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Supplementary Materials for

A long-duration dihydroorotate dehydrogenase inhibitor (DSM265) for prevention and treatment of malaria

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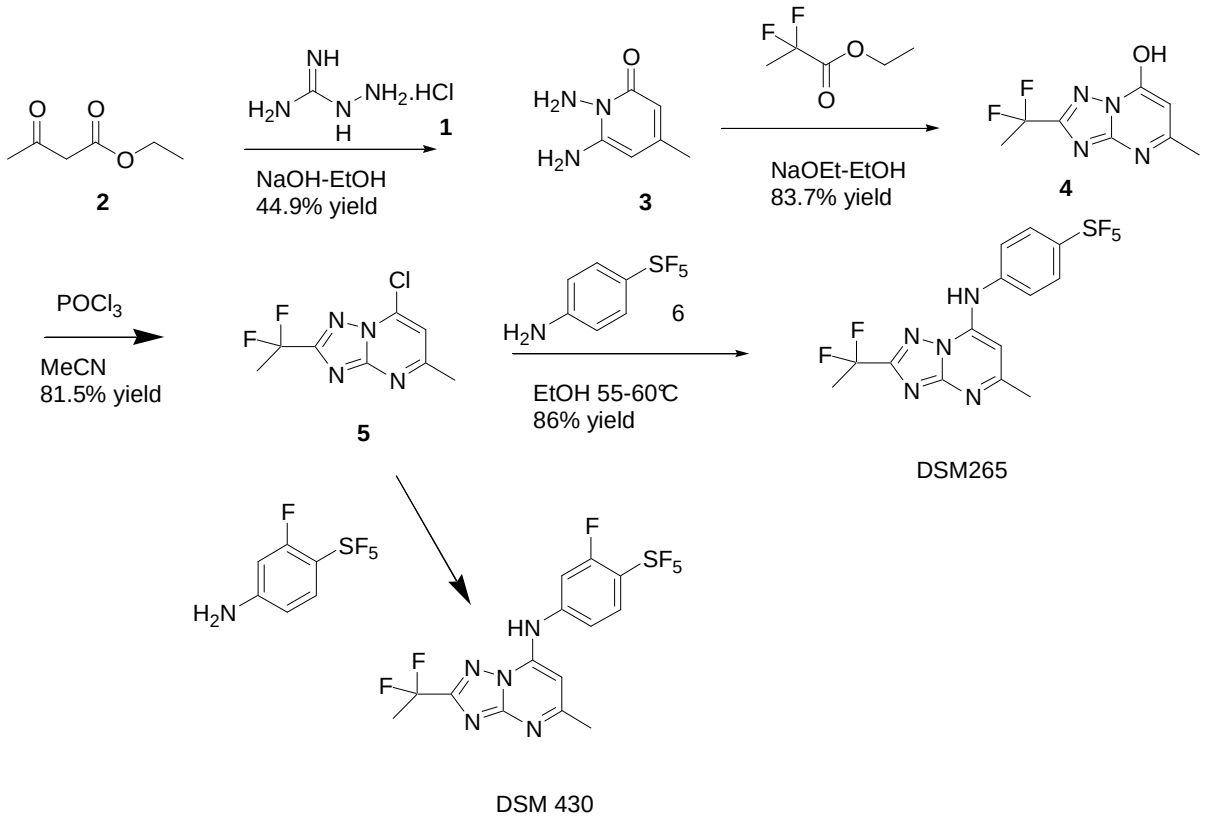
1. Synthesis

Synthesis of DSM265. GMP synthesis of DSM265 was performed under contract by Wuxi App Tec (Shanghai, China) and was achieved on a 12.4 kg scale using chemistry as outlined in scheme 1, which followed our previously published method (*14*). The major impurity resulting from the synthesis was identified as DSM430, which is formed from a fluorinated impurity in the pentafluorosulfanylaniline **6**. DSM430 was present at levels of 0.3-0.5% in early batches when the identification work was done, and it is present at about 0.6% in the GMP batch made on 12.4 kg scale (PT-C11031734-DF1101).

scheme 1



Step 1: Synthesis of 2,3-diamino-6-methylpyrimidine-4(3*H*)-one. Aminoguanidine hydrochloride (29 kg; 1 equivalent) was added to a solution of sodium hydroxide (21.2 kg; 2 eq) in a mixture of water (129 L) and ethanol (47 kg; 58.7 L) and ethyl 3-oxobutanoate (50.41 kg; 1.5 eq) was added to the reaction mass. After the reaction had been completed (about 4 h), ethanol (200.3 L) was added and the reaction mixture cooled to 15-20°C for 3 h. The product was filtered off and washed with ethanol (35 L) and dried under vacuum (50-65^{o}C for 12-16 h) to give the desired product as a light yellow solid (16.5 kg; 44.9% yield). Purity 99.7% by HPLC; water content 0.6%; ROI 4.2%. NMR (400MHz;d6-DMSO) shows peaks at 2.01 (3H,s,Me); 5.32(2H, s, NH_2); 5.53 (1H, s, ring H) and at 7.07 (2H, broad s,NH_2). MS 140.8

Step 2: Synthesis of 2-(1,1-difluoroethyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-ol).
The compound from the previous step (9.5 kg; 1.0 eq) was added to a solution of sodium ethoxide in ethanol, prepared from metallic sodium (3.3 kg) and ethanol (75.4 kg). Ethyl 2,2-difluoropropionate (12.8 kg, 1.3 eq) was then added at RT over 30 min and the mixture heated at 78-84°C until reacted (2-3 h). The reaction mixture was cooled, acidified to pH 2 with 2N HCl (67 kg), and the resultant solid

product was filtered off and dried under vacuum at 50-65°C for 12-16 h to give 12.15 kg of the title compound as a white solid. Assayed yield 83.7%; purity 99.3% by HPLC. NMR (400MHz; $CDCl_3$) shows peaks at 2.30 (3H, t); 2.55 (3H, s); 6.0 (1H, s) and 12.9 ppm (1H, broad s). ^{19}F NMR (DMSO) shows a single peak at -88.53 ppm. MS 214.9.

Step 3: Synthesis of 7-chloro-2-(1,1-difluoroethyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidine. Phosphorus oxychloride (24.15 kg; 3.0 eq) was slowly charged into a solution of the compound from the previous step (11.0 kg of assay 96.6%; 1 eq)) in acetonitrile (45.5 kg) keeping the temperature below 40°C and the mixture was heated to 80-85°C until the reaction was complete (4-5 h). After partial cooling to 40-50°C and evaporation of the excess reagent, the mixture diluted with dichloromethane (120 kg) and was carefully quenched into water (100 L). The layers were separated and the aqueous was extracted with dichloromethane (66 kg) and the combined organic layers were dried over sodium sulphate (10 kg), concentrated, then crystallized from a mixture of ethyl acetate (0.36 kg)and heptane (50 kg) and dried under vacuum (50-60°C for 12-14 h) to give the title compound as a pale brown solid (9.9 kg) in 81.5% assayed yield. Purity by HPLC, 98.9%. NMR (440MHz, $CDCl_3$) shows peaks at 2.13 (3H, t,), 2.70(3H, s), and 7.18 ppm (1H,s)

Synthesis of DSM265. Synthesis of 2-(1,1-difluoroethyl)-5-methyl-[*N*-[4-(pentafluorosulfanyl)phenyl] 1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine (DSM265)

4-(pentafluorosulfanyl)aniline (8.6 kg, 0.97 eq) and the product from step 3 (9.45 kg; 1.0 eq) were both added to ethanol (3116 kg) and the mixture was heated to 55-60°C until the reaction was complete (3.5 h). The solvent was partially evaporated and diluted with ethyl acetate (73 kg) and then concentrated to about 70 L. More ethyl acetate was added (56 kg) and the mixture further evaporated; more ethyl acetate was added (38 kg) the product was crystallized and filtered to give 18.8 kg of wet product. This was dissolved in ethyl acetate (120 kg, washed with 5% sodium carbonate solution (121 kg) and treated with active carbon (0.56 kg) at 75-80°C to remove colour. After filtration to remove carbon the mixture was concentrated to about 20-30 L and heptane (240 L) added; the product crystallized on cooling and was filtered off and dried under vacuum (50-60°C for 12 h) to give the title compound DSM265 (15.55 kg; 86% assayed yield) Purity 99.2%. NMR (400MHz, d6-DMSO) shows peaks at 2.13 (3H, t, J=12Hz); 2.45 (3H, s,); 6.73 (1H); 7.64 (2H, d, J= 5Hz); 7.97 (2H, d, J=5Hz); 10.68 (1H, broad s). The product was recrystallized from a mixture of 1:4 mixture of toluene and heptane (approx. 600 L) to give the desired anhydrous polymorph of DSM265, form 2, which melts at 161°C (12.44 kg; 87.6% yield); purity by HPLC 99.5%.

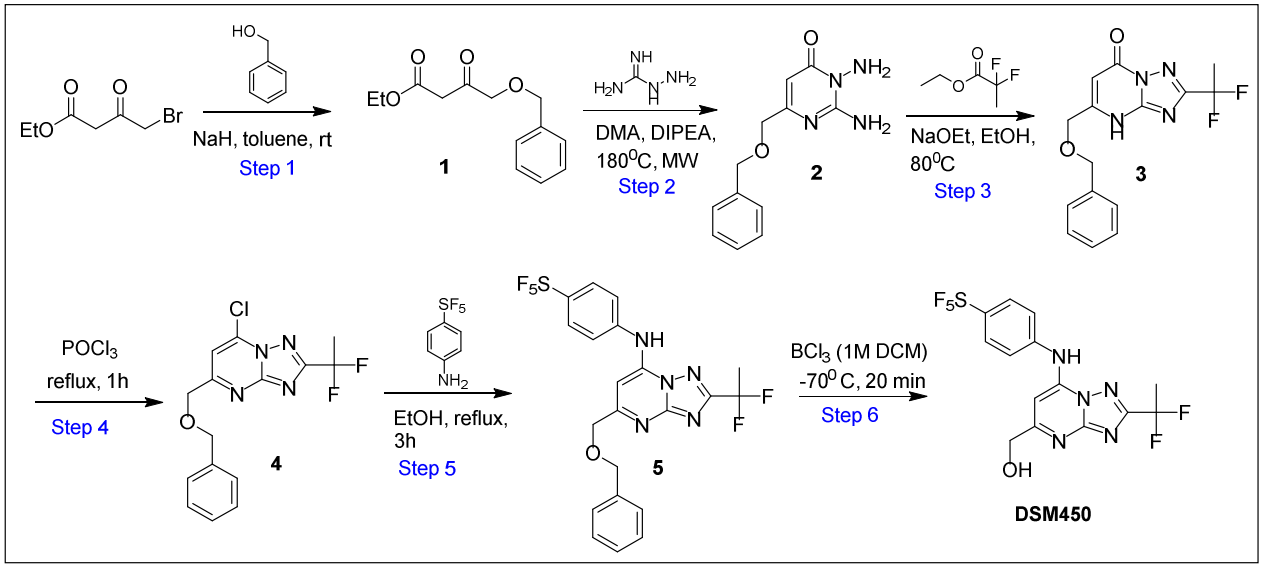
Synthesis of DSM 430. Synthesis of 2-(1,1-difluoroethyl)-*N*-(3-fluoro-4-(pentafluoro-$\lfloor^6$-sulfanyl)phenyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine (DSM430)

Step 1: 3-fluoro-4-pentafluorosulfanyl-aniline was isolated as an impurity in 4-pentafluorosulfanyl-aniline by preparative HPLC, along with the 2-fluoro isomer and characterized by its NMR and MS data. MS 237.9; NMR 7.45 (1H, t); 6.3-6.4 (2H, m); ^{19}F NMR, -73.61 (SF_5); -110.7 (fluoroaryl).

Step 2: Synthesis of DSM 430. 3-fluoro-4-pentafluorosulfanyl-aniline (300 mg) and 7-chloro-2-(1,1-difluoroethyl)-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidine (900 mg)(step 3 above) were heated at 50-55°C in ethanol (40 mL) for 18 h as shown in Scheme 1. The solvent was evaporated and the product crystallized from ethyl acetate to give DSM430 (450 mg) of 99% purity by HPLC; MS 430.1; NMR (D6-DMSO) shows peaks at 2.15(3H, t); 2.51 (3H, s); 6.95 (1H, s); 7.5-7.6 (2H, m) and 8.05 (1H, m). ^{19}F NMR ($CDCl_3$) shows peaks at -104 ppm (aromatic F); -90.5 (CF_3) and -68.8 ppm (SF_5).

Synthesis of DSM450 2-(1,1-difluoroethyl)-7-((4-(pentafluoro-l6-sulfanyl)phenyl)amino)-[1,2,4]triazolo[1,a]pyrimidin-5-yl)methanol. Synthesis was performed under contract by Syngene International, Bangalore, India.

Scheme S2. Synthesis of DSM450



Step 1: To a suspension of NaH (60 percent in mineral oil, 5.84 g, 0.146 mol) in anhydrous toluene was added benzyl alcohol (7.5 g, 0.069 mol) drop wise over 20 min. After stirring for 2 h, ethyl bromoacetoacetate (14 g, 0.0669 mol) was added drop wise over 15 min and the reaction mixture was stirred at RT overnight. After completion, the reaction mixture was slowly quenched with 2 N aq. HCl. The aqueous layer was separated and extracted with EtOAc (2 x 200 mL). The combined EtOAc layers were dried over anhyd.Na_2SO_4 and concentrated *in vacuo*. The crude oil was purified by flash chromatography on silica gel using hexane/EtOAc to give light-yellow oil of intermediate **1** (10.36 g, 70% yield). (m/z): 235.1 (M^-). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33-7.37 (m, 5H), 4.61 (s, 2H), 4.19 (q, J = 7.1 Hz, 2H), 4.16 (s, 2H), 3.59 (s, 2H), 1.29 (t, J = 7.1 Hz, 3H).

Step 2: A reaction mixture of aminoguanidine hydrochloride (1.405 g, 0.0127 mol), ethyl 4-(benzyloxy)-3-oxobutanoate (Int.-1) (3 g, 0.0127 mol) and DIPEA (3.27 g, 0.0254 mol) in DMA (10 mL) was stirred at 180°C for 20 minutes in microwave. After completion of the reaction, water was added and extracted with EtOAc. The EtOAc layer was dried over Na_2SO_4, filtered, concentrated to obtain crude product, which was purified by column chromatography to give intermediate **2** (0.6 g, 19.18% yield). (m/z): 247.2 (M^+) ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.31-7.36 (m, 5H), 7.14 (bs, 2H, NH), 5.72 (s, 1H), 5.35 (s, 2H), 4.56 (s, 2H), 4.14 (s, 2H).

Step 3: Intermediate **2** (0.6 g, 0.00242 mol) was added to a stirred solution of NaOEt prepared freshly from sodium (0.112 g, 0.00484 mol) and ethanol (15 mL), and the mixture was heated at 90 °C for 30 minutes. The reaction mixture was cooled down to room temperature and ethyl 2, 2-difluoropropanoate (0.668 g, 0.00484 mol) was added. The mixture was stirred at room temperature for 30 min before being heated to 90°C for 6 h. The reaction mixture was concentrated to dryness, water (100 mL) was added and the pH was adjusted to 4 by addition of 2N HCl solution leading to precipitation of a white solid. The solid was filtered, washed with water and dried under vacuum to afford Intermediate **3**. The mother liquors were extracted with DCM (5 x 35 mL) and the combined organic layers were dried over Na_2S0_4,

filtered, and concentrated under vacuum to give more Intermediate **3** as a white solid (0.477g 61.3% yield). (m/z): 321.2 (M^+) ^{1}H NMR (400 MHz, MeOD): δ 7.30-7.42 (m, 5H), 6.12 (s, 1H), 4.67 (s, 2H), 4.57 (s, 2H), 2.07 (t, J=18.6Hz, 3H).

Step 4: A suspension of intermediate **3** (0.477 g, 0.00148 mol) in phosphorus oxychloride (0.227 g, 0.00148 mol) was heated to reflux for 1 h. The reaction mixture was added drop wise into ice cold water, neutralized with solid Na_2CO_3, and product was extracted with dichloromethane (3 x 25 mL). The combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. Crude product was purified by flash chromatography on silica gel, eluting with Hexane: EtOAc mixtures from 50:50percent to afford the desired product intermediate **4** (0.250 g, 49.6% yield). (m/z): 339.0 (M^+) ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.61 (s, 1H), 7.34-7.41 (m, 5H), 4.79 (s, 2H), 4.72 (s, 2H), 2.18 (t, J=18.6Hz, 3H).

Step 5: A suspension of Intermediate **4** (0.250 g, 0.000739 mol) and 4-aminophenylsulfur penta fluoride (0.162 g, 0.000739 mol) in ethanol (10 mL) was heated at 50^{o}C for 3 h resulted in the precipitation of a solid. The reaction mixture was concentrated under vacuum, dissolved in dichloromethane (100 mL) and washed with aq. Na_2CO_3 (2 x 50 mL). The organic layer was dried over Na_2S0_4 and filtered. The mixture was concentrated under vacuum to dryness. The residue was purified by silica gel column, eluting with hexane/EtOAc mixtures from 100:0 to 50:50 percent) to afford the title compound intermediate **5** as a white solid (0.270 g, 70.1% yield). (m/z): 522.0 (M^+)^{1}H NMR (400 MHz, $CDCl_3$): δ 7.89 (d, J = 8.8 Hz, 2H), 7.48 (d, J = 8.7 Hz, 2H), 7.33-7.36 (m, 5H), 7.07 (s, 1H), 4.73 (s, 2H), 4.68 (s, 2H), 2.20 (t, J = 18.6 Hz, 3H).

Step 6: Intermediate **5** (0.270 g, 0.000512 mol) was dissolved in anhydrous dichloromethane under argon atmosphere. The solution was cooled to -70^{o}C and borontrichloride (1 M in dichloromethane) (3 mL) was added very slowly. The temperature was maintained for 20 min. After that dichloromethane: methanol (1:1) was added to the reaction mixture very slowly. After 10 min triethylamine (10 mL) was added to the reaction mixture and warmed to room temperature, dried over Na_2SO_4 and concentrated under vacuum. The crude product was purified by Si-gel column chromatography (Eluent CH_2Cl_2: MeOH 10:1) to yield the final product DSM450 (99% purity) as a white solid (0.135 g, 60.53% yield). (m/z): 432.0 (M^+); ^{1}H NMR (400 MHz, DMSO-d6): δ 10.79 (s, 1H), 8.03 (d, J = 8.8 Hz, 2H), 7.69 (d, J = 8.6 Hz, 2H), 6.90 (s, 1H), 5.70 (t, J = 5.84 Hz, 3H), 4.53 (d, J = 5.92 Hz, 2H), 2.13 (t, 19.1 Hz 3H).

2. Materials and Methods

A. Generation of *E. coli* expression constructs for rabbit, monkey, pig and *P. cynomolgi* DHODH and generation of *Pf*DHODH-G181C mutant constructs (UT Southwestern, Dallas, TX, USA)

The genes for rabbit, monkey (*Macaca mulatta*) and *P. cynomolgi* were codon optimized and synthesized by Genescript based on the sequence found in the indicated gene bank accession number (Table S1). Only the truncated soluble enzyme domain was included, which removed the N-terminal mitochondrial membrane-spanning domain from the constructs. Truncations were based on our previously published clones for human and *P. falciparum* DHODH (*14, 38-40*). Genes were synthesized with an *NcoI* restriction site (5'-CC ATG GCA) at the 5'end and *XhoI* restriction site (5'- CTC GAG -3') at the 3' end. Synthesized genes were first cloned into pUC57 vector, then sub-cloned it into pET-28b (Novagen) at the *Nco1* and *Xho1* restriction sites, which produces recombinant proteins with a C-terminal 6-His tag. For mini-pig DHODH, the polymerase chain reaction was used to amplify a truncated gene fragment of mini-pig DHODH from mini-pig (gottingen pig) skeletal muscles cDNA (Zyagen). Forward primer containing *NcoI* restriction site (in bold) 5'- GTG GGC **CAT GGC A**AC AGC CAC GGG GGA TGA GCG TTT -3' and reverse primer containing *XhoI* restriction site (in bold) 5'- CCA CC**C TCG AG**C CTC CGA TGA TCT GCT CCA ATG GC -3' were used to generate a 1125-bp DNA fragment. The PCR fragment was digested with *NcoI* and *XhoI* and ligated to pET-28b (Novagen) to generate the C-terminal 6xHis-tagged protein. Two independent clones were sequenced in their entirety in both directions and the DNA sequence has been submitted to Genbank (KR108306). The full-length sequence had not been previously reported. Expression construct start sequences and amino acid numbers included in the constructs are shown.

New DHODH expression construct start and stop sites

	P. cynomolgi DHODH	rabbit DHODH	monkey DHODH	mini-pig DHODH
Amino acids	194-597	29-395	29-395	29-396
N-terminal start site	MYFESYDPEF	TATGDERFYT	MATGDERFYA	MATATGDERF
C-terminal sequence	GKAHRRGG **LEHHHHHH**	AIGADHRR **LEHHHHHH**	AIGADHRR **LEHHHHHH**	AIGADHRR **LEHHHHHH**
Genbank accession number	XP_004223458	XP_008255781	XP_001104448	KR108306

C-terminal sequence in bold was added as part of the expression tag.

Site-directed mutagenesis: The G181C mutation was created by PCR mutagenesis of the *Pf*DHODH-pRSETb expression plasmid using the Quick-Change site-directed mutagenesis kit (Stratagene) with the following primers: G181C forward 5'- GTT TGA AGT ACA TCG AT**T** GTG AAA TTT GCC ATG ACC TG -3', G181C reverse 5'- CAG GTC ATG GCA AAT TTC AC**A** ATC GAT GTA CTT CAA AC -3'. Mutant enzyme was expressed and purified as described for wild-type *Pf*DHODH.

B. X-ray structure determination of DSM265-PfDHODH complex (UT Southwestern, Dallas, TX, USA)

Crystallization. *Pf*DHODH was purified by Ni^{+2}-agarose chromatography and gel filtration as previously described (*16, 41*). DSM265 solid samples used for crystallization were >99% pure and were used to generate DMSO stocks. Preliminary crystallization conditions were found using the random crystallization screen *Cryos* suite (Nextal). The condition was refined by variation of pH, precipitant, and protein concentrations to find optimal conditions. Crystals grew by the hanging drop vapor diffusion method at 20^{o}C. The crystallization drop was a mixture of an equal volume of the reservoir solution and *Pf*$DHODH_{\Delta 384\text{-}413}$ (20 mg/mL protein in 10 mM Hepes pH 7.8, 20 mM NaCl, 1 mM n-dodecyl-N,N-dimethylamine-N-oxide (LDAO), and 5% (v/v) glycerol) pre-equilibrated with final concentrations of 1 mM DSM265 (0.1 M stock solution in DMSO) and 2 mM dihydroorotate (0.1 M stock solution in DMSO). Crystals with a space group of $P6_4$ grew under conditions of 0.16 M ammonium sulfate, 0.1 M MES, pH 6.5, 18% (w/v) PEG5000, 16% (v/v) glycerol, and 10 mM DTT, while crystals with a space group of $P6_5$ grew under conditions of 0.16 M ammonium sulfate, 0.1 M Sodium Acetate, pH 4.8, 20% (w/v) PEG4000, 24% (v/v) glycerol, and 10 mM DTT. Crystals typically grew in 2 weeks. All commercially available reagents were obtained from Sigma.

Structure determination and refinement. Diffraction data were collected at 100K on beamline 19ID at Advanced Photon Source (APS) using an ADSC Q315 detector. Two independent data sets are reported from two different crystal forms, one which was of higher resolution but when refined, displayed a reduced occupancy of the SF_5 group for the DSM265 ligand (see below), and a second of lower resolution that could be refined to an occupancy of 1 across the entire ligand. The two crystal forms display different space group symmetries and were grown under different conditions (see above). The final refined structures were highly similar (RMSD = 0.132) and the DSM265 binding site was nearly identical between the two independent structure determinations. These data provide cross validation of the final structure and interpretation of the binding site interactions across two different space groups with different lattice contacts (one vs two molecules in the asymmetric unit).

Structure 1: Crystal Form I of *Pf*$DHODH_{\Delta 384\text{-}413}$-DSM265 diffracted to a d_{min} of 2.25 Å and displays the symmetry of space group $P6_4$ with one monomer per asymmetric unit and cell dimensions of a=85.9 Å, c=138.4 Å. Diffraction data were integrated and intensities were scaled with HKL2000 package (*42*), while correcting for effects resulting from radiation damage (*43*) and calculating an optimal error model. Crystallographic phases were solved by molecular replacement using the program Phaser (*44*) with the previously reported structure of *Pf*$DHODH_{\Delta 384\text{-}413}$ bound to DSM1 (PDB ID 3I65) as the search model. Inspection of the data statistics in the *Xtriage* module of the program *Phenix* (*45*) indicated that the data is merohedrally twinned, with a twin law of h,-h-k,-l. Refinement of the model with this twin law decreased R_{free} and resulted in a twin fraction of 0.33. The structure was manually rebuilt using the program COOT (*46*) and refined in PHENIX (*45*) to R_{work} and R_{free} of 0.154 and 0.181 respectively. Refinement of the DSM265 ligand at full occupancy resulted in a large negative peak in a difference electron-density map near the SF_5 group; this peak disappeared when the occupancy of the SF_5 group was set at 0.7. Maps were generated from the first 65 degrees of data and compared to the final 65 degrees of data, and while some weakening of the electron density for the DSM265 ligand occurred in

later frames there was no evidence for X-ray photolysis of the SF_5 group. We concluded that the poor density of the SF_5 group was likely due to degradation (described below) that occurs when DSM265 solutions are exposed to visible light (Table S8A), as all crystallization setup, crystal harvesting and data collection steps were performed under ambient room light. Therefore, we have chosen to refine the structure with a mixture of DSM265 at 70% occupancy, and the degradation product of the ligand minus the SF_5 group at 30% occupancy. The final difference electron density map shows no significant positive or negative peaks around the ligand binding-site. Electron density for the loop containing residues 347-355 was missing and therefore was not modeled. Data processing and structure refinement statistics are shown in Supplementary Table S1.

Structure 2: Crystal Form II of *Pf*DHODH$_{\Delta 384\text{-}413}$-DSM265 diffracted to d_{min} of 2.80 Å and displays the symmetry of space group $P6_5$ with two monomers per asymmetric unit and cell dimensions of a=89.3 Å, c=275.6 Å. Diffraction data were integrated as described above, and this data showed no signs of twinning. Non-crystallographic symmetry restraints were used during refinement. The use of the refined protein coordinates from Crystal Form I as model restraints during refinement improved geometry and R_{free} statistics. In contrast to the above higher resolution data set, this lower resolution data set could be refined to a DSM265 ligand occupancy of 1.0. Data processing and structure refinement statistics are shown in Supplementary Table S1.

C. *P. cynomolgi* liver stage assays (Biomedical Primate Research Centre, Rijswijk, Netherlands)

P. cynomolgi M strain liver-stage parasite assays to test for DSM265 activity were performed as described (*47, 48*). Sporozoites were obtained and used to infect primary rhesus hepatocytes. Briefly, primary rhesus hepatocytes grown in William's E medium (supplemented with glutamax, 10% fetal calf serum, 2% penicillin/streptomycin (P/S), 1% insulin/transferrin/selenium, 1% NaPyruvate, 1% MEM-NEAA, 50 mM β-mercapto-ethanol) were infected with *P. cynomolgi* M strain sporozoites. After hepatocyte invasion, compounds were added in duplicate to the cultures in 3-fold dilutions series from 10 μM to 0.04 μM. After 6 days, cultures were fixed and stained with DAPI anti-*P. cynomolgi* Hsp70 antibody followed by FITC-labeled goat anti-rabbit Ig and the number of exoerythrocytic forms (EEFs) were determined by microscopy using the Operetta high content imager. Survival was calculated as fraction of parasites relative to untreated cells.

D. Sexual stage assays (TropIQ, Nijmegen, Netherlands; Griffith University, Nathan, Australia; Imperial College of Science Technology and Medicine, London, UK)

Sexual stage assays. *P. falciparum* early stage gametocyte viability (*49*); *P. falciparum* gametocyte late stages IV-V (*50*); *P. falciparum* dual gamete formation assay (*17, 51, 52*) and *P. falciparum* oocyst maturation (*49*) assays were performed as described in serum based media.

E. Drug resistance studies (University of Washington, Seattle, WA, USA; Columbia University Medical Centre, New York, NY, USA)

DSM265 resistant Dd2 parasites from $3xEC_{50}$ selections were evaluated to determine if they had acquired point mutations in the DHODH gene. For DHODH gene sequencing, DNA was extracted by parasite lysis with 0.05% saponin using DNAzol (MRC Inc). The *Pf*DHODH gene was PCR amplified

using Phusion (Invitrogen) with primers (CATTTAAGCCCCAAAACATTTTTAC) and (GTGATAGATAGCTCCAGTCGATTTC). PCR products were gel-purified and sequenced using the amplification primers.

F. *Drug combination studies* (Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland)

Possible interactions between DSM265 and other reported antimalarial compounds were evaluated using the fixed-ratio isobolgram method as previously described (*53-55*).

G. Pharmacokinetic studies (SRI International, Menlo Park, CA, USA; AbbVie Inc.; North Chicago, IL, USA; Monash University, Parkville, Australia)

Studies were conducted in mice and rats (Monash University), male beagle dogs (SRI International and AbbVie Inc.), and female cynomolgous monkeys (AbbVie). DSM265 was administered in a range of oral formulations including free-base, tosylate salt, nanomilled preparation (free-base, hydrate), or spray-dried dispersion (SDD). Unless otherwise noted oral doses were administered as a suspension in 0.5% carboxymethylcellulose containing 0.4% Tween 80 (CMC-Tween) as previously described (*14*). In dogs, intravenous administration (1 mg/kg) was conducted as a 10 min infusion in 5% (v/v) DMSO, 70% (v/v) PEG400 in water. In monkeys, DSM265 (1 mg/kg in 10% (v/v) DMSO in PEG400) was administered intravenously as a slow bolus or as an oral dose by gastric intubation. Blood samples were collected into tubes containing anticoagulant (heparin or EDTA) and plasma separated by centrifugation. DSM265 and metabolite plasma levels were quantitated by LC/MS. Dose levels are provided in free-base equivalent amounts. For dog formulation studies, studies were performed in a sequential manner on the same group of dogs with a 1 week washout between doses.

H. Physicochemical, formulation and in vitro ADME assays (Wuxi AppTecCo., LTD, Suzhou, China; Monash University, Parkville, Australia; Molecular Profiles Ltd, Nottingham, UK; Pharmorphix Solid State Services, Cambridge, UK; Bend Research, Bend, OR, USA)

Stability. Solid state stability studies were conducted by Wuxi App Tec Co., LTD using raw material (both DSM265 free base and tosylate salt were tested) packaged into double LDPE bags, secured with cable ties, and stored under the conditions stated. At designated times, samples were withdrawn and assayed for appearance, assay, impurity, water, XRPD, and IR. Photostability studies were conducted according to ICH guidelines by Wuxi App Tec Co., LTD. Solutions were prepared at a concentration of 0.2-0.3 mg/mL in 50:50 (v/v) acetonitrile:water and subjected to simulated sunlight using a Xeon lamp under ID65 light 250 W/m2 for up to 48 h. Degradants present at greater than 1% were evaluated by LC-TOF MS. Control samples were treated identically but were wrapped in aluminum foil to protect them from light.

Solubility. Solubility was assessed at Monash University after 5-6 h incubation of solid material with media at 37°C. Media included fasted state simulated gastric fluid (FaSSGF, pH 1.6), fasted (FaSSIF, pH 6.5) and fed (FeSSIF, pH 5.5) state simulated intestinal fluids prepared as described

(*56*). Analysis was by HPLC (Phenomenex Luna C8(2) column) at 40°C with a mobile phase of water, methanol (45 – 90% over 5 min), and 1% aqueous formic acid at 0.4 mL/min with UV detection (254 nm). DSM265 was quantified by comparison to a calibration curve (0.25-100 μg/mL).

Formulations: Nano-milled formulations of DSM265 were produced by Molecular Profiles Ltd, salt studies were performed by Pharmorphix Solid State Services, and amorphous SDD (containing 25% DSM265:hydroxypropylmethyl cellulose acetate succinate Grade M (HPMCAS-M)) was manufactured by Bend Research.

In vitro metabolism (Monash University). DSM265 (1 μM) was incubated with male Sprague Dawley rat, male beagle dog or pooled human liver microsomes (BD Gentest, Woburn, MA) and analyzed by LC-MS as described (*14*). For human liver microsomes, incubation times were increased to 180 min for more accurate estimation of degradation rates. Metabolic stability of DSM265 (0.2 μM) was also evaluated in cryopreserved hepatocytes (2 x 10^6 viable cells/mL) from the same species as for microsomes (BD Gentest, Woburn, MA). Hepatocytes were prepared as per the supplier's instructions and cell viability determined using trypan-blue exclusion.

Binding studies (Monash University). Binding to plasma proteins, microsomal protein (0.4 mg/mL in microsomal matrix), or in vitro assay media was assessed by ultracentrifugation with LC-MS analysis as described previously (*14*).

Blood to plasma partitioning (Monash University). Partitioning ratios were determined by spiking blood from humans (Australian Red Cross Blood bank), rats or mice with DSM265 and incubating for up to 4 h at 37°C. Samples were centrifuged and plasma concentrations measured by LC-MS (*14*) and compared to a plasma control samples.

I. Analytical quantification of DSM265 plasma levels (Abbvie Inc., North Chicago, IL, USA)

DSM265 and an internal standard were separated from plasma using protein precipitation with acetonitrile. An aliquot of plasma (50 µL, sample or spiked standard) was combined with 5 µL of internal standard (A-1356318 prepared in ethanol) and 400 µL acetonitrile in a 96-well polypropylene plate. Following vortexing, the plate was centrifuged at 2000 g for 10 minutes (4°C). In an automated manner (Tomtec Quadra™), supernatant (100 µL) was transferred to a clean 96-well plate containing an aqueous solution of 0.1% formic acid + 0.025% trifluoroacetic acid (200 µL). Samples were analyzed simultaneously with spiked plasma standards. DSM265 and the internal standard were separated from each other and co-extracted contaminants on a 50 x 3 mm 2.7 µm Ascentis Express column (Supelco) with an acetonitrile: 0.1% formic acid: 0.025% trifluoroacetic acid mobile phase gradient which initiated with 40% acetonitrile, increasing to 70% acetonitrile from 0.1-1 minute, returning to 40% acetonitrile at 1.1 minutes and maintaining 40% acetonitrile through 1.5 minutes; the flow rate was maintained a 0.8 mL/min. Analysis was performed on a Sciex API5000™ Biomolecular Mass Analyzer with a turbo-ionspray interface. Analytes were ionized in the positive ion mode; source temperature 550°C. Detection was in the multiple reaction monitoring (MRM) mode at *m/z* 415.9 → 396.2 for DSM265 and *m/z* 314.052 → 282.1 for the internal standard. DSM265 and internal standard peak areas were determined using Sciex Analyst™ software. The concentration of each sample was calculated by least

squares linear regression analysis of the peak area ratio (parent/ internal standard) of the spiked mouse plasma standards versus concentration. The method provided mean percent accuracy values for the individual DSM265 standard concentrations ranging from 87.6-113.1% for the spiked mouse plasma standards at concentrations of 0.012-24.0 µg/mL (eleven separate concentrations); reproducibility (%CV) ranged from 0.09-11.1% for the analysis of triplicate standards at each concentration. The limit of detection for parent drug and the metabolite was estimated to be ~1 ng/mL from a 0.05 mL sample.

J. Metabolite identification (Abbvie Inc., North Chicago, IL, USA)

Sample preparation. Plasma samples were obtained from the following species given a single oral dose of DSM265 at the level indicated: male CD-1 mice (200 mg/kg), female New Zealand white rabbits (100 mg/kg), female cynomolgus monkeys (1 mg/kg) and male and female beagle dogs (30 mg/kg). Plasma samples were pooled across subjects in equal volume per sampling timepoint: 100 µL each for 3 mice, 75 µL each for two rabbits, 100 µL each for 3 monkeys in the IV or oral group and 50 µL each for 6 dogs. Cross-subject pooled plasma was then subjected to time-weighted sample pooling to generate one pooled plasma sample, which should have a concentration proportional to the pharmacokinetic area-under-the-curve (AUC). The sampling time points used for AUC pooling are: 1, 2, 4, 6, 9, 12 and 24 h for mice; 1, 2, 4, 6, 9, 12, 24, 36, 48, 72 and 96 hr for rabbits; 0.25, 0.5, 1.0, 1.5, 2, 4, 6, 9, 12 and 24 h for monkey (oral group); 0.1, 0.25, 0.5, 1.0, 1.5, 2, 4, 6, 9, 12 and 24 hr for monkey (IV group); 0, 0.5, 1, 1.5, 2, 3, 6, 9, 12, 24, 48, 72 and 96 h for the dog. A total of 5 pooled plasma samples were subjected to solvent extraction with 3 volumes of acetonitrile/methanol: 1/1 (v/v). After three repeating solvent extractions, supernatants were combined and dried using a nitrogen evaporator. Dried samples were reconstituted with water/acetonitrile/methanol: 7/1.5/1.5 (v/v/v). Reconstituted samples (50 µL) were analyzed by accurate LC-MS and MS/MS for metabolite profiling and identification.

HPLC-Mass spectrometry analytical methods. Elution of DSM265 and its metabolites was achieved at RT on a Phenomenex Luna C18, 150 x 2.0 mm 3 micron column with a Phenomenex C18, 4 x 3.0 mm ID guard column. Mobile phases were A: 10 mM ammonium acetate in water, and B: 0.1% formic acid in acetonitrile; the flow rate was maintained at 200 µL/min. The gradient was as below.

Processed plasma samples were profiled on a HPLC-MS system with accurate mass spectrometry analysis. The HPLC system consisted of Thermo Accela 1250 series pump and autosampler. The mass spectrometer used for metabolite characterization was an LTQ-Orbitrap (Thermo Finnigan, San Jose, CA), equipped with electrospray ionization (ESI) source operated in the positive ionization mode. Metabolite exposures relative to DSM265 were estimated based on MS peak area ratios of metabolites to parent. Structures of DSM265 and its metabolites were elucidated by LC-MS/MS using a combination of full scan and product ion scan (MS^2) analyses of plasma samples.

Time (min)	%A	%B
0	95	5
3	95	5
40	40	60
45	10	90
50	10	90
51	95	5
57	95	5

DSM265. A protonated molecular ion at *m/z* 416 was observed at ~41.5 min on HPLC analysis of plasma samples. High resolution mass analysis suggested the formula of $C_{14}H_{12}F_7N_5S$. MS^2 analysis of *m/z* 416 showed key fragment ions at *m/z* 396, 376, 289, 269, 249 and 224. The fragment ion at *m/z* 396 and 376 was formed from the loss of one and two HFs, respectively. Loss of SF_5 radical from the parent ion generated the fragment ion at *m/z* 289, which further gave *m/z* 269 and 249 via the loss of one and two HFs, respectively. The ion at *m/z* 224 was formed from the sequential loss of $C_2H_3F_2$ radical from the ion at *m/z* 289.

M1. M1 was observed at ~27.1 min on HPLC analysis with a protonated molecular ion at *m/z* 592, 176 amu greater than the parent ion. High resolution mass analysis suggested the formula of $C_{20}H_{20}F_7N_5SO_6$. MS^2 analysis of M1 gave parent fragment ion at *m/z* 416, suggesting that M1 was formed via direct glucuronidation of DSM265.

M2 and M3. Two metabolites with the same protonated molecular ion at *m/z* 608 were observed at ~28.6 and 29.4 min, respectively. High resolution mass analysis suggested the formula of $C_{20}H_{20}F_7N_5SO_7$. MS^2 analysis of M2 and M3 shared common fragmentation profiles with key fragment ion observed at *m/z* 432, 16 amu greater than parent ion. M2 and M3 were proposed to be formed via oxidation and glucuronidation of DSM265.

M4. One metabolite with the protonated molecular ion at *m/z* 432 was observed at ~36.3 min. High resolution mass analysis suggested the formula of $C_{14}H_{12}F_7N_5SO$, one oxygen more than parent formula. MS^2 analysis of M4 gave the fragment ion observed at *m/z* 412, which was formed via loss of one HF. M4 was proposed to be formed via mono-oxidation of DSM265.

M5. One metabolite with the protonated molecular ion at *m/z* 430 was observed at ~36.4 min. High resolution mass analysis suggested the formula of $C_{14}H_{10}F_7N_5SO$. MS^2 analysis of M5 gave the fragment ion observed at *m/z* 410, which was formed via loss of one HF. M5 was proposed to be formed via the combination of oxidation and dehydrogenation of DSM265.

Structures of DSM265 and its metabolites, together with the proposed biotransformation pathway are shown in Figure S6 and Tables S10 and S11. Structures of all metabolites are proposed based on accurate mass and MS^2 fragment ions.

K. Rabbit cardiac ventricular wedge assay (GSK, Tres Cantos, Spain)

Inhibition of the hERG channel has been associated with QT prolongation and arrhythmias (*19, 57*). The rabbit ventricular wedge assay has been described previously (*20*) as an alternative method to the

standard patch clamp method to evaluate the potential for a compound to impact the hERG channel. Briefly, a transmural wedge was dissected from the left ventricle of female rabbit hearts (New Zealand White rabbits; 2.0-3.0 kg; sedated with xylazine (6 mg/kg, s.c.), anticoagulated with heparin, and anaesthetised with pentobarbital (50 mg/kg, i.v.) following cannulation and perfusion of the left circumflex/descending branch of the coronary artery with cold cardioplegic solution (24 mM potassium (K^+), buffered with 95% O_2/5% CO_2). The preparation was then placed in a tissue bath and arterially perfused with Tyrode's solution (4 mM K^+, buffered with 95% O_2/5% CO_2, approximately 35.7 ^oC). The preparation was paced at 1 and 0.5 hertz (Hz) (equivalent to 60 and 30 beats per minute (bpm), respectively) using a bipolar silver electrode applied to the endocardial surface, and was exposed to each test concentration for approximately 30 min. Stimulation at 60 bpm is expected to be similar to the resting heart rate in a healthy volunteer, while the lower rate is included to increase the sensitivity of the assay as bradycardia is thought to increase the risk of QT prolongation and related arrhythmias (*19*). A transmural ventricular electrocardiogram (ECG) was recorded from each preparation at the different stimulation frequencies. The parameters measured were QT interval, Tpeak-end (Tp-e), and QRS interval. QT interval represents the time taken for ventricular repolarisation, which is greatly influenced by the human ether-a-go-go related gene current in the rabbit heart. Tp-e is the time from the peak to the end of the T wave of the ECG and is thought to represent transmural dispersion of repolarisation in the heart. QRS interval represents conduction time and is determined by sodium (Na) channel current. From these parameters, the Tp-e/QT ratio was calculated. This ratio has been shown to be increased by drugs known to block hERG and cause TdP arrhythmias (*20, 58*). The QT, Tp-e/QT ratio and any observed TdP arrhythmia data were used to produce a TdP score as described by Liu et al (*20*).

GSK animal use statement: All experiments were approved by the DDW Ethical Committee on Animal Research, performed at the DDW Laboratory Animal Science facilities accredited by AAALAC, and conducted according to European Union legislation and GSK policy on the care and use of animals.

L. Rat toxicology, dog PK, AMES testing and CYP induction (SRI, Menlo Park, CA, USA)

Animals: Sprague-Dawley rats were obtained from Harlan (Livermore, CA), and were 6-7 weeks of age at time of dosing. Beagle dogs were obtained from Marshall Bioresources (North Rose, NY) and were 9-13 months old at time of dosing; dogs had been previously dosed with DSM265 in other studies, and had a washout period of at least 4 weeks before conduct of the studies reported here. Rats were singly housed in hanging polycarbonate cages with hardwood chip bedding and were provided Purina Certified Rodent Chow #5002 and reverse osmosis purified water *ad libitum*. Dogs were singly housed in ~4' x 6' enclosed runs and were provided Harlan Teklad 2025C Certified Global 25% Protein Dog Diet and public supply tap water *ad libitum*. Dogs were fasted overnight before dosing.

All animal studies were conducted in an AAALAC-accredited facility, followed the procedure outlined by the National Research Council (NRC) *Guide for the Care and Use of Laboratory Animals* (1996) and were approved by the Institute Animal Care and Use Committee (IACUC).

Dose administration: Animals were administered drug in vehicle or vehicle alone via oral gavage at a volume of 10 mL/kg or intravenously at 2 mL/kg. DSM265 was administered orally in a vehicle of 0.5% w/v carboxymethylcellulose (Sigma-Aldrich; St. Louis, MO); 0.5% v/v benzyl alcohol (Sigma-Aldrich); 0.4% v/v Tween 80 (Spectrum Chemical; Gardena, CA), and sterile water for injection (Baxter Healthcare; Marion, NC). Intravenous solutions were administered over ~10 min infusion in 5% v/v DMSO (Mallinckrodt; Phillipsburg, NJ), 70% v/v PEG400 (Spectrum Chemicals) and sterile water

(Baxter Healthcare; Deerfield, IL), and were sterile filtered using a Millipore 0.2 μm syringe filter. Dose formulations were prepared fresh daily and delivered at room temperature.

Toxicity endpoints evaluated: Formal clinical observations, including mortality/morbidity, were evaluated daily. Body weights were evaluated pre-dose and at the time of necropsy. Blood was collected 24 h after the 7th dose from the retro-orbital sinus (rats) or cephalic vein (dogs) and evaluated for a standard panel of hematology and clinical chemistry parameters including hematology (hematocrit (HCT), hemoglobin (HGB), red blood cell count (RBC), red blood cell distribution width (RDW), white blood cell count (WBC), WBC differential [neutrophils (ANS), lymphocytes (ALY), monocytes (AMO), eosinophils (AEO), basophils (ABA)], mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCC), platelet count (PLC), mean platelet volume (MPV), reticulocyte count (absolute, REA, and percent, RET)) and clinical chemistry (bilirubin, total (TBI), creatinine (CRE), sodium (SOD), potassium (POT), chloride (CHL), cholesterol (CHO), triglycerides (TRI), glucose (GLU), blood urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), calcium (CAL), phosphorus (PHO), protein, total (TPR), albumin (ALB), globulin (GLO)) parameters.

Electrocardiograms: In dogs, electrocardiograms (ECG), blood pressure (BP) and heart rate (HR) were collected at predose and at several time points out to 24 h. All ECGs were measured from dogs in right lateral recumbency, when possible, using a 4-lead strip recorded at 50 mm/s paper speed at standard sensitivity (10 mm/mV). ECGs were recorded for at least 5 min or as necessary to collect sufficient chart recordings for evaluation.

Plasma drug levels: Blood was collected into tubes containing K_3EDTA, processed to plasma, and then stored frozen at ≤-60°C. Samples were evaluated using an LC-MS/MS method (Waters 2795/Micromass Quattro LC). DSM265 was extracted from plasma samples (100 μl) using acetonitrile (240 μl) in presence of 6 μg/mL diazepam (20 μL, internal standard) in 50% acetonitrile. The mobile phase consisted of MilliQ water with 0.1% (v) formic acid (A) and methanol with 0.1% (v) formic acid (B) used at a flow rate of 0.4 mL/min as a gradient over 7.5 min. The lower limit of quantitation (LLOQ) of the assay was 5 ng/mL.

CYP induction assays: CYP induction assays were a standard commercial assay performed by BD Biosciences (Woburn, MA). Briefly, DSM265 was dissolved in DMSO prior to serial DMSO dilutions, then solutions were added to serum-free BD™ hepatocyte culture medium to obtain final incubation concentrations. The final solvent concentration [0.1% DMSO (v/v)] was constant for all concentrations of DSM265. Omeprazole (OME) and phenobarbital (PB) were used as the positive controls for induction of CYP1A2 and CYP2B6, respectively. Rifampicin (RIF) was used as the positive control for induction of CYP2C9, 2C19, and 3A4. Tamoxifen was used as a positive control for the MTT assay.

Cryopreserved human hepatocytes were plated on 24-well, collagen I-coated plates and maintained in BD^{TM} hepatocyte culture medium. The cultures were maintained for at least 18 hours prior to initial treatment with drugs or controls. During the experimental phase, medium was replaced daily with fresh medium containing the appropriate drugs or controls. Test concentrations were determined by a preliminary MTT cytotoxicity assay. CYPs 1A2, 2B6, 2C9, 2C19, and 3A4 catalytic activities were analyzed *in situ* using appropriate probe substrates (phenacetin, bupropion, diclofenac S-mephenytoin, testosterone) followed by LC-MS/MS analysis. mRNA expression for each CYP isoform was determined by TaqMan® Real Time RT-PCR methods. One-step RT-PCR was used for all CYP isoforms except for CYP2C19 for which a two-step assay was used.

Bacterial mutation assay: *Salmonella typhimurium* LT2 strains (TA1535, TA1537, TA98, and TA100) were obtained from Dr. Bruce Ames (University of California, Berkeley), and *E. coli* strain WP2 (*uvr*A) was obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland). Experiments were performed as described previously for *Salmonella* (*59*) and *E. coli* (*60*) strains in the presence and absence of a metabolic activation mixture containing Aroclor 1254-induced rat-liver microsomes (S9). A dose range-finding experiment was conducted with *Salmonella* strain TA100 in the presence and absence of a rat liver metabolic activation (S9) system to determine a suitable concentration range (156.3 to 5000 μg/plate). For DSM265 there was no evidence of cytotoxicity however precipitate was present at in the 5000 μg/plate and for the SF5-aniline cytotoxicity was observed above 1250 μg/plate dose. The 5000 μg/plate was excluded from further testing for both compounds. Two independent experiments for mutagenicity were conducted with all five tester strains for both compounds at doses ranging from 78.1 to 2500 μg/plate, in the presence and absence of metabolic activation containing 5% S9 (first experiment) and 10% S9 (second experiment). S9 consisted of Aroclor 1254-induced rat liver activation prepared at a concentration of 5% or 10%. DSM265 was twice evaluated for mutagenicity using five tester strains, with three plates per dose level, both with and without S9 mixture. Unless a positive response was obtained in the first assay with 5% S9 mix, the second assay was performed using a 10% S9 mix. The positive controls in the absence of S9 were sodium azide (TA1535 and TA100), 9-aminoacridine hydrochloride (TA1537), 2-nitrofluorene (TA98), and 4-nitroquinoline N-oxide [WP2 (*uvr*A)]. In the presence of S9 for all strains, the positive control was 2-aminoanthracene.

Statistics: Body weights and clinical pathology data were evaluated by one-way ANOVA, followed by Dunnett's test (if the ANOVA was significant). For the bacterial mutagenicity assay, statistics used were: (1) Levene's test to determine if a difference exists among treatment variances; (2) one-tailed Dunnett's *t*-test for comparison of treatments with solvent controls and within-levels pooled variance; and (3) evaluation of dose-relatedness by regression analysis, using a *t*-statistic to test the significance of the regression. Results were considered positive if reproducible and statistically significant ($p < 0.01$) increases in revertants were observed at one or more dose levels; negative if values for the dose levels were not reproducible or significant; or equivocal if results cannot be clearly identified as being positive or negative.

M. Repeat dose exploratory toxicology studies (SRI, Menlo Park, CA, USA; WuXi AppTec Cp. Suzhou, China; Advinus, Bangalore, India)

Exploratory toxicological studies were performed in rats (SRI), mice (WuXi AppTec Cp.) and dogs (Advinus). Rats and dogs were dosed with DSM265 free base orally as a suspension prepared in 0.5% carboxymethylcellulose (CMC) containing 0.4% Tween 80 and 0.5% v/v benzyl alcohol in deionized water. Mice were dosed with the free base as a suspension in soybean oil to improve exposure. Rats were dosed in a single dose escalating study (50 – 500 mg/kg), followed by a 7 day repeat dose phase at 50, 150 or 500 mg/kg/day DSM265 for 7 days. Dogs were dosed first at 100 mg/kg in both the fasted and fed (30 min prior to dosing) state and after noting a significant food effect, a single dose escalating study was performed in the fed state (100 – 500 mg/kg). The repeat dose dog study was then conducted at doses of 30, 120 and 480 mg/kg dosed on alternate days for 10 days (5 doses). Mice were dosed first in a single dose escalating phase (50 – 200 mg/kg) followed by a 7-day repeat dose phase where mice were administered either vehicle, 25, 75 or 200 mg/kg/day of DSM265 for 7 days.

N. Hemolytic toxicity study (SUNY Upstate, Medical University, Syracuse, NY, USA)

Testing for hemolytic toxicity in glucose-6-phosphate dehydrogenase (G6PD) deficient human red blood cells was performed in NOD- mice (n=5 per group) engrafted with blood from a G6PD A- donor (37). Mice were dosed orally for 4 consecutive days with DSM265 tosylate salt (13, 64.8 and 168 mg/kg/day in split doses (b.i.d)) or primaquine (25 mg/kg QD) as a positive control and parameters were followed for a total of 7 days. Hemolysis was monitored via FLOW cytometry using Anti-GlycophorinA-PE for HuRBC assessment and Anti-CD71-FITC + Anti-TER119-PE for MuReticulocyte assessment. Vehicle (0.5% (w/v) sodium carboxymethylcellulose, 0.5% (v/v) benzyl alcohol, (0.4%) v/v Tween 80 in water.) was dosed as a negative control. Toxicokinetic analysis of blood DSM265 levels was performed with the dried blood spot method by Covance Laboratories.

O. Caco-2 permeability (Cyprotex Discovery Ltd, Cheshire, UK)

The permeability of DSM265 was assessed in the apical to basolateral and basolateral to apical direction in Caco-2 cell line monolayers using two different apical/basolateral pH conditions: pH7.4/7.4 and pH6.5/7.4 by as described (*61-63*).

P. In vivo genotoxicity (Covance Laboratories, Ltd, Harrogate, UK)

The in vivo micronuclei study was performed in male CD-1 mice under GLP conditions using standard methods. Five mice were dosed with DSM265 toxylate salt per group (vehicle, 500, 1000 and 2000 mg/kg free-base equivalent) with two doses given 24 h apart by oral gavage in vehicle (0.5% (w/v) CMC + 0.4% Tween 80). Bone marrow was sampled 24 h after last dose and films prepared from cell pellets were stained with the Gollapudi-Kamra modification of the Giemsa stain. Micronuclei in 2000 polychromatic erythrocytes/marrow sample were recorded for all 5 mice per group.

3. Supplemental Figures.

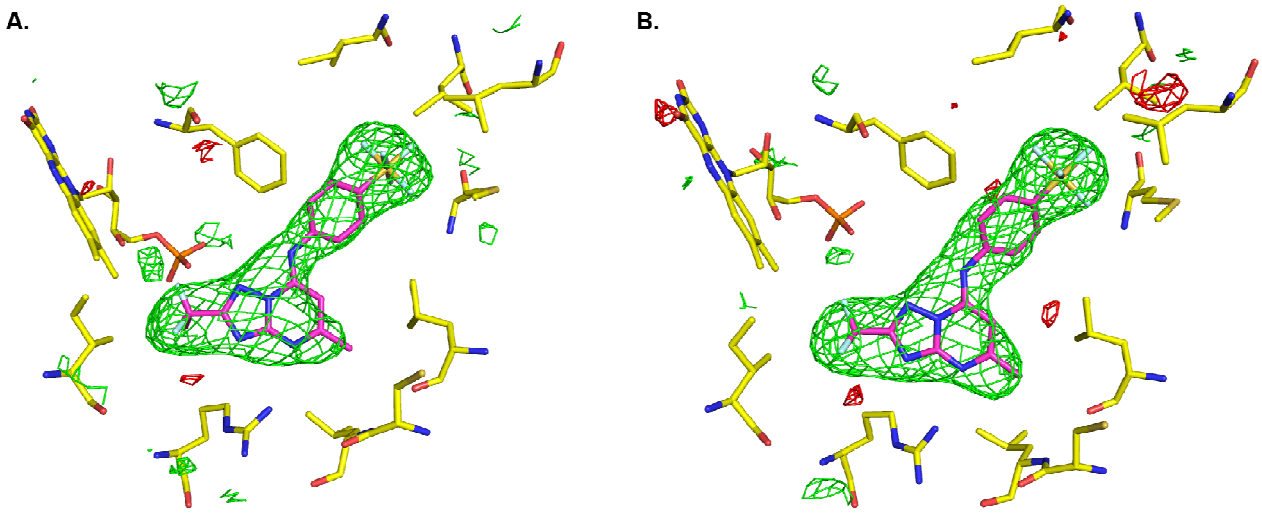


Fig. S1. (Fo-Fc) map for DSM265:*Pf*DHODH binding site. Map shows density (red negative, green positive) for both chain A and chain B of the $P6_5$ space group (2.8 Å resolution) structure contoured to 3σ prior to refinement or inclusion of inhibitor into the model.

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                              181 185
P. falciparum    NPEFFLYDIFLKFCLKYIDGEICHDLFLLLGKYNILPYDTSNDSIYACTNIKHLDFINPF 221
P. vivax         DPEFFLYDVFLKMLLKYVDGETCHELFLLMGKYKLLPYDTGKDNIYSCSEIKGLNFINPF 223
P. cynomolgi     DPEFFLYDVFLKMLLKYVDGETCHDLFLLMGKYSLLPYDTSKDSIYSCSEIKGLKFMNPF 45
P. berghei       NPEFFMYDVFLDFCLNYVDSEVCHDLFLLLGKYGLLPYDTSNDSVYATSDIKNLNFINPF 96
HUMAN            GDERFYAEHLMPTLQGLLDPESAHRLAVRFTSLGLLPRARFQDSDMLEVRVLGHKFRNPV 91
MONKEY           GDERFYAEHLMPALQGLLDPESAHRLAVRFTSLGLLPRARFQDSDMLEVRVLGHKFRNPV 91
DOG              GDEHFYAEHLMPAMQRLLDPESAHRLAVRVTSLGLLPRATFQESDMLEVRVLGHRFRNPI 110
Minipig          GDERFYRELLMPALQGLLDPETAHRLAIRFTSLGLLPRATFQDSDMLEVRVLGHKFRNPV 95
RABBIT           GDERFYTEHLMPALQGLLDPESAHRLAIRFTSLGLLPRARYQDSEMLEVRVLGHKFRNPV 91
RAT              GDDHFYAEYLMPGLQRLLDPESAHRLAVRVTSLGLLPRATFQDSDMLEVKVLGHKFRNPV 62
MOUSE            GDDHFYAEYLMPALQRLLDPESAHRLAVRVISLGLLPRATFQDSNMLEVRVLGHKFRNPV 91
                            46   52  56  60 63

                     227       237 240                       265    272
P. falciparum    GVAAGFDKNGVCIDSILKLGFSFIEIGTITPRGQTGNAKPRIFRDVESRSIINSCGFNNM 281
P. vivax         GVAAGFDKNGVCIDGILKLGFSFIEIGTITPKAQKGNERPRIFRDLETRSIINSCGFNNM 283
P. cynomolgi     GVAAGFDKNGVCIDGILKLGFSFIEVGTITPKAQKGNDKPRIFRDVGTRSIINSCGFNNI 105
P. berghei       GVAAGFDKNGICIDSILKLGFSFIEIGTITPKPQKGNNKPRIFRDVENKSIINACGFNNI 156
HUMAN            GIAAGFDKHGEAVDGLYKMGFGFVEIGSVTPKPQEGNPRPRVFRLPEDQAVINRYGFNSH 151
MONKEY           GIAAGFDKHGEAVDGLYKMGFGFVEIGSVTPKPQEGNPRPRVFRLPEDQAVINRYGFNSH 151
DOG              GIAAGFDKHGEAVDGLYKMGFGFVEIGSVTPKPQEGNPRPRVFRLPEDQAVINRYGFNSH 170
Minipig          GIAAGFDKHGEAVDGLYKMGFGFVEIGSVTPKPQEGNPRPRVFRLPEDQAIINRYGFNSH 155
RABBIT           GVAAGFDKHGEAVDGLYKMGFGFVEVGSVTPQPQEGNPRPRVFRLPEDHAVINRYGFNSH 151
RAT              GIAAGFDKNGEAVDGLYKLGFGFVEVGSVTPQPQEGNPRPRVFRLPEDQAVINRYGFNSH 122
MOUSE            GIAAGFDKHGEAVDGLYKLGFGFVEVGSVTPQPQEGNPRPRVFRLPEDQAVINRYGFNSH 151
                              111                         136

P. falciparum    GCDKVTENLILFRKRQEEDKLLSKHIVGVSIGKNKDTVNIVDDLKYCINKIGRYADYIAI 341
P. vivax         GCDEVCKNLKRFRERQKTDKLLQRHLVGVSLGKNKDSPDILQDLSYCIGKIGRYADYIAI 343
P. cynomolgi     GCDEVTENLRRFREKQKTDKLLQRHLVGVSLGKNKDSADILEDLSYCIARVGRYADYIAI 165
P. berghei       GCDKVTENLINFRKKQEEDKLLSKHIVGVSIGKNKHTENIVDDLKYSIYKIARYADYIAI 216
HUMAN            GLSVVEHRLR-ARQQKQAKLTEDGLPLGVNLGKNKTSVDAAEDYAEGVRVLGPLADYLVV 210
MONKEY           GLSVVEHRLR-ARQQKQAKLTEDGLPLGVNLGKNKTSVDAAEDYAEGVRVLGPLADYLVV 210
DOG              GLSVVEHRLL-ARQEKQARLTEEGLPLGINLGKNKTSVDAAADYTEGVRVLGPLADYLVV 229
Minipig          GLSVVEHRLR-ARQQTQARLTEDGLPLGINLGKNKTSVDAASDYAEGVRVLGPLADYLVV 214
RABBIT           GLSVVEHRLR-ARQQVQAKLTEDGLPLGINLGKNKTSADAATDYAEGVRVLGPLADYLVV 210
RAT              GLSVVEHRLR-ARQQKQAQLTADGLPLGINLGKNKTSEDAAADYAEGVRTLGPLADYLVV 181
MOUSE            GLSAVEHRLR-ARQQKQTQLTTDGLPLGINLGKNKTSVDAAADYVEGVRILGPLADYLVV 210

P. falciparum    NVSSPNTPGLRDNQEAGKLKNIILSVKEEIDNLEKNNIMNDESTYNEDNKIVEKKNNFNK 401
P. vivax         NVSSPNTPGLRDHQKGERLHGIIQRVKEEVAKLDGGGAPLGGATTGGAAMGGATTGEAVV 403
P. cynomolgi     NVSSPNTPGLRDNQQSERLHGIILRVKEEVAKLDGGKAAVGKE-----------VGKAAS 225
P. berghei       NVSSPNTPGLRDNQESNKLKNIILFVKQEINKIE-------QIGHNGET----------- 258
HUMAN            NVSSPNTAGLRSLQGKAELRRLLTKVLQERDGLR-------------------------- 244
MONKEY           NVSSPNTAGLRSLQGKAELRRLLTKVLQERDGLQ-------------------------- 244
DOG              NVSSPNTAGLRSLQGKAELRHLLAKVLQERDALQG------------------------- 264
Minipig          NVSSPNTAGLRSLQGKAELRRLLTKVLQERDALK-------------------------- 248
RABBIT           NVSSPNTAGLRSLQGKAELRCLLTKVLQERDALK-------------------------- 244
RAT              NVSSPNTAGLRSLQGKTELRHLLSKVLQERDALK-------------------------- 215
MOUSE            NVSSPNTAGLRSLQGKTELRRLLSKVLQERDALK-------------------------- 244

P. falciparum    NNSHMMKDAKDNFLWFNTTKKKPLVFVKLAPDLNQEQKKEIADVLLETNIDGMIISNTTT 461
P. vivax         GKAPPDEAATGGEPWANTTKRRPLIFVKLAPDLEEGERKSIANVLLNAEVDGMIICNTTT 463
P. cynomolgi     GKAAVEKPYVGGEPWENTTKRRPLIFVKLAPDLEESEKKKIANVLLKTEVDGMIICNTTT 274
P. berghei       -------------FWMNTIKKKPLVFVKLAPDLENSEKKKIAQVLLDTGIDGMIISNTTI 305
HUMAN            ------------------RVHRPAVLVKIAPDLTSQDKEDIASVVKELGIDGLIVTNTTV 286
MONKEY           ------------------GAHRPAVLVKIAPDLTAQDKEDIASVVKELGIDGLIVTNTTV 286
DOG              -------------------AHKPAVLVKIAPDLTAQDKEDIASVVKELGIDGLIITNTTV 305
Minipig          ------------------VAHKPAVLVKIAPDLTAQDKEDIASVVRELGVDGLIVTNTTV 290
RABBIT           ------------------GAHKPAVLVKIAPDLTAQDKEDIASVVRELGIDGLIITNTTV 286
RAT              ------------------GTRKPAVLVKIAPDLTAQDKEDIASVARELGIDGLIVTNTTV 257
MOUSE            ------------------GPQKPAVLVKIAPDLTAQDKEDIASVARELGIDGLIITNTTV 286

P. falciparum    QIND--IKSFENKKGGVSGAKLKDISTKFICEMYNYTNKQIPIIASGGIFSGLDALEKIE 519
P. vivax         QKFN--IKSFEDKKGGVSGEKLKGVSTHMISQMYNYTNGKIPIIASGGIFTGEDALEKIE 521
P. cynomolgi     QKFN—-IKSFQNKKGGVSGEKLKDISTNFISQMYNYTNKNIPIIASGGIFTGKDALEKIE 334
P. berghei       NKMD--IKSFEDKKGGVSGKKLKDLSTNLISDMYIYTNKQIPIIASGGILTGADALEKIE 363
HUMAN            SRPAGLQGALRSETGGLSGKPLRDLSTQTIREMYALTQGRVPIIGVGGVSSGQDALEKIR 346
MONKEY           SRPAGLQGALRSETGGLSGKPLRDLSTQTIREMYALTQGRVPIIGVGGVSSGQDALEKIR 346
DOG              SRPASLQGALRSEIGGLSGKPLRDLSTQTIREMYALTQGGVPIIGVGGVSSGQDALEKIR 365
Minipig          SRPASLRGALRSETGGLSGRPLRDLSTQTIREMYALTQGSVPIIGVGGISSGQDALEKIR 350
RABBIT           SRPASLQGALRSEAGGLSGKPLRDLSTQTIREMYALTQGKIPIIGVGGVSSGQDALEKIQ 346
RAT              SRPVGLQGALRSETGGLSGKPLRDLSTQTIREMYALTQGRIPIIGVGGVSSGQDALEKIQ 317
MOUSE            SRPVGLQGALRSETGGLSGKPLRDLSTQTIREMYALTQGTIPIIGVGGVSSGQDALEKIQ 346

                       528  532 536
P. falciparum    AGASVCQLYSCLVFNGMKSAVQIKRELNHLLYQRGYYNLKEAIGRKHSKS----- 569
P. vivax         AGASVCQLYSCLVFNGMKAAVRIKRELDHLLYQRGYYKLGDAVGRAHRRAA---- 572
P. cynomolgi     AGASVCQLYSCLVFNGMKAAVRIKRELDHLLYQRGYYKLEDAIGKAHRRGG---- 385
P. berghei       AGASVCQLYSCLVFNGVKSAIQIKREFNNALYQKGYYNLREAIGKKHSNAKSLKV 418
HUMAN            AGASLVQLYTALTFWGPPVVGKVKRELEALLKEQGFGGVTDAIGADHRR------ 395
MONKEY           AGASLVQLYTALTFWGPPVVGRVKRELEALLKEQGFGRVTDAIGADHRR------ 395
DOG              AGASLVQLYTALTYRGPPVVGMIKRELEALLKEQGFTRITDAIGADHRR------ 414
Minipig          AGASLVQLYTALTFRGPPVVGGVKRELEALLKEQGFTTVTDAIGADHRR------ 399
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RABBIT          AGASLVQLYTALTYCGPPVVGKVKRELEALLKEQGFSRVTDAIGADHRR------ 395
RAT             AGASLVQLYTALIFLGPPVVVRVKRELEALLKERGFTTVTDAIGADHRR------ 366
MOUSE           AGASLVQLYTALTFLGPPVVARVKRELEALLKERGFNTVTDAIGVDHRR------ 395
                           364
```

Fig. S2. DHODH sequence alignment. Sequence alignment of select *Plasmodium* and mammalian enzymes. Sequences were obtained from PlasmoDB or the NCBI protein data-base: *P. falciparum* (**PF3D7_0603300**), *P. vivax* (**PVX_113330**), *P. cynomolgi* (**PCYB_115310**), *P. berghei* (**PBANKA_010210**), Human (**NP_001352.2**), rat (**NP_001008553.1**), mouse (**NP_064430.1**), dog (**XP_853399.2**), mini-pig (**KR108306**), rabbit (**XP_008255781**) and monkey (**XP_001104448**). The sequence alignment was generated using the web server **http://www.ebi.ac.uk** and CLUSTAL O(1.2.1) multiple sequence alignment program. Highlighted residues are within the 5Å shell of DSM265 based on the *P. falciparum* structure (Fig. S2). Residues highlighted in green are conserved in the *Plasmodium* enzymes, residues in pink are conserved within the mammalian enzymes and residues in yellow are variable. Amino acid numbers on the top line are based on *Pf*DHODH and amino acid numbers on the bottom line are from the human sequence.

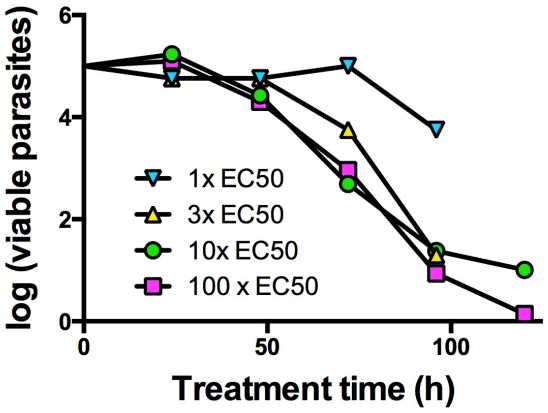


Fig. S3. In vitro parasite killing curves. Killing rate profiles in the presence of different concentrations of DSM265. Parasites were treated with drug for the indicated time and then washed. Drug-free parasites were cultured in 96-well plates by adding fresh erythrocytes and new culture media. The number of viable parasites was determined after 28 days of culturing. Data at 10x and 100x are reproduced from Fig. 2. Number of viable parasites are calculated from two independent experiments using 10-fold serial dilutions. The EC_{50} determined using a 48-hour growth inhibition assay for DSM265 in this study was 0.0046 μg/mL.

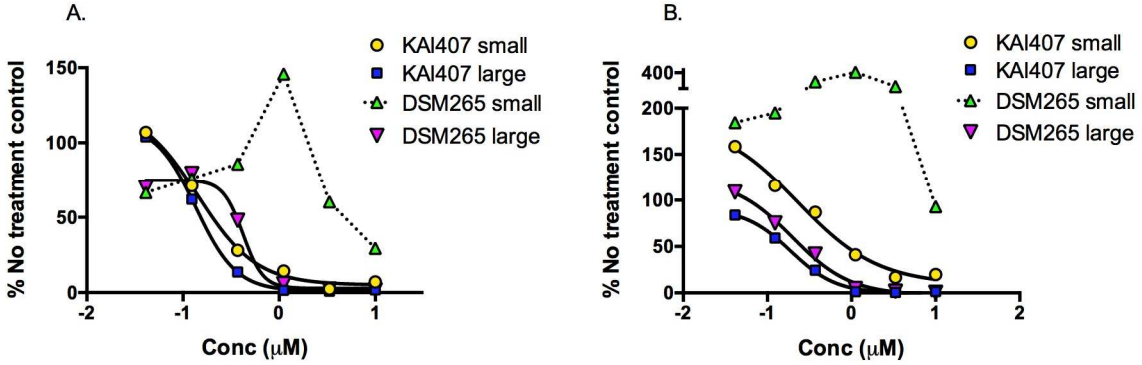


Fig. S4. Activity of DSM265 against *P. cynomolgi* large (liver schizonts) and small (hypnozoite) forms. Two independent assays (A and B) are shown and each assay included duplicate data points. KAI407 was used as the positive control, this compound is active against hypnozoites (EC_{50}s = (A) 0.14 (0.06 – 0.32) μM, (B) 0.23 (0.02 – 2.7) μM) and schizonts (EC_{50}s = (A) 0.14 (0.11 – 0.16) μM, (B) 0.19 (0.04 – 0.81) μM) in vitro with high reproducibility. Development of liver schizonts was inhibited by DSM265, with EC_{50}'s of 0.42 (0.31 – 0.57) μM and 0.20 (0.10 – 0.37) μM in two independent assays (Ave EC_{50} = 0.37 μM (0.13 μg/mL; unbound concentration = 0.028 μg/mL). Data in parenthesis represent the 95% confidence interval of the fit. DSM265 showed no substantial activity against hypnozoites to the top concentration tested (10 μM) and the data were not well fit by any model (dotted line used to connect the data points). Data were fitted to the log(inhibitor) vs response – variable slope (four parameters) equation in Graph Pad Prism (solid black line).

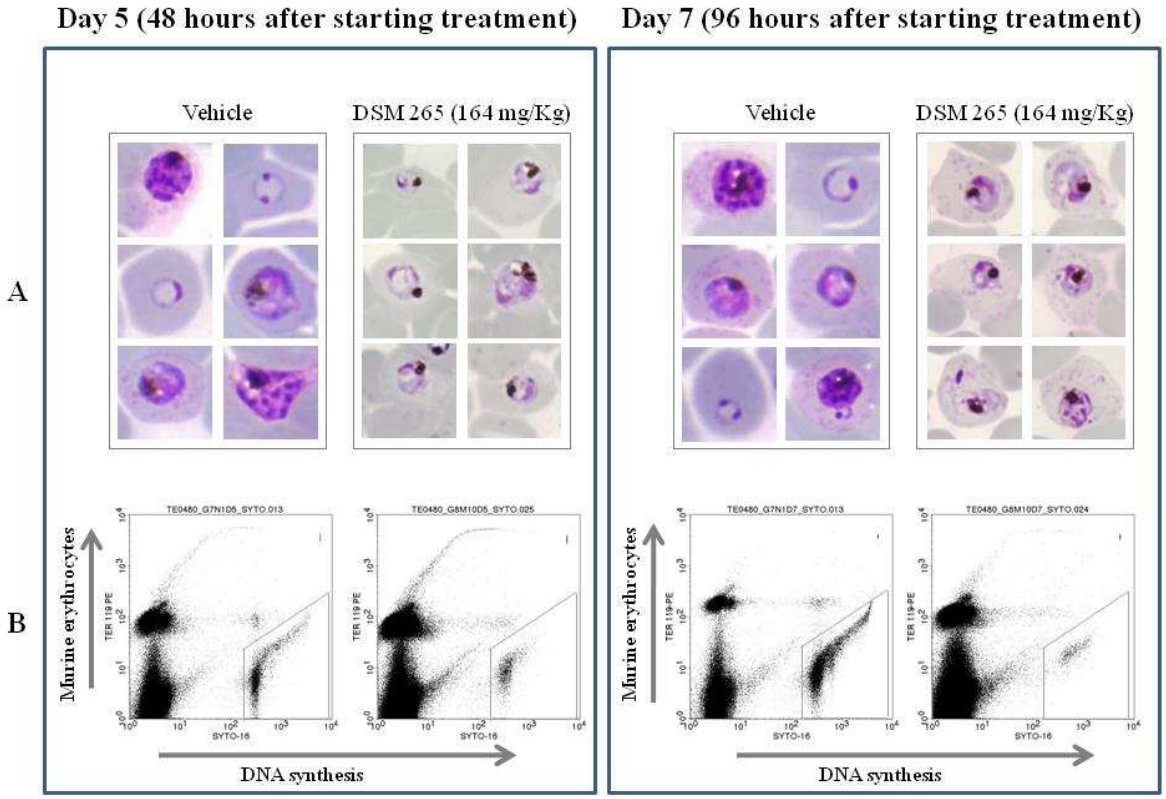
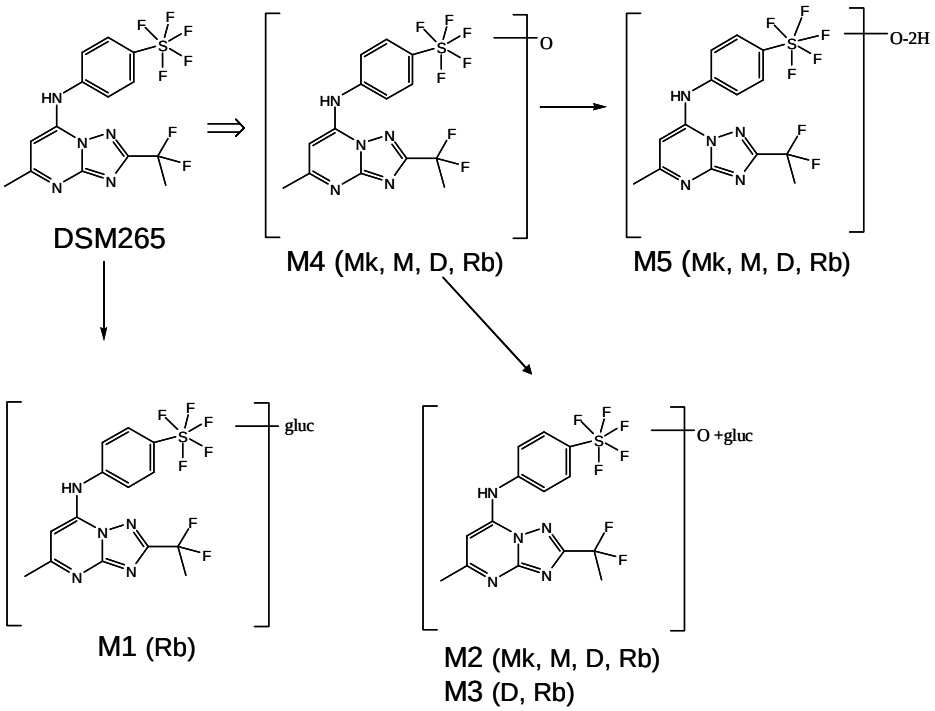


Fig. S5. The effect of DSM265 treatment on *P. falciparum Pf*3D70087/N9 in vivo. A. Peripheral blood smears stained with Giemsa. B. Flow cytometry dot plots from samples of peripheral blood stained with TER-119-Phycoerythrine and SYTO-16. Dots inside the polygonal region represent *P. falciparum*-infected human erythrocytes.



Mk - monkey, M - mouse, D - dog, Rb – rabbit

Fig. S6A. Proposed biotransformation pathways of DSM265 in plasma of mice, rabbits, monkeys, and dogs.

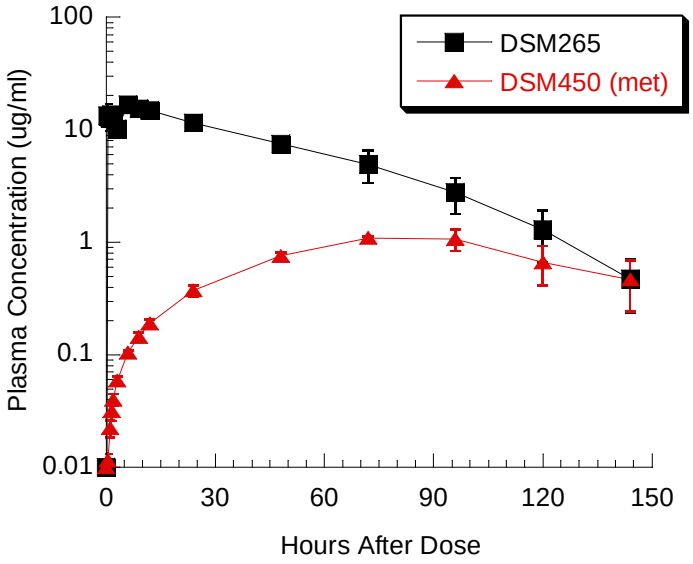


Fig. S6B. Plasma concentrations of DSM265 and DSM450 (hydroxy metabolite). Data show exposure for a 30 mg/kg single oral dose in dogs (n=3, mean ± SEM).

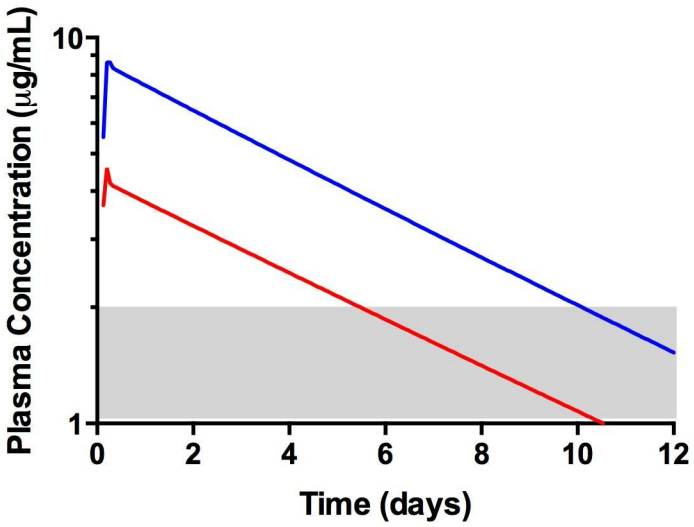


Fig. S7. Simulated human plasma profiles using a PBPK model (GastroPlus). Simulations were performed for 200 (red) and 400 (blue) mg single oral doses. The shaded area represents the approximate MPC of 1-2 μg/mL.

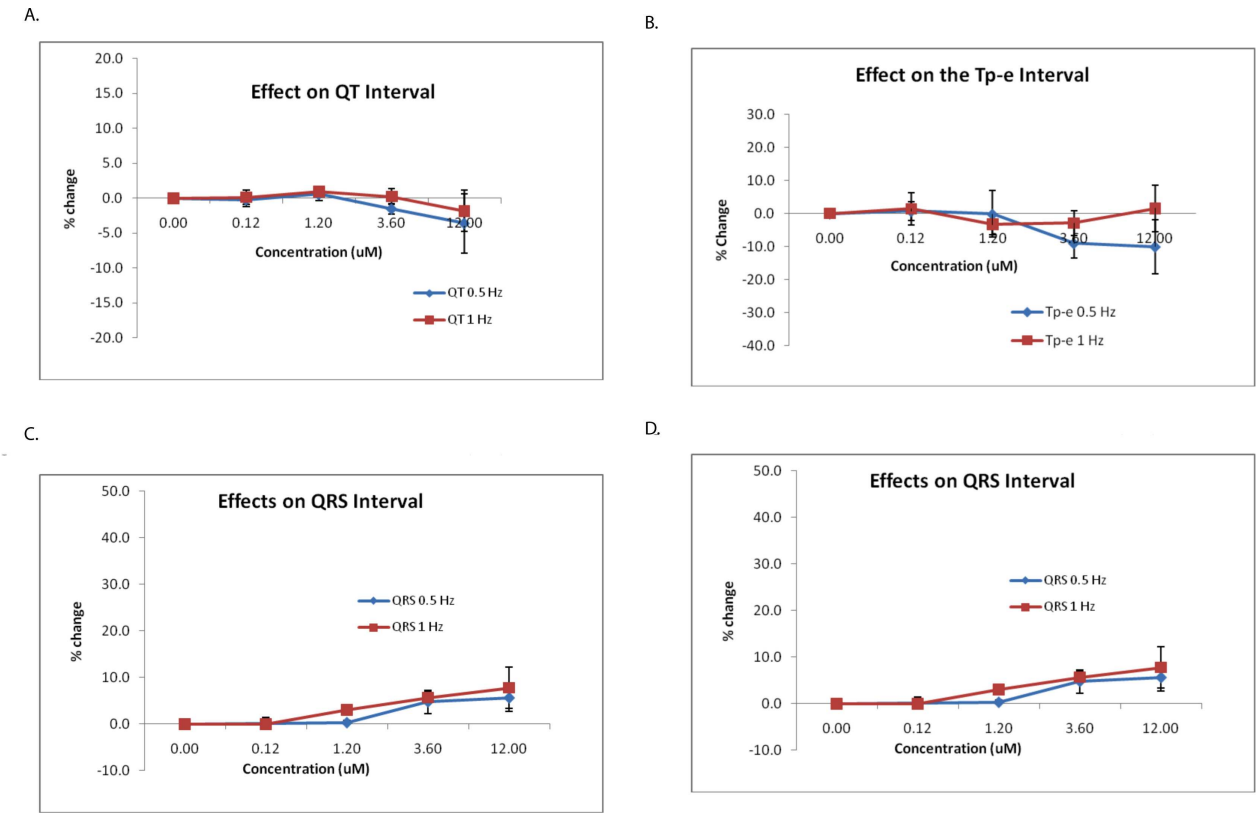


Fig. S8. Effect on ECG in the rabbit cardiac ventricular wedge assay. A. Effect of DSM265 on the QT interval at 1 Hz and 0.5 Hz respectively (n=3), B. Effect of DSM265 on Tp-e interval at 1 Hz and 0.5 Hz respectively (n=3), C. Effects of DSM265 on the QRS interval (n=3), D. Effects of DSM265 on the TdP score (n=3). The TdP scores at 5 µg/mL DSM265 were -0.3 ±0.6 and -1±0.6 at 1 Hz and 0.5 Hz, respectively. These values are well below the threshold levels found previously to be associated with a risk of TdP arrhythmia. Compounds that produced TdP scores of 2.5 or more were associated with QTc prolongation and TdP in humans at nominal concentrations that were up to 30 times the associated clinical unbound C_{max} (*20*).

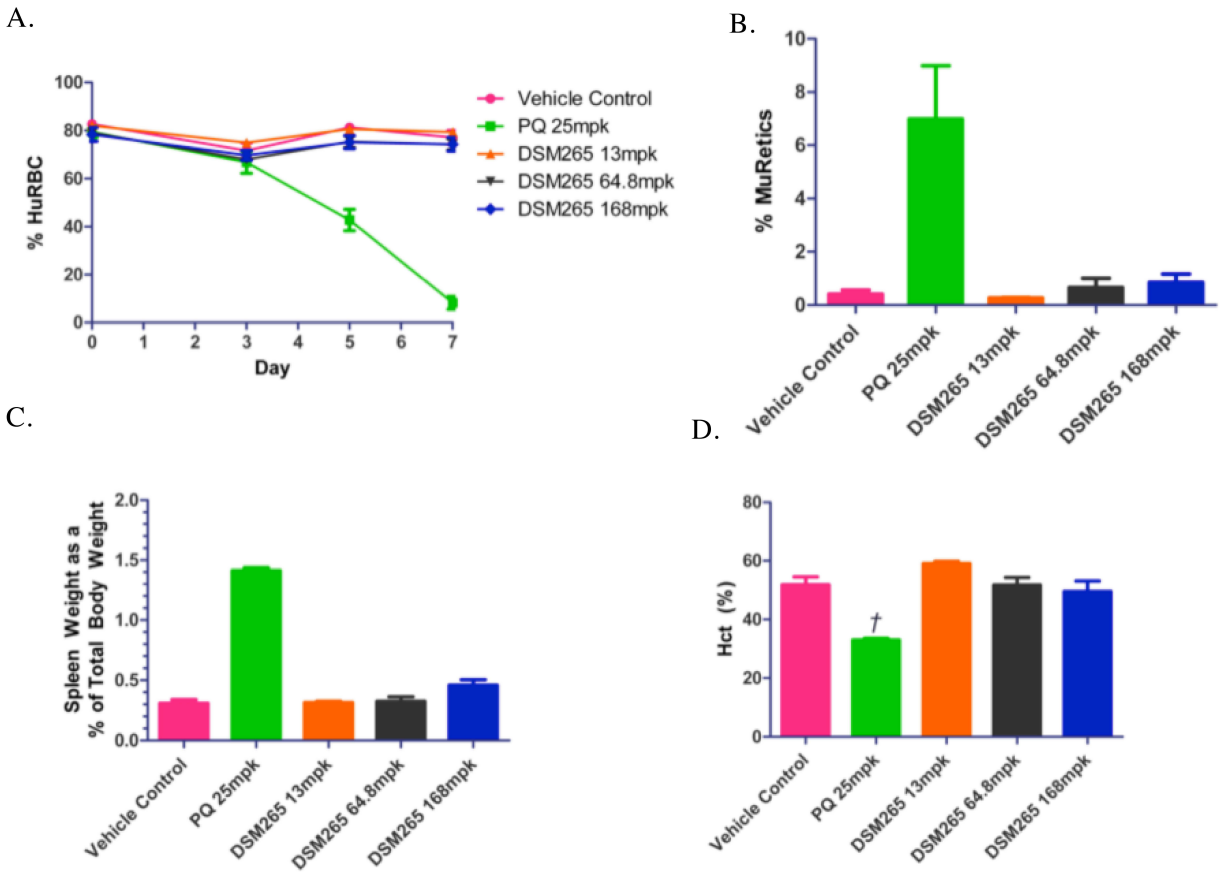


Fig. S9. **Evaluation of the effects of DSM265 on G6PD-deficient human RBCs engrafted into a NOD-SCID mouse.** DSM265 tosylate salt was dosed for 4 days orally at doses of 13, 64.8 and 168 mg/kg (mpk) b.i.d. for 5 mice per dose group where data shown are mean ± SEM. DSM265 was not hemolytic in this model and all parameters were statistically equivalent to the vehicle control values (One way ANOVA with Bonferroni's Multiple Comparison Test). In contrast primaquine (PQ)(25 mg/kg QD for 3 days) displayed significant changes in all tested parameters, consistent with its hemolytic effects on G6PD deficient human RBCs. A. Treatment Phase Kinetics of %HuRBC. B. Peripheral blood levels of mouse reticulocytes on day 7. C. Spleen Weight. D. HCT levels on Day 7 of treatment. DSM265 blood levels at the 168 mg/kg dose were 12.4 μg/mL 23 hr post the 1st dose.

4. Supplemental Tables

Table S1. *Pf*DHODH-DSM265 x-ray diffraction data and refinement statistics.

Data collection		
Crystal form	I	II
Space group	$P6_4$	$P6_5$
Cell constants a, c (Å)	85.93, 128.43	89.26, 275.61
Wavelength (Å)	0.97918	0.97912
Resolution range (Å)	39.2 – 2.25 (2.29 – 2.25)	44.1 – 2.80 (2.85 – 2.80)
Unique reflections	26,460 (1,181)	30,850 (1,513)
Multiplicity	5.9 (5.9)	7.5 (6.7)
Data completeness (%)	95.9 (87.1)	99.8 (100.0)
R_{merge} (%)[a]	5.7 (100)	6.7 (100)
R_{pim}(%)[b]	2.6 (43.7)	3.0 (68.4)
I/σ(I)	20.9 (1.2)	26.3 (1.1)
Wilson B-value (Å^2)	29.3	44.0
Refinement statistics		
Resolution range (Å)	39.2 – 2.25 (2.36 – 2.25)	44.1 – 2.79 (2.86 – 2.79)
No. of reflections R_{work}/R_{free}	21,810/1,178 (948/51)	27,318 (1,987)
Data completeness (%)	79.3 (28.0)	88.7 (29.0)
Twin law/twin fraction	h,-h-k,-l/0.33	NA
Atoms (non-H protein/cofactors/DSM256/solvent)	2,914/42/27/85	5,915/84/54/NA
R_{work} (%)	16.3 (23.6)	23.4 (30.8)
R_{free} (%)	18.0 (30.1)	25.4 (29.3)
R.m.s.d. bond length (Å)	0.009	0.004
R.m.s.d. bond angle (°)	1.05	1.06
Mean B-value (Å^2) (protein/cofactors/DSM256/solvent)	33.2/37.5/51.6/30.9	58.1/19.1/36.4/NA
Ramachandran plot (%) (favored/additional/disallowed)[c]	96.4/3.6/0.0	96.5/3.4/0.1
Missing residues	160, 347-355, 567-569	A: 160-161, 566-569 B: 160-161, 565-569

Data for the outermost shell are given in parentheses.

[a]$R_{merge} = 100\ \Sigma_h\Sigma_i|I_{h,i} — \langle I_h\rangle|/\Sigma_h\Sigma_i\langle I_{h,i}\rangle$, where the outer sum (h) is over the unique reflections and the inner sum (i) is over the set of independent observations of each unique reflection.

[b]$R_{pim} = 100\ \Sigma_h\Sigma_i\,[1/(n_h - 1)]^{1/2}|I_{h,i} — \langle I_h\rangle|/\Sigma_h\Sigma_i\langle I_{h,i}\rangle$, where n_h is the number of observations of reflections h as defined in (*43*)

[c]As defined by the validation suite MolProbity (*64*)

Table S2. In vitro antimalarial activity of DSM265.

P. falciparum	Resistance	EC_{50} (μg/mL)
NF54[a]	-	0.0018, 0.0020
HB3	Pyrimethamine	0.00095, 0.00071
3D7	-	0.0020, 0.0015, 0.0017, 0.0033, 0.0022 mean ± SD = 0.0021±0.071, n=5
K1[a]	Chloroquine, Pyrimethamine Sulphadoxine Cycloguanil	0.0026, 0.0021
TM90C2A[a]	Chloroquine Pyrimethamine Mefloquine	0.0024, 0.0035
7G8[a]	Chloroquine Pyrimethamine Cycloguanil	0.0028, 0.0028
D6[a]	Mefloquine	0.00066, 0.0012
W2[a]	Chloroquine	0.0016, 0.0027
Dd2[b]	Chloroquine Pyrimethamine Sulphadoxine Cycloguanil Mefloquine	0.0013, 0.0031, 0.00095 mean ± SD = 0.0018 ± 0.0012, n=3
V1/S[a]	Chloroquine Pyrimethamine Sulphadoxine Cycloguanil	0.0037, 0.0037, 0.0050 mean ± SD = 0.0041± 0.00075, n=3

Cell lines are resistant to the listed drugs and are described in (*18*). Culture-adapted *Plasmodium falciparum* strains using standard albumax media. Minimally n=2 replicates were collected. Standard deviation (SD) is shown for n>3, and replicate data is shown for all data sets. [a]Standard hypoxanthine based assay conducted at Swiss TPH (*65*). [b]Collected using the Syber green method, Rathod lab (*16*). Assay methods are described in the methods section of the main paper.

Table S3A. Blood pharmacokinetic data for DSM265 in SCID mice.

Dose (mg/kg)	C_{max} (µg/mL)	C_{max}/Dose (µg/mL per mg/kg)	T_{max} (h)	C_{23h} (µg/mL)	$AUC_{0\text{-}10\ h}$ (µg.h/mL)	$DNAUC_{0\text{-}10h}$ (µg.h/mL) per mg/kg
0.5 (0.5)	0.105	0.21	4	0.033	0.83	1.7
1.0 (1.0)	0.163	0.16	4	0.051	1.4	1.4
2.5 (1.5)	0.703	0.46	4	0.262	5.5	3.6
5.0 (6.4)	**0.996**	**0.15**	**4**	**0.529**	**8.5**	**1.3**
10 (9.0)	1.95	0.22	4	1.13	16.2	1.8
20 (19)	4.33	0.22	8	4.88	36.3	1.8
30 (32)	13.7	0.46	10	16.5	113.9	3.8
40 (45)	10.3	0.23	4	14.4	80.0	1.8
50 (51)	13.4	0.27	10	19.7	95.6	1.9
75 (82)	21.3	0.26	10	33.3	127.7	1.5

Doses of DSM265 (expressed as free base) administered every 12 h. Experimentally determined doses are shown in parenthesis. Maximum rates of parasite clearance were observed at and above doses of 6.4 mg/kg/12 h. **C_{23h}** data were collected 23 h post the last dose on day 4 of the study.

Table S3B. SCID mouse in vivo antimalarial activity.

	ED_{90} (mg/kg/day)	AUC_{ED90} (µg.h/mL/day)
DSM265	3.0	10.8
Chlroq	4.3	1.0
Mefloq	7.7	8.2

Table S3C. SCID mouse parasitemia.

DSM265 dose mg/kg/12 h	0			0.5	1.5	6.4	9	19	32	44	51	82
Day						**%Parasitemia**						
0	0.01			0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
3	0.48	0.57	0.4	0.4	0.39	0.51	0.43	0.41	0.44	0.48	0.47	0.49
4	0.87	0.98	0.83	0.93	0.49	0.54	0.46	0.37	0.37	0.38	0.43	0.44
5	2.18	2.64	2.17	2.2	0.45	0.36	0.38	0.29	0.26	0.29	0.31	0.31
6	3.63	3.97	3.84	4.39	0.64	0.14	0.18	0.18	0.15	0.17	0.18	0.18
7	8.41	7.7	8.28	9.15	0.7	0.07	0.03	0.05	0.05	0.06	0.06	0.11

Table S3D. SCID mouse pharmacokinetic individual time point data.

	DSM265 Blood Levels (µg/ml)								
DSM265 dose mg/kg/12 h	0.5	1.5	6.4	9	19	32	44	51	82
Time (h)									
0.25	0.032	0.32	0.451	0.433	0.972	1.82	1.57	2.79	2.85
1	0.083	0.557	0.809	1.15	3.03	8.58	4.92	6.38	7.66
4	0.105	0.703	0.996	1.95	3.68	12.8	10.3	10.4	12
8	0.073	0.488	0.878	1.76	4.33	12.8	8.63	10.8	16.2
10	0.073	0.436	0.691	1.7	4.25	13.7	8.17	13.4	21.3

Table S4A. Selection for DSM265-resistant parasites in *P. falciparum* Dd2: Rathod laboratory.

Inoculum	DSM265 ng/mL	Day of recrudescence	Frequency (# of flasks)	Clone # (EC_{50} ng/mL)
Parasites selected in bulk cultures/flasks				
2×10^6	8.3	N/A	0 of 3	N/A
2×10^7	8.3	28 - 30	5 of 6	D3 (2.7), Cl1 (8.3), Cl2 (9.9)
2×10^7	20	N/A	0/3	N/A
Parasites selected in 96 well plates instead of bulk cultures				
2×10^6	8.3	30 - 35	3/96 positive wells	D9 (9.8)
2×10^7	8.3	28 - 40	8/96 positive wells	A8 (19), H9 (19)

Dd2 wild-type EC_{50} = 1.3 - 2.5 ng/mL. N/A – no clones obtained

Table S4B. Selection for atovaquone-resistant parasites in *P. falciparum* Dd2: Rathod laboratory.

Inoculum	Atq ng/mL	Day of recrudescence	Frequency (# of flasks)
Parasites selected in bulk cultures/flasks			
2×10^7	3.7	N/A	0/3
2×10^7	1.9	29	3/3
2×10^7	0.73	16	3/3
2×10^7	0.37	11, 11, 16	3/3

EC_{50} Atovaquone (Atq) = 0.11 ng/mL.

Table S4C. Selection for DSM265-resistant parasites in *P. falciparum* Dd2: Fidock laboratory.

Inoculum	Selection concentration	Day of recrudescence	Frequency (# of flasks)	Clone # (EC_{50} ng/mL)
2×10^8	9.5 ng/mL	10	3 of 3	R4 (8.3) R5 (12)
2×10^7	9.5 ng/mL	10	3 of 3	nd
2×10^6	9.5 ng/mL	10	3 of 3	R10 (9.6)
2×10^5	9.5 ng/mL	27	2 of 3	R14 (11)
2×10^4	9.5 ng/mL	N/A	0 of 3	N/A
2×10^9	15 ng/mL	27	3 of 3	R1B C1a (58 ng/mL)
2×10^9	25 ng/mL	24	1 of 1	R1A (71 ng/mL)

Dd2 wild-type EC_{50} = 3.1 ng/mL, n=3. N/A – no clones obtained, nd, not determined.

Table S4D. Selection for atovaquone-resistant parasites in *P. falciparum* Dd2: Fidock laboratory.

Inoculum	Day of recrudescence	Frequency (# of flasks)	Clone # (EC_{50} ng/mL)
2×10^8	18, 21, 21, 24,30	5 of 5	F1 (5.3)
2×10^7	18, 18	2 of 5	nd
2×10^6	N/A	0 of 3	N/A
2×10^5	N/A	0 of 3	N/A
2×10^4	N/A	0 of 3	N/A

Dd2 wild-type EC_{50} = 0.55 ng/mL, n=3. Atovaquone selection concentration = $3xEC_{50}$.
N/A – no clones obtained, nd, not determined.

Table S4E. Selection for DSM265-resistant parasites in *P. falciparum* K1: Fidock laboratory.

Inoculum	Day of recrudescence	Frequency (# of flasks)
2×10^9	21	1 of 1
2×10^8	28	3 of 3
2×10^7	N/A	0 of 3
2×10^6	N/A	0 of 3

Wild-type K1 EC_{50} = 6.2 ng/mL, n=3. DSM265 selection concentration = 4xEC_{50} (25 ng/mL). N/A – no clones obtained.

Table S4F. Selection for DSM265 and atovaquone *P. falciparum* HB3: Rathod laboratory.

Parasite clone	DSM265 (ng/mL)	Inoculum	Day of recrudescence	Frequencey (# of flasks)
HB3	42	10^7		0 of 3
HB3	21	10^7		0 of 3
HB3	8.3	10^7		0 of 3
HB3	0.42	10^7		0 of 3
	Atq (ng/mL)			
HB3	3.7	10^7		0 of 3
HB3	0.37	10^7	28, 21	2 of 3
	Control			
HB3	0.1 % DMSO	10	15, 15, 15	3 of 3

HB3 Selections were performed in bulk culture format. EC_{50} DSM265 = 0.71 ng/mL; EC_{50} Atovaquone (Atq) = 0.059 ng/mL.

Table S5. Summary of DSM265-resistant clones: Analysis of parasites in whole cell assays.

Cell line	DSM265 EC_{50} (µg/mL)	Fold change	Atovaquone EC_{50} (µg/mL)	Artemisinin EC_{50} (µg/mL)	*Gene copy #
Dd2 parent	2.2 ± 0.87	control	0.14 (0.12 - 0.14)	1.9 (1.5 – 2.5)	1
R10 Cla	1.7 (1.4 – 2.0)	none	0.10 (0.08 - 0.14)	1.9 (1.8- 2.2)	1.3
R10 Clb	58 (50 – 71)	26	0.23 (0.21 - 0.26)	1.9 (.04 - 90)	1.1
R1B Cla	58 (94 – 110)	26	0.077 (0.037–0.15)	1.9 (1.6 – 2.3)	1.0
R1A Clb	71 (92 – 120)	32	0.22 (0.17 – 0.29)	2.2 (1.5 – 2.5)	1.1
K1 parent	3.7 (3.7 – 4.2)	control	0.18 (0.14 - 0.22)	1.2 (1.1 – 1.3)	1
K1 60 Cul 3	66 (58 - 79)	20	0.22 (0.19 - 0.24)	1.7 (1.5- 1.9)	1.3
K1 60 Cul 4	27 (24 - 31)	7	0.14 (0.05 - 0.35)	1.9 (1.6 – 2.2)	5.1; 6.2
K1 60 Cul 5	460 (210 - 950)	120	0.081 (0.07 - 0.01)	0.90 (.81 – 1.0)	1
K1 60 Cul 6	910 (620 - 1200)	240	0.14 (0.12 - 0.16)	1.5 (1.5 - 1.6)	0.9

Values in parentheses represent the 95% confidence interval. All data were collected in triplicate and then used for a global fit, except for wild-type Dd2 data where 4 independent experiments (each including 3 replicates) have been averaged and error represents the standard deviation. K1 and Dd2 were found to have equivalent copies of DHODH (data not shown). * Normalized copy number determined by qPCR of gDNA from 11 replicates. Dd2 isolates that were characterized are clonal lines but the K1 data were obtained on bulk cultures isolated in the 10^8 and 10^9 parasite selections, so these data are from mixed populations. EC_{50}'s generated on bulk cultures can represent transient events and can vary from those that are measured for clonal isolates derived from them.

Table S6. Kinetic analysis of *Pf*DHODH mutants. The G181C mutation in *Pf*DHODH was generated by site-directed mutagenesis and the effect on the DSM265 IC_{50} evaluated. The IC_{50} is shown in ng/mL with the 95% confidence interval displayed for the fit to 3 replicates data points. For k_{cat} and K_m, the standard error of the fit is shown for 3 replicates. wt, wild-type *Pf*DHODH. Data were collected using the direct assay with the inclusion of glucose oxidase and catalyze to eliminate background oxidase activity as described (3).

Enzyme	IC_{50} (ng/mL)	k_{cat} (s^{-1})	K_m (CoQ) µM	K_m (DHO) µM
wt	9.5 (7.5 – 9.5)	12 ± 1.7	22 ± 12	58 ± 9.8
G181C	120 (87 – 160)	6.2 ± 0.6	21 ± 7.2	39 ± 5.6

Table S7. Drug combination analysis. Sum or fractional inhibitory concentration $\sum FIC$ for DSM265 with potential partner drugs. Data were collected at three EC_{50} drug ratios (1 +3; 1 + 1; 3 + 1). Data from two replicates was averaged first and the data shown are the mean $\sum FIC$ data ± standard deviation for the three drug ratios. Synergism is concluded when $\sum FIC$ <0.8; antagonism when $\sum FIC$ > 1.5. The respective EC_{50} values in ng/mL against unsynchronized NF54 parasites were 2.6 (DSM265), 2.3 (OZ439), 1.2 (Artemether), 5.1 (Chloroquine), 4.5 (Pyronaridine), 8.5 (Piperaquine), 4.9 (Lumefantrine), 5.8 (Mefloquine), 0.38 (Atovaquone), 0.7 (Dihydroartemisinin), 440 (Proguanil), 0.22 (P218) and 5.7 (Pyrimethamine).

Drug Combination	**Mean $\sum FIC$ K1**	**Mean $\sum FIC$ NF54**	**Interaction**
Atovoquone + proguanil	0.2 ± 0.06	0.4 ± 0.10	Synergistic
DSM265 + OZ439	1.3 ± 0.25	1.4 ± 0.15	Additive
DSM265 + artemether	1.2 ± 0.20	1.4 ± 0.20	Additive
DSM265 + dihydroartemisinin	1.3 ± 0.10	1.4 ± 0.10	Additive
DSM265 + chloroquine	1.3 ± 0.1	1.4 ± 0.20	Additive
DSM265 + piperaquine	1.4 ± 0.20	1.4 ± 0.10	Additive
DSM265 + pyronaridine	1.3 ± 0.15	1.5 ± 0.30	Additive
DSM265 + mefloquine	1.4 ± 0.10	1.5 ± 0.20	Additive
DSM265 + Lumefantrine	1.2 ± 0.20	1.3 ± 0.10	Additive
DSM265 + atovaquone	0.8 ± 0.0	0.9 ± 0.06	Additive
DSM265 + proguanil	1.0 ± 0.1	1.1 ± 0.10	Additive
DSM265 + P218	1.4 ± 0.15	1.3 ± 0.10	Additive
DSM265 + pyrimethamine	1.0 ± 0.06	1.2 ± 0.06	Additive

Data were collected at STI

Table S8A. Stability data for DSM265 free base and tosylate salt.

Form	Storage Condition	Storage Time	Result
Free base, anhydrate - solid	30°C/65% RH	24 months	No chemical degradation detected; some absorption of water (1%) detected
Free base anhydrate - solid or tosylate salt - solid	Simulated sunlight (250W/m^2), room temperature	48 hours	No degradation detected
Free base - solution or Tosylate salt - solution (50:50 acetonitrile/water)	Simulated sunlight (250W/m^2), room temperature	48 hours	23 and 33% degradation, respectively observed[a]; no degradation in control sample protected from light

[a]loss of SF_5, replacement of SF_5 by hydroxyl and loss of para-SF_5-benzyl detected

Table S8B. Solubility data for DSM265 free base.

Media	Solubility (µg/mL)
FaSSIF[a]	5.1
FeSSIF[b]	27.6
SGF[c]	6.8

[a] Fasted state simulated intestinal fluid (pH 6.5)
[b] Fed state simulated intestinal fluid (pH 5.5)
[c] Simulated gastric fluid (pH 1.6)
study conditions (5-6 h at 37°C)

Table S9. In vitro ADME data for DSM265.

Species	Human	Mouse	SCID mouse	Rat	Dog	RPMI 1640 media 0.5% Albumax II	RPMI 1640 media 10% human serum	DMEM media 10% FCS
Plasma protein binding (%)	99.9	99.7	NA	97.7	99.4	83.1	99.6	78.5
Blood to plasma ratio	0.7	0.7	0.5	0.8	NA	---	---	---
Microsomal protein binding (%)	72	66	---	66	NA	---	---	---
In vitro CL_{int} microsomes (µL/min/mg protein)	4.3	2.8	---	1.8	<1.4	---	---	---
In vitro CL_{int} hepatocytes (µL/min/10^6 cells)	0.5	0.5	---	0.8	0.4	---	---	---

Albumax and RPMI media were used for blood stage in vitro assays; DMEM or similar media with 10% FCS were used for in vitro liver stage assays. NA=not available. In vitro studies with individual human cytochrome P450 (CYP) enzymes (Supersomes™) indicated a low extent of metabolism in the presence of CYP2C8 (0.032 µL/min/pmol CYP) and CYP2C19 (0.039 µL/min/pmol CYP), while no measurable loss was observed with the other tested CYP isoforms, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 or CYP3A4.

Table S10. In vivo metabolite identification. Retention time, molecular ion, chemical formula and characteristic fragment ions of DSM265 and its metabolites.

Met ID	RT (min)	m/z	Chemical formula	Key Fragments (MS^2)
DSM265	41.5	416.07744	$C_{14}H_{12}F_7N_5S$	396, 376, 289, 269, 249,224
M1	27.1	592.10953	$C_{20}H_{20}F_7N_5SO_6$	416
M2	28.6	608.10444	$C_{20}H_{20}F_7N_5SO_7$	432
M3	29.4	608.10444	$C_{20}H_{20}F_7N_5SO_7$	432
M4	36.3	432.07235	$C_{14}H_{12}F_7N_5SO$	412
M5	36.4	430.05670	$C14H_{10}F_7N_5SO$	410

Table S11. Relative plasma exposures of DSM265 metabolites in mice, rabbits, monkeys, and dogs.

Met ID	Biotransformation	AUC Ratio (metabolite/parent) (%)				
		Rabbit	Mice	Dog	Monkey (IV)	Monkey (PO)
DSM265	DSM265 (A-1400550)	100	100	100	100	100
M1	Glucuronidation	0.09	ND	ND	ND	ND
M2	Hydroxylation + Glucuronidation	0.04	3.7	0.03	0.04	0.03
M3	Hydroxylation + Glucuronidation	0.05	ND	0.07	ND	ND
M4	Hydroxylation	3.5	26.8	17.7	8.5	9.7
M5	Hydroxylation + Dehydrogenation	0.007	0.2	0.1	0.009	0.01

ND - not detected

Table S12. DSM265 plasma pharmacokinetics after a single intravenous dose in mice, rats, dogs, and monkey.

Species	Dose[a] (mg/kg)	$T_{1/2}$ (h)	V_{ss} (L/kg)	$AUC_{0-\infty}$ (μg·h/mL)	CL_p (L/hr·kg)	n
Mouse	1	3.4	2.3	2.03	0.49	2
Rat	0.9	10.1	3.2	4.22	0.22	2
Rat	2.6	12.6	5.9	6.62	0.39	2
Dog	1	8.7, 11.1[c]	n.c.	16.7±5.3	0.054, 0.087[c]	3
Monkey	1	45.1±12[b]	1.3±0.4	46.9±7.3	0.022±0.004	3

Data are expressed as mean values; [a]free base equivalent; [b] Evidence of enterohepatic recirculation. [c]data recorded for only two animals. For n>2, standard deviation of the mean is shown, replicate data are reported otherwise. Mouse and rat data were previously reported (*14*) and are included for comparison.

Table S13. DSM265 plasma pharmacokinetics after a single oral dose of DSM265 in mice, rats, dogs, and monkeys.

Species	Salt form	Dose[a] (mg/kg)	$T_{1/2}$ (h)	C_{max} (μg/mL)	T_{max} (h)	$AUC_{0-\infty}$ (μg·h/mL)	F[b] (%)	n
Mouse	base	0.4	4.1	0.025	0.5	0.188	23	2
	base	2	3.5	0.183	1.0	1.13	28	2
	base	10	2.4, 2.6	0.96, 1.64	1.0, 2.0	6.5, 12.4	32, 61	2
	base	50	3.2	3.96	2.0	14.1	14	2
	tosylate	25[c]	3.0	5.51±1.15	1.0	34.2±3.2	67	3
	tosylate	200[c]	2.0	18.9±1.35	4.0	292±24.5	72	3
Rat	base	2.1	11.6	0.31	3.3	6.5	66	2
	base	10.1	18.0	1.02	4.5	33.8	70	2
	base	18.8	15.5	1.64	7.0	50.6	57	2
	base	46.7	27.9	2.66	17.0	134	61	2
	tosylate	25	21	2.88, 2.47	10, 10	84, 71 (0-48 h)	86	2
Dog	base	3[d]	21.4	0.36±0.091	8.0±0.0	10.2±2.5	20.3±5.0[e]	3
	base	30[d]	19.7	2.64±2.1	13.3±9.2	80.2±72	16.0±14[e]	3
	base	300[d]	17.4	6.49±7.3	18.7±9.2	362.5±471	7.2±9.4[e]	3
Monkey	base	1[d]	c.n.c.	2.15±1.07	1.0±0.5	70.0±12	>100[e]	3

All data are expressed as mean values; [a]free base equivalent; [b]oral bioavailability; [c]CD-1 mice; all other mouse studies in Swiss Outbred mice; [d] DSM265 was administered to fasted dogs and food was reinstated 4 h post-dosing. [d] bioavailability estimate confounded by enterohepatic recirculation. Vehicle for mice, rats, dogs: 0.5% w/v CMC, 0.5% v/v benzyl alcohol, 0.4% Tween 80; monkey: 10% DMSO in PEG 400. Oral doses were administered as aqueous suspensions. c.n.c. = could not determine. For n>2, either standard deviation (monkey and dog) or standard error of the mean (mouse) are provided. Mouse 0.4, 2, 10, 50 mg/kg and rat 2.1, 18.8 and 46.7 mg/kg data taken from (*14*) and are include for comparison.

Table S14. Effect of salt form, formulation, and food on DSM265 plasma pharmacokinetics after oral dosing in beagle dogs.

Formulation	Dose (mg/kg)	Fed State[c]	C_{max} (μg/mL)	T_{max} (h)	$T_{1/2}$ (h)	$AUC_{0\text{-}inf}$ (μg·h/mL)	n
Free base, suspension	10	fasted	0.54±0.23	1.6±0.6	22.4±27	20.5±16	12
Free base, suspension	10	fed	0.92±0.55	9.2±4.3	22.5±11	43.4±39	6
Free base, suspension	30	fasted	1.44±0.50	1.0±0.4	40.2±22	86.5±47	6
Free base, suspension	30	fed	7.72±0.42	8.0±0.0	nd	398±48	4
Tosylate salt, susp	30	fasted	2.61±0.97	1.4±0.5	21.1±12	89.3±61	6
Toslylate salt, susp	30	fed	13, 7.2	1, 2	66, 18	657, 165	2
Free base	84	fasted	4.1, 1.8	8, 1	61, 39	165, 53[d]	2
Free base	84	fed	17, 18	4, 8	108, 61	1018, 939[d]	2
Free base, hydrate[a]	10	fasted	2.8±0.44.	5.7±9	20.7±11	130±59	6
SprayDried Dispersion[b]	10	fasted	6.3±1.8	1.1±.44	13.6±8.2	166±89	6
SprayDried Dispersion[b]	10	fed	9.54±2.6	3.0±4.5	16.2±8.1	269±133	6
SprayDried Dispersion[b]	30	fasted	18.5±1.2	2.8±2.8	18.1±11.9	888±204	3

All data expressed as mean data. [a]nanomilled; [b]Spray Dried Dispersion (SDD) selected for the Phase 1 clinical study and was dosed in suspension in Methocel A4M. [c]For fed dogs, food provided 30 min prior to dosing; for fasted dogs, food provided 12 h after dosing. [d]AUC was calculated from 0 – 72 h. Data are shown as the mean ± standard deviation except for n<3, where replicate data are provided. nd, not determined.

Table S15. Safety pharmacology.

Assay	Compound	Result
AMES[a]	DSM265	None mutagenic 5 strain test ± metabolic activation
AMES[a]	SF5-aniline	None mutagenic 5 strain test ± metabolic activation. The finding that the SF_5-aniline was Ames negative differs from that for the closely related pCF_3-aniline, which was reported to be Ames positive (*66*).
Micronucleus test	DSM265	No increase in micronucleated polychromatic erythrocytes. Male mice dosed twice 24 h apart with vehicle, 500, 1000 and 2000 mg/kg. The average plasma concentration of DSM265 was 21 μg/mL 4 h after the first dose.
CYP Inhibition	DSM265	CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 (Midazolam or Testosterone as probe substrates), IC_{50} all > 10 μg/mL
CYP Induction	DSM265	No induction of CYP1A2, CYP2B6, CYP2C9, CYP2C19, and CYP3A4 at enzyme or mRNA level tested at 4.2 μg/mL in human hepatocytes (SRI)
hERG	DSM265	IC_{50} = 2.9 μg/mL[b]; IC_{50} = 0.66 μg/mL[c].
Rabbit Wedge	DSM265	No TdP risk at maximum concentration tested; > 5 μg/mL
CEREP	DSM265	Panel Express S panel of human receptors tested at 4.2 μg/mL; all showed less than 25% inhibition except A3 (35%), NK3 (27%), 5-HT2b (29%), Cl- channel (GABA-gated)(28%)
Kinase Panel	DSM265	Panel of 120 human kinases tested at 2.1 μg/mL; all were below 25% inhibition except PKBb (65%), Scrc (47%) and SRPK1 (43%).
G6PDH	DSM265	No effect; DSM265 was not hemolytic or significantly different from the vehicle control at doses up to 168 mg/kg/day for 4 d (blood levels = 12 μg/mL at 23 h after the first dose).

[a] Ames tests were performed by SRI and both compounds were Ames negative ($p < 0.01$) by Dunnett's test. CYP Induction assays were performed by SRI. CYP inhibition was performed by Cyprotex Discovery Ltd, United Kingdom. hERG analysis was performed in two separate labs: [b]PatchXpress assay performed at GSK and [c]IonWorks patch clamp electrophysiology at Essen Labs. Rabbit Wedge studies were performed by GSK. CEREP assays (Express S profile) were performed by CEREP (France), 25% inhibition is the cut off for significance. Kinase Panel screening was performed at the University of Dundee. The weak activity towards PkBb, Scrc and SRPK1 (Table S15), the activities of which would not be expected to impact these enzymes in vivo given the high human plasma protein binding of DSM265 (Table S9).

Table S16. Exploratory toxicology studies (non-GLP) in rodents and dogs.

Species	PO Dose (mg/kg/day) and Duration	Vehicle	Gender/#of animals	Findings
[a]Mouse (CD1)	**Repeat dose,** 25, 75, 200 mg/kg/day for 7 days	Soybean oil suspension	M/123	No macroscopic or microscopic findings. MTD > 200 mg/kg
[b]Rat (Sprague Dawley)	**Repeat dose**, 50, 150, 500 mg/kg/day for 7 days	0.5% w/v CMC: 0.5% v/v benzyl alcohol: 0.4% Tween 80	M/3, F/3	MTD = 50 mg/kg/day. TK analysis not done as early stage exploratory study.
[c]Dog (Beagle)	**Single dose**, 10, 30, 60, 120, 240, 500	0.5% w/v CMC: 0.5% v/v benzyl alcohol: 0.4% Tween 80	M/6	No adverse clinical signs, no alterations in clinical pathology parameters. MTD ≥500 mg/kg after single dose administration.
[d]Dog (Beagle)	**Repeat dose**, 30, 120, 480 mg/kg for 10 days dosed on alternate days (5 total doses); dose in Fed state	0.5% w/v CMC: 0.5% v/v benzyl alcohol: 0.4% Tween 80	M/4	Instances of vomiting, slight weight loss and decreased food consumption in top dose dogs. MTD > 480 mg/kg
[e]Dog (Beagle)	**CVS**: telemetry ECG, Blood Pressure, Clinical observation. Single dose, 30, 300 mg/kg	Vehicle: 0.5% w/v CMC; 0.5% v/v benzyl alcohol; 0.4% Tween 80	M/6	No clinical symptoms. All ECGs within normal limits at all time-points. No adverse BP recordings associated with DSM265. Plasma C_{24h} at the high dose was 1.3 µg/mL

DSM265 was dosed as the free base for all above studies. [a]Study performed by Wuxi (study number 127-006); [b] Study performed by SRI (study number M755-10a); [c] Study performed by SRI (study number M810-10); [d]Study performed by Advinus (study number N1276); [e] Study performed by SRI (study number M811-10).

Table S17A. Toxicokinetic parameters on days 1 and 7 in a mouse 7-day toxicology study.

Dose (mg/kg)	C_{max} (µg/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-24h} (µg.h/mL)	Cav (µg/mL)
Day 1					
25 (29)	6.15±0.29	1.0	1.9	53.2±5.3	2.21
75 (66)	10.1±1.6	4.0	2.7	126±3.1	5.23
200 (233)	15.9±1.0	4.0	3.8	222±3.7	9.26
Day 7					
25 (26)	5.03±1.02	1.0	2.3	41.9±2.6	1.75
75 (84)	10.4±2.8	1.0	2.7	123±3.1	5.15
200 (280)	20.9±2.0	1.0	3.2	280±5.7	11.7

Nominal doses with measured doses in parenthesis. n=3 animals per group data show mean ± SEM. 123 male mice were assigned to four study groups: control, 25, 75 and 200 mg/kg. Data shown are plasma levels. Study was performed under contract at Wuxi.

Table S17B. Individual mouse plasma concentrations 7-day toxicology study 25 mg/kg.

Dosing Day	Nominal Dose (mg/kg)	Meas. Dose (mg/kg)	Time (h)	Plasma Concentration (μM)			Mean	SD
				Sample 1	Sample 2	Sample 3		
1	25	28.9	1	14.4	13.7	16.1	14.7	1.2
			4	11.4	11.8	14.0	12.4	1.4
			8	4.7	8.0	3.8	5.5	2.2
			24	0.02	0.02	0.02	0.02	0.00
4	25	20.2	1	20.4	15.8	19.6	18.6	2.5
7	25	20.2	1	9.0	17.4	9.8	12.1	4.6
			4	6.0	10.5	9.0	8.5	2.3
			8	5.9	5.3	2.8	4.7	1.6
			24	0.01	0.06	0.03	0.03	0.03

Note: data are shown in μM, whereas data in Table S17A are in μg/ml

Table S17C. Individual mouse plasma concentrations 7-day toxicology study 75 mg/kg.

Dosing Day	Nominal Dose (mg/kg)	Meas. Dose (mg/kg)	Time (h)	Plasma Concentration (μM)			Mean	SD
				Sample 1	Sample 2	Sample 3		
1	75	66.4	1	28.7	24.3	19.3	24.1	4.7
			4	19.7	21.3	31.7	24.2	6.5
			8	16.4	19.4	14.6	16.8	2.4
			24	0.15	0.09	0.64	0.29	0.30
4	75	71.5	1	17.5	19.2	16.8	17.8	1.2
7	75	84.0	1	25.6	28.2	21.5	25.1	3.4
			4	23.9	20.1	17.1	20.4	3.4
			8	12.9	21.1	18.0	17.3	4.1
			24	0.59	0.20	0.10	0.30	0.26

Note: data are shown in μM, whereas data in Table S17A are in μg/ml

Table S17D. Individual mouse plasma concentrations 7-day toxicology study 200 mg/kg.

Dosing Day	Nominal Dose (mg/kg)	Meas. Dose (mg/kg)	Time (h)	Plasma Concentration (μM)			Mean	SD
				Sample 1	Sample 2	Sample 3		
1	200	233	1	20.2	29.7	34.3	28.1	7.2
			4	34.3	42.4	37.9	38.2	4.1
			8	37.2	32.8	29.2	33.1	4.0
			24	1.87	0.47	3.00	1.78	1.27
4	200	289	1	32.7	35.2	21.6	29.8	7.2
7	200	280	1	41.3	51.6	57.6	50.2	8.2
			4	34.8	39.0	47.1	40.3	6.3
			8	44.9	44.2	37.7	42.3	4.0
			24	0.93	2.55	0.33	1.27	1.15

Note: data are shown in μM, whereas data in Table S17A are in μg/ml

Table S18A. Toxicokinetic data from a 10-day toxicology study in male beagle dogs.

	Dose	30 mg/kg		120 mg/kg		480 mg/kg	
		Ave Plasma Concentration µg/ml (n = 4 dogs)					
Day	**Time (h)**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1	predose	ND	ND	ND	ND	ND	ND
	1	2.28	1.10	5.27	0.889	9.34	5.52
	2	3.43	1.34	6.68	1.82	13.0	5.48
	4	5.89	2.03	10.2	2.32	17.1	3.65
	8	7.72	0.419	10.9	2.26	19.9	2.49
	24	4.69	1.25	7.89	1.83	15.1	4.19
	48	3.26	0.311	7.47	2.03	16.2	4.81
3	predose	3.40	0.464	7.22	1.49	18.3	5.98
5	predose	2.85	1.02	10.6	5.27	22.8	9.38
7	predose	2.03	1.42	12.0	6.18	22.5	11.9
9	predose	1.61	1.27	13.6	9.79	25.8	13.6
	24	2.75	1.72	16.0	10.8	29.9	14.1
	48	1.20	1.10	13.7	10.5	26.5	16.0

Dogs were dosed in the fed state at the indicated levels every other day. Nominal doses are shown. Study was performed under contract at Advinus. Data shown are plasma levels. Mean data n=4 dogs per group with standard deviation (SD).

Table S18B. Toxicokinetic parameters day 1 of toxicology study in male beagle dogs.

Dose	**C_{max} (µg/ml)**	**T_{max} (h)**	**$AUC_{0\text{-}48h}$ (µg.h/ml)**	**Cav (µg/ml)**
30 mg/kg	7.72 ± 0.419	8	232 ± 27.3	4.8 ± 0.569
120 mg/kg	10.9 ± 2.29	8	399 ± 76.4	4.15 ± 1.57
480 mg/kg	20.2 ± 2.74	8	786 ± 153	16.4 ± 3.20

Average data n=4 dogs per group showing mean ± standard deviation. Data shown are plasma levels.

Table. S19. Primary data supporting Fig. 2.

Fig. 2A

DHODH	Pfalciparum			Human			Dog			mouse			Rat			Rabbit		
ug/ml	Rate (s-1)			Rate (s-1)			Rate (s-1)			Rate (s-1)			Rate (s-1)			Rate (s-1)		
0.0000	10.3	10.4	10.0	12.6	11.9	10.8	13.5	13.7	12.6	6.2	5.5	6.0	15.5	15.4	14.9	15.9	15.2	15.1
0.0004	9.0	9.0	9.3				13.1	12.1	14.1	6.1	6.8	6.2	15.3	14.7	15.7	15.6	15.3	15.4
0.0012	7.7	8.1	8.1				14.0	12.4	13.8	6.3	6.0	6.2	14.9	14.1	16.8	15.5	15.7	14.9
0.0042	6.9	5.5	7.7				13.4	13.4	14.0	6.7	7.1	6.0	13.8	14.7	16.0	15.4	15.6	15.5
0.012	4.3	4.4	3.8	12.8	12.0	12.0	13.4	12.3	13.6	6.6	6.0	7.7	14.5	13.5	16.6	15.6	15.5	15.7
0.042	2.8	2.5	2.8	12.9	11.4	11.4	13.3	13.2	13.7	6.2	6.0	5.9	13.6	13.7	14.2	15.7	15.3	15.3
0.12	1.3	1.4	1.7	12.5	12.1	11.2	14.3	14.3	13.0	5.3	6.1	5.0	13.3	14.7	13.7	13.1	13.9	14.0
0.42	0.89	1.0	1.1	11.1	12.0	11.4	13.4	12.9	12.0	4.5	4.7	4.5	9.3	11.3	9.4	13.1	13.6	13.8
1.2	0.67	0.78	0.83	11.6	11.7	10.9	11.5	12.3	10.9	4.3	3.1	2.7	6.9	6.9	5.9	13.0	13.5	13.5
4.2	0.61	0.54	0.60	10.7	10.4	11.0	10.9	8.8	8.3	1.3	1.4	1.4	3.1	3.5	3.2	13.0	13.3	12.8
12	0.42	0.41	0.40	9.9	9.1	8.3	6.9	5.6	5.2	0.68	0.63	1.1	1.8	1.9	1.8	12.5	12.9	11.8
42	0.21	0.19	0.20	7.5	6.8	6.6	3.9	3.0	2.8	0.29	0.30	0.52	1.1	1.0	1.2	10.9	10.9	10.2

Fig. 2B P. falciparum DHODH

	DSM265			DSM450			DSM430		
ug/ml	Rate (s-1)			Rate (s-1)			Rate (s-1)		
0.0000	10.3	10.4	10.0	12.4	12.5	12.8	12.6	12.8	12.8
0.0004	9.0	9.0	9.3	12.4	12.1	12.7	11.3	12.7	12.1
0.0012	7.7	8.1	8.1	12.4	11.9	12.3	11.1	10.9	11.9
0.0042	6.9	5.5	7.7	10.7	10.9	11.3	10.0	8.3	9.4
0.012	4.3	4.4	3.8	8.86	9.8	9.7	6.7	5.5	6.8
0.042	2.8	2.5	2.8	6.48	7.5	7.1	3.4	3.4	3.8
0.12	1.3	1.4	1.7	3.99	4.1	4.5	2.9	3.1	3.1
0.42	0.89	1.0	1.1	3.73	3.4	3.4	2.3	2.4	2.6
1.2	0.67	0.78	0.83	3.14	3.4	3.0	2.2	2.2	2.2
4.2	0.61	0.54	0.60	2.94	2.8	2.9	1.7	1.6	1.5
12	0.42	0.41	0.40	2.56	2.3	2.3	0.9	0.9	0.83
42	0.21	0.19	0.20	1.42	1.3	1.4	0.5	0.4	0.44

Data for DSM265 is the same as Fig. 2A

Fig. 2C

	control	Atq	Art	Pyr	DSM265
total parasites	228	241	201	216	214
Parasitemia (%)	1.5	1.3	1.1	1.5	1.5
Rings (%)	0	0	0	0.9	10.7
YT (%)	21.1	98.8	2.5	10.2	87.9
MT(%)	78.9	0	0	88.9	0
Schizonts (%)	0	0	0	0	0
Pyknotic (%)	0	1.2	97.5	0	1.4

Fig. 2D

time (h)	DSM265 10 x IC50			DSM265 100 x IC50			Art			Ato			Pyr			Chl		
	Ave	SD	n	Ave	SD	n	Ave	SD	n	Ave	SD	n	Ave	SD	n	Ave	SD	n
0	5	0.32	4	5	0.32	4	5	0.31	4	5	0.33	4	5	0.31	4	5	0.31	4
24	5.23	0.51	4	5.1	0.31	4	0.38	0.38	4	4.77	0.53	4	4.05	0.54	4	2.87	0.46	4
48	4.43	0.27	4	4.3	0.44	4	0.19	0.1	4	4.37	0.32	4	1.9	0.31	4	0.5	0.24	4
72	2.69	0	4	2.96	0.69	4	0.14	0	4	3.44	0.31	4	0.79	0.29	4	0.06	0	4
96	1.38	0.29	4	0.94	0.56	4	0.14	0	4	1.49	0.59	4	0.24	0.12	4	0.06	0	4
120	1.01	0.29	4	0.14	0	4	0.14	0	4	0.19	0	4	0.14	0	4	0.06	0	4

Table. S20. Primary data supporting Fig. 3 (A and B).

Fig. 3A

			DSM265			Atq
Dose (µg/ml)	**DMSO**	**0.0042**	**0.042**	**0.42**	**4.2**	**0.0018**
			EEF/10K hepatocytes			
	158	127	142	133	96	120
	174	153	145	160	103	100
	180	160	150	183	90	95
Mean	171	147	146	159	96	105
SD	11	17	4	25	7	13

Fig. 3B

DSM265(µg/ml)	**mean**	**SEM**	**n**
0.0000042	100	3.85	3
0.0042	85.9	5.88	3
0.042	85.4	1.37	3
0.42	92.9	8.47	3
4.2	56.4	2.20	3

Data derived from above data for Fig. 3A

Table. S21. Primary data supporting Fig. 5.

A. Free base Suspension in 0.5% CMC/0.4% Tween 80; Fasted dogs

	Plasma Concentration (µg/ml)														
Dog #	0	0.5	1	1.5	2	3	6	9	12	24	48	72	96	120	144 h
Dog 139	0.005	0.333	0.387	0.380	0.443	0.231	0.213	0.147	0.137	0.303	0.128	0.221	0.139	0.106	0.107
Dog 140	0.000	0.154	0.255	0.401	0.440	0.414	0.124	0.141	0.123	0.292	0.027	0.020	0.006	0.003	0.002
Dog 141	0.000	0.186	0.373	0.379	0.309	0.327	0.205	0.135	0.161	0.355	0.188	0.233	0.131	0.094	0.121
Dog 142	0.002	0.203	0.314	0.294	0.368	0.188	0.244	0.234	0.191	0.259	0.058	0.014	0.004	0.000	0.000
Dog 143	0.000	0.106	0.311	0.272	0.297	0.307	0.207	0.165	0.162	0.226	0.092	0.108	0.074	0.051	0.053
Dog 144	0.000	0.255	0.358	0.287	0.379	0.237	0.171	0.149	0.162	0.343	0.126	0.104	0.068	0.028	0.028
Dog 139	0.457	0.786	1.080	1.120	0.956	0.809	0.636	0.459	0.522	0.834	0.346	0.275	0.171	0.080	0.082
Dog 140	0.000	0.343	0.740	0.584	0.495	0.348	0.165	0.124	0.195	0.129	0.010	0.005	0.000	0.000	0.000
Dog 141	0.178	0.478	0.793	0.766	0.606	0.695	0.374	0.342	0.317	0.442	0.229	0.153	0.066	0.040	0.040
Dog 142	0.000	0.374	0.452	0.405	0.337	0.170	0.172	0.130	0.149	0.076	0.011	0.003	0.000	0.000	0.000
Dog 143	0.049	0.462	0.481	0.499	0.489	0.220	0.120	0.111	0.123	0.094	0.049	0.030	0.011	0.007	0.004
Dog 144	0.002	0.326	0.454	0.348	0.361	0.535	0.225	0.231	0.207	0.122	0.023	0.013	0.000	0.000	0.000
Mean	0.058	0.334	0.500	0.478	0.457	0.373	0.238	0.197	0.204	0.290	0.107	0.098	0.056	0.034	0.036
SD	0.136	0.184	0.246	0.246	0.181	0.206	0.142	0.105	0.113	0.207	0.103	0.100	0.062	0.040	0.045

B. Free base Suspension in 0.5% CMC/0.4% Tween 80; FED dogs

5876419	0.134	0.283	0.383	0.439	0.472	0.678	1.51	1.71	1.83	1.40	0.913	0.547	0.265	0.225	0.162
1760328	0.003	0.090	0.271	0.571	0.612	0.737	0.768	0.775	0.822	0.620	0.196	0.063	0.014	0.005	0.002
5872391	0.167	0.295	0.401	0.460	0.529	0.728	1.03	1.35	1.23	1.12	0.614	0.376	0.161	0.100	0.069
1827384	0.005	0.230	0.371	0.383	0.407	0.400	0.326	0.345	0.483	0.338	0.147	0.053	0.009	0.005	0.000
5878616	0.013	0.123	0.176	0.208	0.282	0.382	0.408	0.548	0.425	0.293	0.155	0.093	0.036	0.023	0.015
5888387	0.025	0.488	0.496	0.393	0.371	0.267	0.175	0.136	0.110	0.136	0.071	0.025	0.012	0.010	0.005
Mean	0.058	0.251	0.350	0.409	0.445	0.532	0.703	0.811	0.817	0.651	0.349	0.193	0.083	0.061	0.042
SD	0.073	0.143	0.111	0.119	0.118	0.206	0.504	0.607	0.626	0.505	0.337	0.216	0.107	0.088	0.064

C. SDD: 25% DSM265: HPMCAS-M; suspension in Methocel A4M); fasted

Dog 139	0.014	8.86	7.65	5.94	5.30	4.35	3.50	2.07	1.55	3.73	2.26	3.02	1.270	0.904	0.407
Dog 140	0.013	4.37	7.75	8.07	5.13	2.30	1.98	1.60	1.70	2.25	0.63	0.09	0.006	0.001	0.000
Dog 141	0.082	1.46	4.32	3.85	2.29	2.53	1.95	1.43	1.32	3.06	1.56	1.83	0.968	0.523	0.414
Dog 142	0.021	1.02	4.05	5.74	4.66	2.55	2.99	3.44	2.60	2.81	1.15	0.48	0.036	0.006	0.001
Dog 143	0.007	4.04	5.77	4.35	3.46	1.87	1.75	1.32	1.25	1.12	0.82	1.12	0.410	0.169	0.068
Dog 144	0.010	2.56	4.82	2.70	2.48	2.88	1.80	1.45	1.68	2.02	0.51	0.23	0.084	0.025	0.006
Mean	0.024	3.72	5.73	5.11	3.89	2.75	2.33	1.89	1.68	2.50	1.16	1.13	0.462	0.271	0.149
SD	0.028	2.85	1.64	1.89	1.33	0.85	0.73	0.81	0.49	0.91	0.66	1.13	0.537	0.368	0.204

D. SDD: 25% DSM265: HPMCAS-M; suspension in Methocel A4M); FED dogs

5876419	0.630	7.01	5.79	4.60	4.86	4.60	4.78	6.07	7.67	4.52	5.38	3.40	1.72	1.21	0.645
1760328	0.018	8.26	10.90	10.70	9.51	7.22	7.22	6.86	5.07	3.88	2.18	0.644	0.136	0.021	0.007
5872391	0.260	10.40	8.47	6.67	6.34	5.63	4.88	5.35	5.92	4.50	2.62	1.08	0.605	0.305	0.129
1827384	0.066	13.70	11.10	8.12	7.20	5.91	5.22	5.00	5.77	3.76	1.89	0.602	0.101	0.022	0.009
5878616	0.052	5.39	7.42	6.87	6.02	5.06	3.32	2.67	3.39	2.45	1.42	0.520	0.266	0.121	0.047
5888387	0.044	2.93	3.13	3.19	4.31	7.13	5.65	4.10	2.92	2.05	0.88	0.159	0.038	0.013	0.006
Mean	0.178	7.95	7.80	6.69	6.37	5.92	5.18	5.01	5.12	3.53	2.40	1.07	0.478	0.282	0.140
SD	0.238	3.79	3.06	2.64	1.85	1.07	1.27	1.48	1.76	1.04	1.58	1.18	0.641	0.468	0.252