SUPPORTING INFORMATION.

Triazolopyrimidine-based dihydroorotate dehydrogenase inhibitors with potent and selective activity against the malaria parasite, *Plasmodium falciparum*

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Content: Experimental section describing enzyme and parasite assays, and general chemical and analytical methods. HPLC traces demonstrating purity of compounds 7 and 20.

Experimental Methods.

Steady-State Kinetic Analysis. Steady-state kinetic assays were performed as follows. The reduction of 2,6-dichloroindophenol (DCIP; 0.12 mM) was followed at 600 nm (ε=18.8 $mM^{-1}cm^{-1}$) using enzyme (E_T = 5 - 10 nM) and substrates (0.2 mM L-dihydroorotate and 0.02 mM CoQ_D) in assay buffer (100 mM HEPES, pH 8.0, 150 mM NaCl, 10% Glycerol, 0.1%Triton) at 20°C. Background CoQ-independent oxidase activity (<3% of activity) was subtracted from the data prior to analysis. For potent compounds activity was confirmed by the direct detection of orotic acid at 296 nm (ε_{296} = 4.30 $mM^{-1}cm^{-1}$), which was also used for K_I determination. Substrate and buffer conditions were as above except oxygen was depleted by the inclusion of an oxidase/catalase system (0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, and 50 mM glucose).

Pre-steady-state kinetic analysis by stopped flow spectroscopy. Rapid kinetic analysis was performed as described previously.[30] The transition of FMN between the oxidized and reduced state was monitored at 485 nm on a Bio-Logic SFM-3 stopped-flow instrument. For DHO-dependent reactions, enzyme (final concentration 20 μM) was mixed with DHO (125 μM final concentration) in assay buffer at 4 °C. For CoQ_D-dependent reactions, oxygen was depleted from the reactions using the oxidase/catalase system (described above) and by bubbling with nitrogen. Oxidized enzyme (45 μM) was reduced with a limiting amount of DHO (30 μM) before loading on to the stopped-flow instrument. Enzyme reduced in this manner (final concentration 10 μM) was then mixed with CoQ_D (100 μM final concentration). For inhibitor analysis enzyme (10 or 20 μM final concentration) was pre-mixed with inhibitor (50 μM final concentration) prior to reaction with substrates and monitoring of the reaction.

P. falciparum cell culture. Parasite clones 3D7, FCR3, K1, Dd2, HB3 and D6 were propagated in Gibco-Invitrogen RPMI-1640 supplemented with 20% human type A+ plasma and 2% (w/v) red blood cells.[34] Low-passage L1210 mouse leukemia cells (American Type Culture Collection) were also grown in plasma-supplemented RPMI-1640. Blood products were obtained from Biochemed Services, Virginia. To study inhibition of cell proliferation [^{3}H]-hypoxanthine uptake was measured in drug-treated *P. falciparum* infected-erythrocytes and L1210 cells described previously.[15]

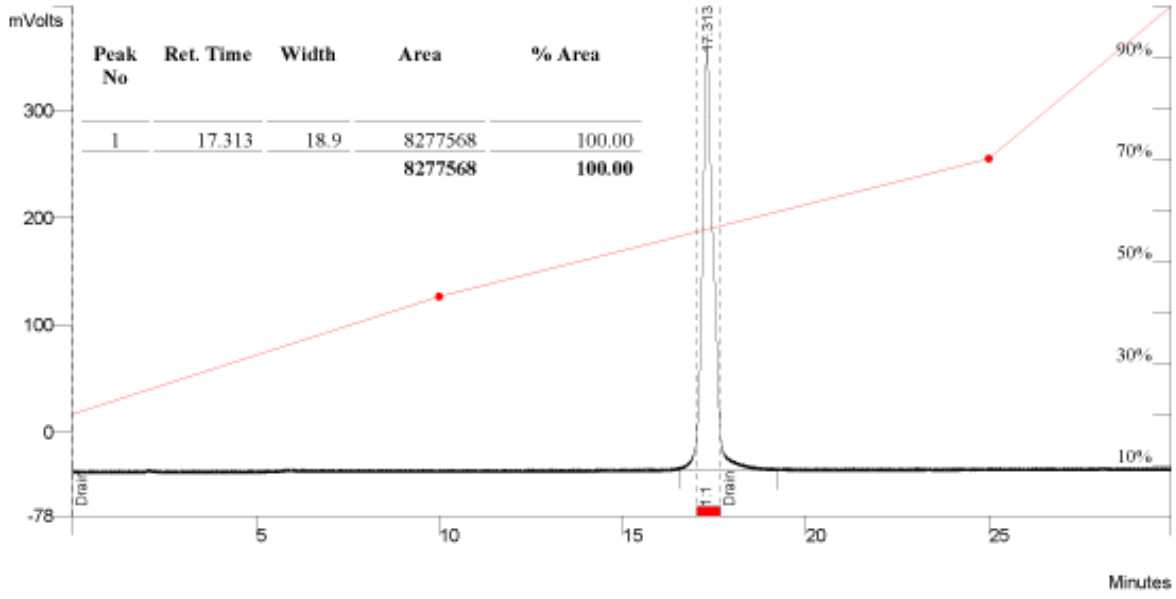
Curve fitting and error analysis. Enzyme IC_{50} and parasite EC_{50} data were determined over a range of inhibitor concentrations using triplicate data points at each concentration. IC_{50} values were determined using the graphing program Prism (GraphPad). Data reported in Table 1 represents the average of at least 2 independent experiments.

General Chemistry. Unless otherwise indicated, all anhydrous solvents were commercially obtained and stored under nitrogen. Reactions were performed under an atmosphere of dry nitrogen in oven-dried glassware and were monitored for completeness by thin layer chromatography (TLC) using silica gel 60 F-254 (0.25 mm) plates with detection with UV light.

General Analytical Methods. ^{1}H NMR spectra were recorded on dilute solutions in $CDCl_3$ or DMSO-d_6 at 300 MHz. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane (TMS). Coupling constants (*J*) are reported in Hz. Electrospray ionization mass spectra were acquired on a Bruker Esquire Liquid Chromatograph-Ion Trap Mass Spectrometer. Flash chromatography was carried out with silica gel (32-63 μm). Melting points were taken in capillary tubes (Mel Temp apparatus) and are uncorrected. Compound **7** was crystallized in CH_2Cl_2/CH_3OH. Compounds were subjected to HPLC analysis on an automated Varian Prep star system using a gradient of 20% MeOH to 100% MeOH (with 0.1% trifluoroacetic acid) over 30 min, using a YMC S5 ODS column (20 × 100 mm, Waters, Inc.).

Figure 1S. A. HPLC trace for compound 7. B. HPLC trace for compound 20. Purified compounds eluted as single peak, and these peak fractions had the expected *Pf*DHODH inhibitor activity (IC_{50} = 0.05 and 0.1 μM respectively). The HPLC method is described in the Experimental Section.

A.



B.