Discovery of Antimalarial Azetidine-2-carbonitriles That Inhibit *P. falciparum* Dihydroorotate Dehydrogenase

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Supporting Information

ABSTRACT: Dihydroorotate dehydrogenase (DHODH) is an enzyme necessary for pyrimidine biosynthesis in protozoan parasites of the genus *Plasmodium*, the causative agents of malaria. We recently reported the identification of novel compounds derived from diversity-oriented synthesis with activity in multiple stages of the malaria parasite life cycle. Here, we report the optimization of a potent series of antimalarial inhibitors consisting of azetidine-2-carbonitriles, which we had previously shown to target *P. falciparum* DHODH in a biochemical assay. Optimized compound BRD9185 (27) has *in vitro* activity against multidrug-resistant blood-stage parasites (EC$_{50}$ = 0.016 μM) and is curative after just three doses in a *P. berghei* mouse model. BRD9185 has a long half-life (15 h) and low clearance in mice and represents a new structural class of DHODH inhibitors with potential as antimalarial drugs.

KEYWORDS: BRD7539, BRD9185, DHODH, malaria, diversity-oriented synthesis, *Plasmodium falciparum*

Malaria is a global health concern with nearly 200 million cases annually, many of which occur in sub-Saharan Africa. The disease is caused by parasitic protozoans of the genus *Plasmodium* and transmitted by female *Anopheles* mosquitoes. Malaria is treatable using chemotherapy, but reduced efficacy of first-line treatments artemisinin and its derivatives at the Cambodia-Thailand border underscores the need for new, safe, and effective antimalarial therapies.

Moreover, while most current antimalarial drugs target asexual blood-stage parasites, next-generation antimalarials should ideally also target the liver- and/or sexual blood-stage parasites to impede parasite replication and transmission from host-to-vector, respectively. New antimalarial candidates have entered clinical trials in this regard, including one that targets dihydroorotate dehydrogenase (DHODH).

DHODH catalyzes the flavin mononucleotide (FMN)-dependent oxidation of l-dihydroorotate to orotate as the fourth step in *de novo* pyrimidine biosynthesis. While most organisms use both *de novo* and salvage pathways to generate pyrimidines, *Plasmodium* parasites lack the necessary genes for the latter, making *de novo* pyrimidine synthesis an essential pathway for the parasite. One compound in the antimalarial pipeline, DSM265, has progressed to phase-II clinical trials and has activity against both asexual blood-stage and liver-stage parasites. DSM265 and secondary candidate DSM421 (Chart 1) comprise a class of selective and potent antimalarial DHODH inhibitors. These triazolopyrimidines remain the most well-studied and clinically relevant antimalarial DHODH inhibitors to date, but 5-benzimidazolyl-thiophene-2-carboxamides and 7-arylimidopyrazolo[1,5-α]pyrimidines have also been reported.

We recently identified numerous compounds with multistage activity by growth-inhibition phenotypic screening of 100,000 compounds prepared in advance using diversity-oriented synthesis (DOS). The DOS collection was synthesized using modern asymmetric organic chemistry to impart three-dimensional topographical features using the build–couple–pair strategy. The success of this strategy in revealing novel therapeutic targets is illustrated by the discovery of small-molecule antimalarial inhibitors of phenylalanyl-tRNA synthetase.

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multidrug-resistant asexual blood-stage (P. falciparum, Dd2 strain) was reported to have potent activity against both multiresistant human (Hs) DHODH (IC_{50} > 50 μM). BRD7539 was reported to have potent activity against both multiresistant asexual blood-stage (P. falciparum) and liver-stage (P. berghei) parasites but no activity against sexual blood-stage (P. falciparum, stages IV–V, IC_{50} > 20 μM) parasites. BRD7539 is an azetidine carbonitrile with three contiguous possible stereoisomers are active. The clinical relevance of DHODH inhibitors and the selectivity and potency of BRD7539 arising directly from a high-throughput screen encouraged us to pursue this series further. Here, we report our efforts to optimize this compound and to evaluate this series in vivo.

To confirm the biological activity of BRD7539 and to explore structure–activity relationships (SARs) of the scaffold, we resynthesized core structure 1 (Scheme 1) as reported.

**Scheme 1. Elaboration of the Azetidine-2-carbonitrile Scaffold**

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Reagents and conditions: (a) Pd(PPh3)4, 1,3-dimethylbarbituric acid, 2:1 EtOH/DCM, 40 °C, 1 h, 92%; (b) propylisocyanate, DIPEA, DCM, 23 °C, 1 h, 96%; (c) trifluoroacetic acid, Et3SiH, DCM, 23 °C, 1 h, 87%; (d) 1-ethynyl-3-fluorobenzene, XPhos Pd-G3, Et3N, MeCN, 70 °C, 6 h, 91%.
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Deallylation of the protected azetidine core and sequential capping of the nitrogen with propyl isocyanate gave urea 2. Trypt cyclodeprotection followed by a Heck alkenylation or Suzuki cross-coupling afforded the para-Br position and served as a route to most analogues. Biological activity of BRD7539 was confirmed in 20-point dose (n ≥ 2 independent experiments in triplicate) against a multiresistant strain (P. falciparum, Dd2 strain) using a phenotypic blood-stage growth inhibition assay that models a human blood-stage infection. Additionally, in vitro stability against mice and human microsomes for 1 h was used as a guide to identify analogues with potential in vivo stability.

Our initial SAR focused primarily on the acetylene (R_1) region of BRD7539 as this was the most facile point of diversification to explore and a possible toxicity concern (Table 1). Activity was assessed using the phenotypic blood-stage growth inhibition assay. The activity of BRD7539 was reconfirmed in dose, and in vitro activity was maintained with a wide variety of hydrophobic acetylens (4–6, 9, 10). Heteroaromatic 2- and 3-pyridyl analogues (7–8) showed significant loss in activity compared to aromatic analogues. Interestingly, cis-alkene (11) and alkane (12) derivatives of BRD7539 showed only a slight loss in activity, suggesting that the acetylene was not necessary. Indeed, unsubstituted biaryl 13 is essentially equipotent to BRD7539 while removing the distal ring (17, Scheme S1) abolished activity, indicating the need for a large hydrophobic region in the scaffold. Having removed the acetylenic toxicity concern, we decided to use this region to modulate mouse and human microsomal stability while maintaining activity. Improved stability should correlate with favorable pharmacokinetic (PK) properties. Analogues bearing a 4-pyridyl (22) and 4-methanesulfonyl (23) distal aryl were synthesized in an effort to improve solubility but were inactive in vitro. CF_3-substitution (24–26) was found to impart greater in vitro microsomal stability than methoxy substituents (28–30). Ultimately, we found that the addition of two −CF_3 groups on the distal phenyl ring (BRD9185, 27) to be comparable in activity and microsomal stability to BRD7539.

We briefly sought to evaluate the role of the primary alcohol (R_2) and secondary nitrile (R_3) on activity (Table 2). Analogues were synthesized from BRD7539 in 1–3 steps or from commercially available starting materials (Schemes S2–S7). Modifying the nitrile to a methyl ester (32) or alcohol (33) abolished activity. This is unsurprising given our previous result that the 2S,3S,4R diastereomer, which only differs from BRD7539 at the nitrile-bearing stereocenter, is inactive, hinting at the importance of this functional group. This also illustrates the subtle but significant role stereochemistry can have on small molecule–protein interactions and highlights the strength of diversity-oriented synthesis in identifying key interactions. Any modification of the primary alcohol, including methylation (34) or conversion to a primary amine (36), resulted in large loss in activity.

To confirm that our lead compound 27 inhibits P/DHODH despite removal of the acetylenic motif, we performed biochemical assays against both recombinant P. falciparum DHODH.
and human DHODH enzyme (Table 3, Figure S1). Similar to hit compound BRD7539, 27 is a potent inhibitor of PfDHODH (IC$_{50}$ = 0.012 μM) but not HsDHODH (IC$_{50}$ > 50 μM), suggesting that this class of DHODH inhibitors provides selectivity between orthologues. The IC$_{50}$ of selected analogues against PfDHODH was also shown to track well with Dd2 EC$_{50}$ (Table S1). To assess the suitability of BRD9185 further for in vivo use, we measured plasma protein binding and obtained mouse PK data. Lead compound 27 is highly protein bound in both mouse and human plasma (>99%) and is a highly bioavailable (94%), long half-life (15 h) compound in mice (PO 5 mg/kg; IV 1 mg/kg) with low clearance (0.40 mL/min/kg). Notably, the DNAUC$_{0-24}$ of 27 is >54.3 μM, higher than the EC$_{50}$ in vitro.

Based on the promising PK properties, we were interested in evaluating the efficacy of 27 in vivo (Figure 1). We used a blood-stage model with the rodent malaria parasite P. berghei that expresses luciferase and treated infected CD-1 mice for 3 days with 66.6 mg/kg of 27 or vehicle (70% PEG300, 30% solution of 5% dextrose in H$_2$O). Bioluminescence intensity was used to measure parasite growth, and artesunate was used as a positive control. No parasites were detected after 30 days in mice treated with 27, suggesting that the analogue achieved a sterile cure for P. berghei. These results are particularly interesting in light of the properties of DSM265 and the triazolopyrimidine series, which are not effective in the P. berghei (Pb) model due to poor binding to the PbDHODH enzyme.$^9$,$^{14}$ This raises the possibility that 27 has a different mechanism of action from DSM265 on PfDHODH. However, 27 binds competitively with decylubiquinone (Figure S2), the same proposed binding site as DSM265.$^5$ X-ray crystallography studies are underway to gain insights into binding features of these compounds.

Table 1. Activity of BRD7539 Analogues at R$_1$ Position against Dd2 Parasites

| Cmpd | R$_1$ | EC$_{50}$ (μM) | MH$^b$ |
|------|------|---------------|--------|
| BRD7539 | | 0.010 | 86/99 |
| 3 | Br | 0.249 | - |
| 4 | F | 0.083 | 85/85 |
| 5 | F | 0.013 | 100/100 |
| 6 | | 0.016 | - |
| 7 | | 5.640 | - |
| 8 | Cl | 0.427 | - |
| 9 | MeO | 0.020 | 85/96 |
| 10 | | 0.016 | 45/94 |
| 11 | F | 0.035 | 56/87 |
| 12 | | 0.046 | 0/14 |
| 13 | | 0.019 | - |
| 14 | | 0.051 | - |
| 15 | | 0.106 | - |
| 16 | | 0.019 | - |

EC$_{50}$ values are the mean of at least two independent experiments. Mouse (M) and human (H) microsomal stability (% remaining after 1 h). Data were obtained from a single experiment performed in duplicate and calculated from a six-point curve over 1 h.

Table 2. Activity of BRD7539 Analogues at R$_2$ and R$_3$ Positions against Dd2 Parasites

| Cmpd | R$_2$ | R$_3$ | EC$_{50}$ (μM) |
|------|------|------|---------------|
| 32 | OH | OMe | 1.629 |
| 33 | - | OH | 16.200 |
| 34 | OMe | CN | 1.913 |
| 35 | N$_2$ | - | 0.390 |
| 36 | OAc | - | 4.440 |
| 37 | | OMe | 0.102 |
| 38 | | Me | 0.307 |

EC$_{50}$ values are the mean of at least two independent experiments.

and human DHODH enzyme (Table 3, Figure S1). Similar to hit compound BRD7539, 27 is a potent inhibitor of PfDHODH (IC$_{50}$ = 0.012 μM) but not HsDHODH (IC$_{50}$ > 50 μM), suggesting that this class of DHODH inhibitors provides selectivity between orthologues. The IC$_{50}$ of selected analogues against PfDHODH was also shown to track well
Table 3. Key Properties of Lead Compound 27

| Property               | Value         |
|------------------------|---------------|
| in vitro enzyme inhibition, IC₅₀<sup>a</sup> |               |
| PfDHODH (μM)           | 0.012         |
| HsDHODH (μM)           | >50           |
| plasma protein binding<sup>b</sup> |               |
| mouse (%)              | 99.3          |
| human (%)              | >99.0         |
| t₁/₂ (h)               | 15.2          |
| Cₜ₀ (μM)               | 4.9           |
| Cₚ₅₀ (μM)              | 16.9          |
| DNAUC<sub>∞</sub> (μM·h) | >54.3        |
| Vₕ (L/kg)              | 0.37          |
| CL (mL/min/kg)         | 0.40          |
| F (%)                  | 94            |

<sup>a</sup>Mean of a single experiment in triplicate. <sup>b</sup>Single experiment, calculated from a six-point curve over 1 h. t₁/₂, terminal half-life; Cₜ₀, initial serum concentration at t = 0; Cₚ₅₀, peak serum concentration; DNAUC<sub>∞</sub>, dose-normalized area under the plasma concentration vs time curve following PO dosing; Vₕ, volume of distribution at steady state; CL, plasma clearance; F, bioavailability. IV dosing in 5% DMSO/10% cremophor/85% H₂O at 0.25 mg/mL (1 mg/kg). PO dosing in 70% PEG300/30% (5% dextrose in H₂O) at 0.50 mg/mL calculated from a six-point curve over 1 h.

These data collectively show that azetidine-2-carboxanilides comprise a promising, potent, and selective new class of inhibitors of PfDHODH. In contrast to other antimalarial DHODH inhibitors to date, compound 27 exhibits a sterile cure in an in vivo P. berghei model after just three doses. Additional efforts assessing the inhibition of azetidine-2-carboxanilides against DHODH from other Plasmodium species and evaluating efficacy of this series in the humanized NSG mouse P. falciparum model are underway.

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