, 100, 100
CBL0253	3.2	36
Primaquine	30	75, 95
	40	100, 99, 100

^a Multiple values indicate the results for more than one independent experiment.

FIG 2 *In vivo* efficacy of xenomycins against luciferase-expressing *P. berghei*. Representative images of mice from groups ($n = 5$) that were treated with vehicle, xenomycin CBL0252 (16 mg/kg/day), or chloroquine (40 mg/kg/day) for 5 days and then monitored for luminescence are shown.

myelosuppression and immunosuppression. An ongoing clinical phase 1 dose escalation study in patients with solid tumor malignancies has not revealed any dose-limiting toxicities in the first seven cohorts of subjects.

The antimalarial activity of selected xenomycins *in vivo* was tested using a well-established mouse model of *P. berghei* infection of Swiss-Webster mice. Compounds were administered orally at ca. 80% of the respective repetitive MTD value determined previously. Taking advantage of the development of transgenic *P. berghei* expressing luciferase (9), infection with this parasite was monitored by injection of the luciferase (Fig. 2).

At 3 days after infection with *P. berghei* expressing luciferase, treatment with xenomycins by oral gavage started for five consecutive days, with no apparent toxicity and no weight loss observed. After the 5 days of treatment, the mice were anesthetized and imaged to quantify the luciferase signal, which is proportional to the parasite load. A group treated with vehicle was used as a control for infection. The antimalarial drug chloroquine was used as a positive control. Some compounds were tested twice in independent experiments. Several xenomycins showed strong antiparasmodial activity in mice, comparable to the standard drug chloroquine (Fig. 2 and Table 2).

Xenomycins show strong anti-*P. berghei* liver-stage activity *in vivo*. To determine whether xenomycins have activity against liver-stage *Plasmodium*, we used *Anopheles stephensi* mosquitoes infected with *P. berghei*-luc. Mice were anesthetized before individual exposure to *P. berghei*-infected mosquitoes for 15 to 20 min. Selected xenomycins were administered orally 1 day before infection and on the following 2 days. Primaquine, the most com-

TABLE 5 Activity of xenomycin leads against different *P. falciparum* strains

Strain	Mean IC ₅₀ (nM) $\pm$ SEM	
	CBL0207	CBL0252
3D7	30 $\pm$ 3.3	27 $\pm$ 1.7
W2	223 $\pm$ 29.6	133 $\pm$ 4.5
V1S	285 $\pm$ 3.5	60 $\pm$ 4.7

monly used anti-liver-stage drug for malaria treatment, was used as a positive control in each experiment. Since liver infection by *Plasmodium* is reduced to a limited number of hepatocytes, it is barely detectable even by luminescence imaging. Therefore, mice were left for 5 days after infection (2 days after the end of treatment) to allow for initial blood-stage development, which is proportional to the original parasite load in the liver.

Several xenomycins showed very strong activity in the inhibition of liver infection in mice (Table 3). Radical cure was achieved after treatment with CBL0207, which achieved sterile clearance in the liver, since mice remained negative for blood-stage infection 14 days after treatment. Primaquine presented lower efficacy (three of five mice became positive for blood stage infection after 3 days, and all five mice were positive after 14 days). Treatment with CBL0252 did not result in sterile clearance in any of the mice.

Considering the high antiparasmodial activity in the different stages of the life cycle, good solubility, and acceptable metabolic stability in the presence of rat and human liver microsomes, two lead compounds from the xenomycin family, CBL0207 and CBL0252, were selected as lead candidates for further analysis (Table 4). These compounds also showed high activity against different strains of *P. falciparum* (Table 5).

Xenomycins effectively block transmission of *P. berghei* to mosquitoes. We next tested the ability of the selected xenomycin leads to eliminate gametocytes in *P. berghei*-infected mice. CBL0207, CBL0252, and primaquine (as a control) were administered orally to mice infected with *P. berghei*-luc at day 3 after infection (10^7 infected erythrocytes intraperitoneally), when they present ca. 1% gametocytemia. Since the gametocyte half-life is very short (11), gametocytes need to be quantified shortly after treatment to ensure that any decrease is due to gametocidal activity and not a consequence of the elimination of the precursor asexual blood stages. Using Giemsa-stained blood smears for each mouse, gametocytes were quantified by microscopy after 2 days of treatment. CBL0207 presented a significant activity, comparable to primaquine, whereas low significant activity was observed for CBL0252 (Table 6).

TABLE 4 Metabolic stability and solubility of xenomycin leads

Compound	Metabolic stability in the presence of liver microsomes (% intact after 60 min of incubation)		Solubility (mg/ml)	hERG inhibition ^a (IC ₅₀ [μ M])	Repetitive (five daily administrations) oral MTD (mg/kg)
	Rat	Human			
CBL0207	41.1	81.7	8 (in the presence of 300 mg/ml Captisol)	ND	20
CBL0252	27	86	Water, 9.6; PBS, 0.94; saline, 1.74	8.9	20

^a hERG is the potassium ion channel encoded by the "human Ether-a-go-go-Related Gene." ND, not determined.

TABLE 6 Activity of xenomycin leads against *P. berghei* gametocytes *in vivo*

Compound	Dose (mg/kg/day)	Avg % reduction over control (vehicle) $\pm$ SD ^a
CBL0207	16	68 $\pm$ 10*
CBL0252	16	25 $\pm$ 46†
Primaquine	40	85 $\pm$ 7*

^a Averages for groups of five mice are shown. *, $P > 0.0001$; †, $P > 0.05$.

To determine whether xenomycin leads effectively inhibit transmission of malaria, we let groups of 50 noninfected *Anopheles* mosquitoes feed on the control and treated groups of five mice each on day 2 posttreatment. The midgut oocysts of these mosquitoes were quantified by their luminescence signal (12) to determine their level of infection and therefore their capacity to transmit malaria. We observed that CBL0207 reduced mosquito midgut *Plasmodium* oocysts by 99% and was thus as effective as primaquine. CBL0252 also presented high activity in reducing oocysts (Fig. 3, Table 7). This effect had not been observed in gametocyte counts (Table 6), probably because nonviable gametocytes affected by drug treatments still remain in the blood for a period of time but are not morphologically distinguishable.

Xenomycins inhibit *P. falciparum* intra-erythrocytic development. To characterize in more detail the effect of the selected xenomycin leads on *P. falciparum*-infected erythrocytes, we added these compounds to *in vitro*-synchronized cultures and followed their development. When xenomycins were added to cultures of ring/trophozoite stages for 24 h (Fig. 4A), we observed an almost complete arrest in these stages, indicating that xenomycins affect early intra-erythrocytic parasite development. The addition of xenomycins to cultures in the schizont/ring stages for 18 h (Fig. 4B) did not affect the progression from schizonts into rings, indicating that merozoite release and reinvasion of new erythrocytes was not affected. The transition from rings into trophozoites was again inhibited.

DISCUSSION

Despite intensive research efforts in the vaccine development area, chemotherapy remains the basis for effective interventions in malaria prevention, treatment, and control. However, the emergence

FIG 3 Xenomycins inhibit *P. berghei* transmission to mosquitoes. Quantification of luciferase signal in *Anopheles stephensi* midguts that were fed on mice infected with luciferase-expressing *P. berghei* and treated with vehicle or the indicated compounds was performed. Oocyst numbers are proportional to the luciferase signal.

TABLE 7 Activity of xenomycin leads against transmission to mosquitoes *in vivo*

Compound	Dose (mg/kg/day)	Avg % reduction over vehicle control $\pm$ SD ^a
CBL0207	16	99 $\pm$ 2
CBL0252	16	96 $\pm$ 3
Primaquine	40	99 $\pm$ 2

^a Averages of 10 groups of five mosquitoes for each condition are shown.

and spread of resistance to currently available drugs is a major concern, especially after the appearance of artemisinin resistance in Southeast Asia (13). The search for new antimalarials is guided primarily by the need to discover novel compounds with not only differing mechanisms of action from those currently available but that are also less susceptible to the development of resistances by *Plasmodium* in the field. In addition, the desirable attributes for novel antimalarials include improved characteristics, such as fast parasite clearance (>6 log total parasite reduction), long duration effect (between two and 4 weeks), and activity against liver stages, hypnozoites, and gametocytes (3). Drugs targeting different stages of the parasite vertebrate life cycle are ideal, since a single compound may be effective in prevention, treatment, and control of malaria. Recently, a family of compounds targeting *Plasmodium* PI(4)K has been reported to have activity against all stages of the *Plasmodium* vertebrate life cycle (14, 15), providing the first evidence that an antimalarial drug with multistage activity may be developed for clinical use.

Our finding that xenomycins also inhibit every stage of the *Plasmodium* vertebrate life cycle introduces a novel chemical class of compounds with high antimalarial potential. Although still unknown, the mechanism of action of xenomycins on *Plasmodium* is likely to be similar to their effect on cancer cells, where binding to DNA leads to trapping of the chromatin remodeling complex FACT (facilitates chromatin transcription) (6), which is critical for the survival of tumors, and probably other rapidly dividing cells, such as *Plasmodium*. Unlike other known DNA intercalators, many of these compounds have the special characteristic of being nongenotoxic and nonmutagenic (6), which allows their development as antimalarial drugs. Being structurally distinct, xenomycins share their biological effects on mammalian cells with the old antimalarial drug quinacrine (6–8), suggesting that the mechanisms of their antimalarial effects could also be similar. It is noteworthy that xenomycins do not possess the negative properties of quinacrine that determined discontinuation of its clinical use, namely, photosensitizing activity (16) and high levels of accumulation in skin resulting in yellow discoloration (17).

Another important characteristic of xenomycins is the dual activity against both asexual and sexual *Plasmodium* blood stages. This would result in simultaneous cure and inhibition of disease transmission, an essential feature for eradication campaigns (3). In addition, the effective elimination of liver-stage *Plasmodium* may indicate a possible activity of xenomycins against hypnozoites, which is another stage of the parasite in great need of improved chemotherapy (18).

Antimalarial target product profiles have recently been reevaluated to include not only rapid and effective activity against asexual blood-stage *Plasmodium* but also transmission-blocking and prophylactic activities (18). In this context, xenomycins stand as

FIG 4 Inhibition of growth in synchronized *P. falciparum* cultures treated with xenomycins. *P. falciparum* cultures synchronized at ring/trophozoite (A) or schizont/ring (B) stages at the beginning of the experiment were incubated with vehicle (DMSO 0.1%) as a control (C), quinacrine (Q), CBL207, or CBL252 (all at 10 μ M) for 24 h (A) or 18 h (B). The panels show representative images of parasites at the indicated time points. Quantification results for parasite densities at 24 h (A) or 18 h (B) after addition of the compounds are shown. The quantification of the parasite stages shows the results for rings (white bars), trophozoites (gray bars), and schizonts (black bars).

antimalarial candidates with potential activity in prevention, treatment and elimination of this disease.

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