**In Vitro** Antimalarial Activity of Novel Semisynthetic Nocathiacin I Antibiotics

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Presently, the arsenal of antimalarial drugs is limited and needs to be replenished. We evaluated the potential antimalarial activity of two water-soluble derivatives of nocathiacin (BMS461996 and BMS411886) against the asexual blood stages of *Plasmodium falciparum*. Nocathiacins are a thiazole peptide group of antibiotics, are structurally related to thiostrepton, have potent activity against a wide spectrum of multidrug-resistant Gram-positive bacteria, and inhibit protein synthesis. The *in vitro* growth inhibition assay was done using three laboratory strains of *P. falciparum* displaying various levels of chloroquine (CQ) susceptibility. Our results indicate that BMS461996 has potent antimalarial activity and inhibits parasite growth with mean 50% inhibitory concentrations (IC50s) of 51.55 nM for *P. falciparum* 3D7 (CQ susceptible), 85.67 nM for *P. falciparum* Dd2 (accelerated resistance to multiple drugs [ARMD]), and 99.44 nM for *P. falciparum* K1 (resistant to CQ, pyrimethamine, and sulfadoxine). Similar results at approximately 7-fold higher IC50s were obtained with BMS411886 than with BMS461996. We also tested the effect of BMS491996 on gametocytes; our results show that at a 20-fold excess of the mean IC50 gametocytes were deformed with a pyknitic nucleus and growth of stage I to IV gametocytes was arrested. This preliminary study shows a significant potential for nocathiacin analogues to be developed as antimalarial drug candidates and to warrant further investigation.

Parasitic and tropical diseases continue to pose major public health problems. There were an estimated 198 million episodes of malaria in 2013, resulting in approximately 584,000 deaths (1). The causative agents are obligate intracellular protozoan parasites belonging to the phylum Apicomplexa and the genus *Plasmodium*. This parasite exhibits a complex life cycle, multiplying asexually within the human host to precipitate morbid efficacies over the past 4 years (2). Currently, there is no vaccine ing an individual to complete its life cycle.

Efforts to develop effective vaccines have resulted in modest successes over the past 4 years (2). Currently, there is no vaccine available on the market. The increasing drug resistance of current antimalarials worldwide has resulted in a need to continuously explore, identify, and develop new drugs that exert their antimalarial effects by targeting novel sites (3). These novel targets/mechanisms include but are not limited to the protein synthesis machinery, DNA replication, exflagellation, and proteasome pathways. All of these biochemical processes are critical for parasite survival, development, and transition from the asexual blood stages to gametocytes (4). The current antibiotics for the treatment of malaria infection include doxycycline, azithromycin, tetracycline, and clindamycin (5). These antibiotics interfere with apicoplast function and result in the slow killing of parasites termed “delayed death.” In delayed death, parasite growth is arrested at the schizont stage during the second replication cycle following antibiotic treatment. On the other hand, ciprofloxacin, rifampin, and thiostrepton have an immediate killing effect (6). Doxycycline, a widely used antimalarial, is contraindicated for treatment of malaria in children under the age of 8 and pregnant women as it interferes with bone growth. Clindamycin is considered safe for treatment of uncomplicated malaria in pregnant women and children under the age of 5 years. However, the appearance of clindamycin resistance in Amazonian *Plasmodium falciparum* has been reported (7).

Nocathiacin, a thiazole peptide class antibiotic, is isolated from *Nocardiopsis* species (8). This antibiotic and its derivatives were developed by Bristol-Myers Squibb (BMS) to treat multidrug-resistant bacterial infections, including methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*, *Enterococcus*, and *Mycobacterium tuberculosis* infections. The two derivatives BMS411886 and BMS461996 are water soluble and structurally related to thiostrepton (Fig. 1). There are three categories of ribosomes: mitochondrial, plastid, and nuclear. The nuclear genome has five complete sets of rRNA genes and one partial cassette, located on 6 separate chromosomes. The mitochondrial genome has fragmented rRNA genes and appears to be functional as all the fragments are expressed during asexual stages (9, 10). The plastid genome has one functional ribosome unit, and many of the antibiotics used for malaria treatment target apicoplast ribosomes (11). It has been shown that thiostrepton, a thiazolyl peptide antibiotic, has antiplasmodial activity and inhibits parasite growth by targeting the apicoplast ribosomes and has an immediate effect (6, 12, 13). A major limitation with thiostrepton is poor aqueous solubility. Recently, thiostrepton derivatives have shown a potential for further development as antimalarials with a 10-fold increase in 50% inhibitory concentrations (IC50s) and gametocyto
cidal activity by dually acting on two independent targets (14). The two derivatives BMS411886 and BMS461996 inhibit bacterial protein synthesis by binding to the large subunit with direct interaction with 23S rRNA at the same site as the L11 protein. This mechanically stalls ribosomal functioning and inhibits growth (15). The limited copy number of ribosomal genes in the Apicomplexa parasite and single copy of L11 ribosomal protein make it a promising drug target which has not been exploited to its fullest potential.

Here, we present the first study to explore the potential in vitro antimalarial activity of the nocathiacin I derivatives BMS411886 and BMS461996. Investigation of these drugs provides a promising approach to the discovery of novel antimalarial agents which possess potent in vitro antimalarial activity against chloroquine (CQ)-susceptible and -resistant P. falciparum strains.

MATERIALS AND METHODS

Parasite culture and plate setup. (i) Plasmodium culture. Parasite lines (P. falciparum 3D7, P. falciparum Dd2, and P. falciparum K1) were cultured by the method of Trager and Jenson with minor modifications (16). Briefly, cultures were maintained in O-positive human erythrocytes (RBCs), suspended at 2% hematocrit in RPMI 1640 medium containing 10% heat-inactivated pooled serum. Prior to experiments, the culture was synchronized twice at 48-h intervals using the sorbitol treatment (17). After 96 h, parasitemia was determined using light microscopy and by counting at least 1,000 erythrocytes on Giemsa-stained thin blood smears. The culture was diluted to 1% parasitemia and 4% hematocrit in complete medium (RPMI 1640 medium containing 10% heat-inactivated pooled serum).

(ii) Stock solutions of test drugs. All of the test drugs were prepared in sterile water at a stock concentration of 10 mM and stored at −80°C until further use. The stock vial was thawed and diluted to a working concentration of 20 μM using complete medium.

(iii) Plate setup. The working stock concentration was 2-fold serially diluted in triplicate test wells to yield concentrations ranging from 10 μM to 0 μM. To each well, the Plasmodium culture was added to a final parasitemia of 1% and 2% hematocrit. In addition, several wells contained nonparasitized RBCs at 4% hematocrit to serve as reference controls, and CQ was used as a positive control at a final concentration of 20 μM, which is known to inhibit 100% parasite growth even in resistant Plasmodium species. All the plates were incubated for 72 h at 37°C. Each concentration was set in triplicate, and experiments were repeated independently at least three times to validate the results obtained.

N′,N′-Dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene) methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine (SYBR Green I) fluorescence assay. An SYBR Green I fluorescence assay was performed as described previously (18). Briefly, after 72 h of incubation, 100 μl of the lysis buffer containing SYBR Green I (20 mM Tris [pH 7.5], 5 mM EDTA, 0.008% saponin [wt/vol], 0.08% Triton X-100 [vol/vol], and 2× SYBR Green I concentration) was added to the test and control wells. The samples were incubated in the dark for 1 h. Fluorescence was read using a BMS FluorStar microplate reader. The background fluorescence was subtracted using wells that contained only lysis buffer as well as control wells that contained nonparasitized RBCs.

Determination of IC_{50}. The fluorescence counts obtained were analyzed using GraphPad Prism (San Diego, CA) software. Growth inhibition curves were generated by plotting the percentage of parasite growth inhibition against the logarithm of the drug concentration (nanomolar concentration) and curve fitting using nonlinear regression (sigmoidal dose response/variable slope with a four-parameter equation) to estimate the 50% decline in growth compared to that in drug-free control wells. Results are expressed as the concentrations at which 50% of parasite growth is inhibited (IC_{50}) and the standard errors of mean (SEM) are plotted as error bars (n = 3).

In vitro parasitidal activity. The synchronous parasite cultures were incubated in the presence of BMS461996 for 48 h at concentrations of 100 nM and 1 μM. After 24 h, thin smears were prepared, stained with Giemsa stain, and observed under the microscope. After 48 h, cells were washed twice with complete RPMI medium without drug, and cultured in the absence of the nocathiacin derivative. The medium was replenished daily and monitored for a total of 2 weeks by preparation of thin Giemsa-stained smears daily for 14 days.

Gametocyte and the effect of the drug. The Plasmodium falciparum 3D7 culture was initiated at 4% hematocrit and a parasitemia of 0.5% rings (synchronized twice using the sorbitol method). Fifty percent of the spent medium was replenished daily, and the parasite stage was monitored. On day 4, stage I gametocytes appeared in the culture and were divided into three groups. Group 1 was the untreated control; the other two groups were exposed to 100 nM and 1 μM concentrations of...
RESULTS

Growth inhibition. The MICs (MIC_{50}) for BMS461996 and BMS411886 were determined by the *in vitro* growth inhibition assay. Table 1 shows the mean IC_{50} values for BMS461996, BMS411886, thiostrepton, thiostrepton derivative S231/[14], micrococcin, CQ, doxycycline, and anisomycin. *P. falciparum* strains with different CQ susceptibility were treated with nocathiacin analogues and CQ in the range of 10 μM to 0 nM, and IC_{50}s were calculated using the dose-response curve (Fig. 2). The nocathiacin analogue BMS461996 was found to be highly potent against all plasmodial strains used in this study, including the multidrug-resistant *P. falciparum* K1 parasite line (Fig. 2). In contrast to BMS491996, CQ showed a 5.5-fold higher mean IC_{50} against the *P. falciparum* K1 strain (Table 2 and Fig. 2C). These MIC values are minimally affected by human serum as all of the growth inhibition assays were done using RPMI medium supplemented with 10% heat-inactivated human serum.

**In vitro parasiticidal activity.** To determine the parasiticidal activity, asynchronous *P. falciparum* cultures at 1% parasitemia and 4% hematocrit were incubated for 48 h in the absence and presence of BMS461996 at 100 nM and 1 μM concentrations and subsequently washed and further incubated in the absence of the drug for an additional 12 days with regular medium changes. The parasites were observed at 24- and 48-h time points after exposure to the drug and in the absence of the drug by Giemsa staining of thin smears followed by light microscopy. As shown in Fig. 3, *P. falciparum* growth was arrested in the cultures treated with 100 nM BMS461996 at the 24-h time point, whereas the untreated control parasite culture progressed normally and appeared healthy after 24 h. This observation was also true for both the 100 nM and 1 μM 48-h exposures. Over a 12-day observation period, no viable parasites were observed in the cultures treated with BMS461996 for 48 h, whereas the untreated culture declined after 8 days due to high parasitemia. This result suggests that BMS461996 inhibited growth and killed the parasites within 48 h.

**Effect on gametocyte development.** The gametocytes were induced in culture, and stage 1 gametocytes were incubated in the presence and absence of BMS461996 at concentrations of 100 nM and 1 μM and observed for 5 days. As shown in Fig. 4, the effect of BMS461996 on gametocyte growth was assessed by light microscopy of thin blood smears over a period of 5 days. The control gametocyte culture developed normally, and the morphology of mature gametocytes was compared with that of the gametocytes treated with BMS491996. The nocathiacin derivative, BMS461996, had no effect on gametocyte development at 100 nM concentration, whereas gametocytes grown in the presence of 1 μM BMS491996 were completely deformed with pyknotic nuclei.

DISCUSSION

The spread of drug resistance is a continuing threat to malaria control in developing countries. With CQ resistance originating in the 1950s in Southeast Asia and later in the 1970s, its spread to the rest of the world led a major thrust to develop new antimalarial drugs (19). In 2014, oral artemisinin-based monotherapy (oAMT) was identified as a primary cause of increasing drug resistance, and the WHO recommended its withdrawal. Presently, there are reports demonstrating that parasite clearance is delayed despite artemisinin being administered in combination with other drugs such as sulfadoxine-pyrimethamine, lumefantrine, CQ, and amodiaquine (20, 21, 22). This is also supported by a large-scale

### TABLE 1 Mean IC_{50}s against *P. falciparum* 3D7

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean IC_{50} (nM)</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>BMS461996</td>
<td>51.55</td>
<td>This paper</td>
</tr>
<tr>
<td>BMS411886</td>
<td>333.2</td>
<td>This paper</td>
</tr>
<tr>
<td>Thiostrepton</td>
<td>8,900</td>
<td>14</td>
</tr>
<tr>
<td>Thiostrepton derivative S231/[14]</td>
<td>1,000</td>
<td>14</td>
</tr>
<tr>
<td>Micrococcin</td>
<td>35</td>
<td>26</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>40.35</td>
<td>This paper</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>3389</td>
<td>This paper</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>31.80</td>
<td>This paper</td>
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BMS461996, respectively. Thin smears were prepared, stained with Giemsa, and monitored over a period of 5 days.
An ongoing study conducted by the Tracking Resistance to Artemisinin Collaboration (TRAC), which shows that artemisinin resistance has already spread from southern Vietnam to central Myanmar (23). One way to overcome this global problem, in a collective effort to eradicate malaria, is to continuously develop and identify new antimalarial drug candidates with novel chemical scaffolds. A drug candidate, nocathiacin I, and its semisynthetic analogues are the subject of this study. These nocathiacin analogues were initially developed to treat MRSA and other multidrug-resistant Gram-positive bacteria and displayed potent bactericidal activity in the nanomolar range comparable to that of vancomycin, the only drug that is currently available to treat MRSA (15, 24). These analogues possess high selectivity to bacterial protein synthesis and minimal toxicity to mammalian cells, a desirable trait for drug candidates (M. Pucci, personal communication). Since there are few antibiotics that target the plasmodial ribosome in nanomolar concentrations, this study demonstrates the potential of nocathiacin for development as an antimalarial antibiotic.

Structurally, the nocathiacin class of antibiotics is related to the thiazole peptide antibiotic thiostrepton. Our results indicate that BMS461996 and BMS411886 have excellent antiplasmodial activities in the nanomolar range and compare favorably with other antimalarials such as thiostrepton, doxycycline, tetracycline, clindamycin, CQ, and the universal protein synthesis inhibitor anisomycin (Table 1). It is intriguing, though, how subtle differences in structure resulted in the 10-fold difference in mean IC_{50} values of BMS461996 and BMS411886. In a recent report, thiostrepton and its derivative have been shown to rapidly eliminate parasites by dually targeting the apicoplast protein synthesis and proteasome in the micromolar range (4, 6, 14, 25). In comparison to thiostrepton, BMS461996 is water soluble and thus is biologically accessible for parasiticidal activity and results in irreversible growth inhibition within the first growth cycle, being therefore immediately effective. The thiazole peptide antibiotics are known to exert their effects by binding to the GTPase-associated region of the ribosome/L11 complex, which blocks the binding region of elongation factor G (EF-G) and does not allow translocation of the growing peptide/tRNA complex in the ribosome to occur. The ability of BMS461996 to eliminate parasites within the first cycle might involve additional targets, potentially the proteasome pathway along with apicoplast and/or mitochondrial protein synthesis, similar to thiostrepton. Identifying the pathway of BMS461996 as an antimalarial antibiotic requires further investigation.

To evaluate the antiplasmodial activity of BMS461996 against CQ resistance parasites, we used the P. falciparum Dd2 strain that has an accelerated resistance to multiple drugs (ARDM) profile and the P. falciparum K1 strain that displayed resistance to CQ, pyrimethamine, and sulfadoxine. Both strains displayed mean

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Chloroquine Mean IC_{50} (nM)</th>
<th>95% confidence interval</th>
<th>BMS411886 Mean IC_{50} (nM)</th>
<th>95% confidence interval</th>
<th>BMS461996 Mean IC_{50} (nM)</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. falciparum Dd2</td>
<td>327.4</td>
<td>86.95–105.5</td>
<td>676.7</td>
<td>99.61–107.0</td>
<td>85.67</td>
<td>76.06–96.49</td>
</tr>
<tr>
<td>P. falciparum K1</td>
<td>553.4</td>
<td>473.2–644.5</td>
<td>645.5</td>
<td>610.3–682.6</td>
<td>99.44</td>
<td>81.63–121.1</td>
</tr>
</tbody>
</table>

*Tested against three laboratory parasite lines, Plasmodium falciparum 3D7, a CQ-sensitive line, P. falciparum Dd2, a line with ARMD, and P. falciparum K1, a line resistant to CQ, pyrimethamine, and sulfadoxine.

![FIG 3 In vitro effect of the nocathacin derivative BMS461996 on the asexual blood stages of P. falciparum 3D7 after 24 h at the 100 nM concentration. Thin smears were stained with Giemsa stain. (A) Untreated control culture; (B) culture treated with 100 nM BMS461996. Similar results were observed at the 48-h time point.](http://aac.asm.org/).
IC\textsubscript{50}s of 80.68 nM and 90.01 nM, respectively, for BMS461996, and we found no significant difference in comparison to that for the CQ-susceptible strain. In contrast to BMS461996, the mean IC\textsubscript{50} for CQ is \(\approx 4.8\) and is 7-fold higher for \emph{P. falciparum} Dd2 and \emph{P. falciparum} K1, respectively (Table 2). We also investigated the effect of nocathiacin on \emph{P. falciparum} gametocytes. Our preliminary data show that BMS461996 has a deleterious effect on gametocytes at a concentration of 1 \(\mu\)M, which is a 20-fold excess of the 50% inhibitory concentration; the growth of stages I to IV gametocytes was arrested. Further, the transmission-blocking activity of BMS461996 needs to be assessed by testing whether drug-treated stage V gametocytes are producing viable sporozoites in \emph{Anopheles} mosquitoes, and this is outside the scope of the current manuscript.

In summary, nocathiacin displayed \emph{in vitro} parasiticidal activity in nanomolar concentrations against \emph{P. falciparum}. BMS461996 showed 170-fold and 20-fold increased activities in comparison to those of thiostrepton and its derivatives. This study strongly suggests that the semisynthetic nocathiacin derivative BMS461996 shows potential for further development as an anti-malarial agent and its transmission blocking activity is worth investigating.

**ACKNOWLEDGMENTS**

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**REFERENCES**


