Supporting Information for:

Discovery of Potent and Fast-Acting Antimalarial Bis-1,2,4-Triazines

Daniel L. Priebbenow[†], Mitch Mathiew[‡], Da-Hua Shi[‡], Jitendra R. Harjani[‡], Julia G. Beveridge[‡], Marina Chavchich[ǂ], Michael D. Edstein[ǂ], Sandra Duffy[ʊ], Vicky M. Avery[ʊ], Robert T. Jacobs[○], Stephen Brand[○], David M. Shackleford[£], Wen Wang[£], Longjin Zhong[£], Given Lee[£], Erin Tay[£], Helena Barker[£], Elly Crighton[£], Karen L. White[£], Susan A. Charman[£], Amanda De Paoli[¥], Darren J. Creek[¥], Jonathan B. Baell[‡§]*

[†]School of Chemistry, The University of Melbourne, Parkville VIC, 3010, Australia

[‡]Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University (Parkville Campus), 381 Royal Parade, Parkville, VIC 3052, Australia

[ǂ] The Department of Drug Evaluation, Australian Defence Force Malaria and Infectious Disease Institute, Brisbane, QLD 4051, Australia

[ʊ]Discovery Biology, Griffith University, Nathan QLD 4111, Australia

[○]Medicines for Malaria Venture, CH-1215 Geneva, Switzerland.

[£]Centre for Drug Candidate Optimisation, Monash Institute of Pharmaceutical Sciences, Monash University (Parkville Campus), 381 Royal Parade, Parkville, VIC 3052, Australia

[¥] Drug Delivery Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University (Parkville Campus), 381 Royal Parade, Parkville, VIC 3052, Australia

[§]ARC Centre for Fragment-Based Design, Monash University (Parkville Campus), 381 Royal Parade, Parkville, VIC 3052, Australia

Email**:** jonathan.baell@monash.edu

Table of Contents:

Methodology **S2**

References **S7**

METHODOLOGY

In vitro Parasitology

Testing against the asexual blood stages of *P. falciparum* 3D7 *in vitro* was undertaken using our well validated assay, as previously reported.[S1]

Stability and Metabolite ID in Liver Microsomes and Cryopreserved Hepatocytes

Hepatic microsomes (Sekisui XenoTech, LLC) were suspended in pH 7.4 buffer (0.4 mg/mL microsomal protein), and the metabolic process initiated by addition of the NADPH-regenerating buffer system. Substrate depletion, and in some cases metabolite formation, was monitored using a Waters Xevo G2 QTOF MS coupled to a Waters Acquity UPLC. For metabolite identification, separate incubations were conducted using modified conditions (2 mg/mL microsomal protein and 50 µM substrate), and LC-MS analysis included MS/MS (CID) spectral analysis.

Cryopreserved hepatocytes (Sekisui XenoTech, LLC) were first purified by centrifugation using a Percoll gradient and cell viability assessed by trypan blue exclusion. Cells were resuspended in Krebs-Henseleit buffer at a concentration of 0.5 x 10^6 viable cells/mL, spiked with compound, and incubated at 37°C in a humidified incubator with 7.5% CO_2 for 4 hours. Substrate depletion was monitored and analyzed as described for microsomes. Validation studies confirmed that pH 7.4 and cell viability were both maintained throughout the incubation period.

The first-order degradation rate constant for substrate loss in both microsomes and hepatocytes was used to determine the *in vitro* intrinsic clearance value (CL_{int}) in each test system.[S2] To compare the data from microsomes and hepatocytes, the CL_{int} from each *in vitro* test system was scaled using published scaling factors[S3] and corrected for the fraction unbound ($f_{u,inc}$) to the *in vitro* test systems[S4,S5] to obtain predicted *in vivo* unbound intrinsic clearance values ($CL_{int,unb}$, mL/min/kg).

and

$$f_{u,inc} \text{ hepatocytes} = 1 / (C_{hep} \times 10^{(0.40 \times \text{LogP/D} - 1.38)} + 1)$$

$$f_{u,inc} \text{ microsomes} = 1/(C_{mic} \times 10^{(0.56 \times \text{LogP/D} - 1.41)} + 1)$$

where C_{hep} and C_{mic} are the total concentrations of hepatocytes (10^6 cells/mL) and microsomes (mg/mL) present in the incubations. Log P is used for bases with pKa > 7 and neutral compounds, whereas Log $D_{7.4}$ is used for acids with pKa < 5.5.

The unbound *in vivo* intrinsic clearance was then calculated by:

$$\textit{in vivo } CL_{int,unb} \text{ (mL/min/kg)} = CL_{int,\text{in vitro}} /1000 * \text{PBSF} / f_{u,inc}$$

where $CL_{int,\text{in vitro}}$ is µL/min/10^6 cells or µL/min /mg protein from the *in vitro* assay, 1000 µL/mL is a unit conversion, and PBSF is the physiologically-based scaling factor (10^6 cells/kg or mg protein[S3]).

Plasma Protein Binding

Protein binding was determined via rapid equilibrium dialysis (RED), using a method based on that reported previously[S6]. Rat plasma was spiked with **23** and dialysed for 6 h against pH 7.4 phosphate-buffered saline (100 mM sodium phosphate and 40 mM NaCl). Concentrations of **23** in dialysate (C_u) and donor (C_T) samples at the end of the dialysis period were determined via LC-MS, and the fraction unbound was calculated ($f_u = C_u / C_T$) assuming that the system was at distributional steady state by 6 h.

Blood Stability

Volumes of freshly collected whole blood from a male Sprague Dawley rat and a male Swiss mouse were spiked with **23,** briefly mixed and dispensed into aliquots that were maintained at 37°C under a 5% CO_2 atmosphere over the course of 240 min incubation period. At designated time points, samples (n=3 per species) were snap-frozen on dry ice and stored frozen for analysis by LC-MS.

Caco-2 Permeability

Permeability of **23** across Caco-2 monolayers was assessed using the procedure described previously[S7], with pH 7.4 Hanks balanced salt solution containing 20 mM HEPES in both the apical and basolateral chambers. Compound flux was assessed over a period of 120 minutes, with samples taken from the acceptor chamber at multiple time points. At each sample time, the volume of acceptor solution removed was replaced with blank transport buffer and acceptor concentrations were corrected for the dilution that occurred with buffer replacement in the data analysis. Samples from the donor chamber were taken at the start and end of the experiment. All samples were stored frozen at -80°C until analysis by LC-MS.

Functional integrity of the monolayer and activity of P-gp/MRP-mediated efflux was confirmed on the basis of TEER values and permeability data for marker compounds (lucifer yellow, naproxen and rhodamine 123). Apparent permeability coefficients (P_{app}) values and efflux ratio were calculated as described previously[S7].

Cytochrome P450 Inhibition

Cytochrome (CYP) P450 inhibition studies were conducted using human liver microsomes with a substrate-specific interaction approach which relies on the formation of a metabolite that is mediated by a specific CYP isoform. The assay conditions employed for each CYP isoform were based on those reported previously.[S8]

Pharmacokinetic Properties of 23 in Mice and Rats

In vivo pharmacokinetic studies were performed in accordance with Australian National Health and Medical Research Council Guidelines, with all procedures approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee.

The pharmacokinetic properties of **23** were assessed using male Swiss outbred mice (24−33 g) that had full access to food and water before dosing and during the sampling period. Compound **23** was administered by a bolus IV injection into the tail vein at a dose of 2.7 mg kg^{-1} or orally by gavage at doses of 2.8 and 27 mg kg^{-1}. Groups of 12 (IV) or 9 (PO) mice were used for each dose group, with 3 samples taken by submandibular bleed from each mouse and 3 mice used for each time point. Blood samples were collected into tubes containing heparin and a stabilization cocktail for up to 24 h post dose. Samples were centrifuged for the collection of plasma which was stored at -20°C until analysis.

The pharmacokinetic properties of **23** were also assessed using male Sprague Dawley rats (283−298 g). Surgeries for the insertion of cannulas in the carotid artery (for blood sampling) and jugular vein (for IV administration only) were performed under general anaesthesia on the day prior to dosing. Rats had access to food immediately post-surgery, and were fasted overnight prior to dosing, and access to food was reinstated 4-hours post-dose. Water was available ad libitum at all times. Compound **23** was administered intravenously as a 10-minute constant rate infusion at a dose of 3 mg kg^{-1} or orally by gavage at a dose of 10 mg kg^{-1}. Blood samples were collected (via a Culex Automated Blood Sampler into heparinised tubes) up to 24 h post dose, and these were centrifuged for the collection of plasma which was stored at -20°C until analysis. The concentration of **23** in blood was also determined in a sample collected from each rat at 1 h after IV administration for the purposes of determining the whole blood to plasma partitioning ratio.

PK Studies Following ABT Pre-dosing to Rats

To evaluate the contribution of CYP450-mediated metabolism to the *in vivo* elimination of **23**, the systemic clearance of **23** (3 mg/kg) was measured in rats (n=3) that were pre-dosed with the pan-CYP inhibitor, 1-aminbenzotriazole (ABT).[S9] All procedures were as described above for rat pharmacokinetic studies, with the exception that 100 mg/kg of ABT (as a solution in 0.9% saline; 10 mL/kg) was administered orally by gavage 2 hours prior to commencing the IV infusion of **23**.

Bile Cannulated Studies in Rats

To evaluate whether direct excretion into bile was a significant *in vivo* clearance pathway for **23**, the fraction of a 3 mg/kg IV dose recovered in bile was determined in anesthetised bile-cannulated rats (n=3). All procedures were as defined above for rat pharmacokinetic studies, with the exception that on the day of dosing, rats were anaesthetised (inhaled isoflurane) and had a cannula inserted into the bile duct prior to IV infusion of **23**. Animals remained anaesthetised for the remainder of the experiment, and intermittent plasma samples and total bile were collected up to 4 hours post-dose.

LC-MS analysis of in vivo samples

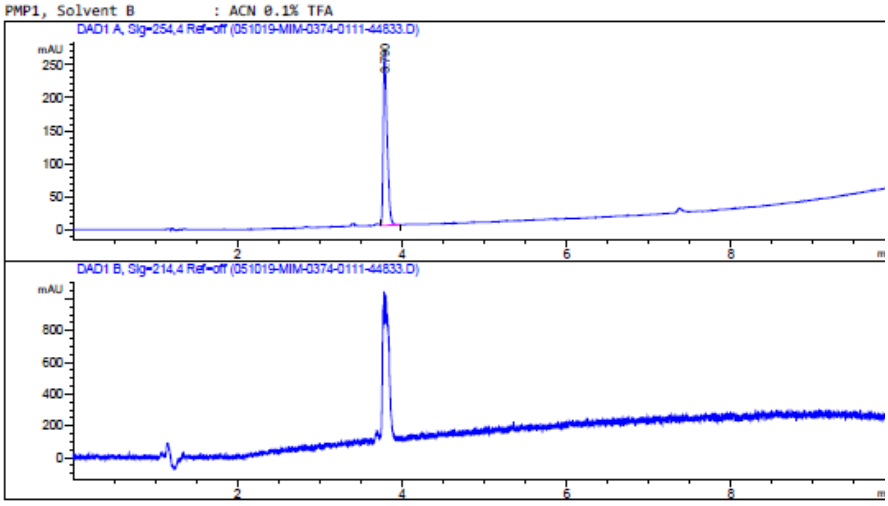
Concentrations of **23** in samples collected during *in vivo* PK studies (plasma, blood, urine, bile) were measured using a Waters Xevo TQS Micro mass spectrometer coupled to a Waters Acquity UPLC. Analyte detection conducted was in positive electrospray ionisation multiple-reaction monitoring mode (transition 306.20>71.96; cone voltage 25 V; CID 25V), and chromatographic separation was accomplished using a Supelco Ascentic Express RP amide column (50x2.1 mm, 2.7 µm) with an acetonitrile-water gradient containing 0.05% formic acid. Quantitation was conducted by comparing the response for **23** in samples was a set of calibration standards prepared by spiking solvent standard solutions into the same blank matrix as the samples. Sample processing was via protein precipitation with acetonitrile (2:1 volume ratio for plasma, blood and faeces) or dilution with 50/50 acetonitrile/water (for urine and bile). Assay performance was confirmed by the assessment of linearity, accuracy and precision for each run.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) to Assess Cytotoxicity.

Human embryonic kidney 293 (HEK293) cells were maintained in complete DMEM medium (DMEM containing 2mM GlutaMAX, supplemented with 10% Foetal Bovine Serum and 1% penicillin-streptomycin antibiotics). 24 hours prior to compound exposure, HEK293 cells were seeded ($8x10^4$ cells/well) into flat bottom 96-well plates (Corning) and incubated at 37 °C, 5% CO_2. Serial dilutions of test compounds ranging from 1mM - 3.9 µM were incubated at standard conditions for 24 hours. After compound exposure, the media was removed from the wells and replaced with an equal volume of MTT media (complete DMEM medium containing 0.5mg/ml MTT, Sigma). Plates were incubated for an additional 3-4 hrs at standard conditions, after which MTT media was removed and an equal volume of DMSO added to dissolve formazan crystals. The total absorbance was measured at 570 nm (EnSight plate reader, PerkinElmer). Raw data was blank adjusted, normalised to DMSO control to obtain % viability data and IC_{50} values were calculated by via a 4-parameter logistic curve fitting in GraphPad Prism. Data is representative of 3 biological replicates with 3 technical repeats.

HPLC Trace of Compound 23

```
================================================================
Acq. Operator   :
Acq. Instrument : 1260ANALYTICAL                Location : P2-F-08
Injection Date  : 5/10/2019 7:46:11 PM
                                                Inj Volume : 2.000 µl
Method          : C:\CHEM32\1\METHODS\ROUTINE_PEAK PURITY GRAD METHOD#1.M
Last changed    : 5/10/2019 7:45:31 PM
                  (modified after loading)
Method Info     : Standard Peak Purity Gradient Method for Monash Agilent Analytical

Sample Info     : Easy-Access Method: 'PP Gradient'

================================================================

Instrument Conditions   :    At Start          At Stop
Column Temp. (left)     :      35.0             35.0   °C
Column Temp. (right)    :      35.0             35.0   °C
Pressure                :     187.1             83.1   bar
Flow                    :       1.000            1.000 ml/min

Detector Lamp Burn Times: Current On-Time  Accumulated On-Time
DAD 1, UV Lamp          :       6.42          5678.4   h

Solvent Description     :
PMP1, Solvent A         : Water 0.1% TFA
PMP1, Solvent B         : ACN 0.1% TFA
```




```
================================================================
                      Area Percent Report
================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Do not use Multiplier & Dilution Factor with ISTDs


Signal 1: DAD1 A, Sig=254,4 Ref=off

Peak RetTime Type  Width     Area      Height     Area
  #   [min]       [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1   3.790 VB    0.0484  863.09167  262.35428 100.0000

Totals :                   863.09167  262.35428
```

REFERENCES

S1 Duffy, S. Avery, VM, Development and optimization of a novel 384-well anti-malarial imaging assay validated for high-throughput screening. *Am J Trop Med Hyg* **2012**, *86*, 84-92

S2 Obach RS. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab. Dispos.* **1999**, 27**:**1350-1359.

S3 Ring et al., PHRMA CPCDC initiative on predictive models of human pharmacokinetics, part 3: comparative assessment of prediction methods of human clearance. *J. Pharm. Sci.*, **2011**, *100*, 4090-4110.

S4 Austin RP, Barton P, Cockroft SL, Wenlock MC and Riley RJ. The Influence of Nonspecific Microsomal Binding on Apparent Intrinsic Clearance and Its Prediction from Physicochemical Properties. *Drug Metab. Dispos.* **2002**; *30*,1497-1503.

S5 Austin RP, Barton P, Sarfaz M and Riley RJ. The binding of drugs to hepatocytes and its relationship to physicochemical properties. *Drug Metab. Dispos.* **2005**, *33*, 419-425.

S6 Curran RE, Claxton CRJ, Hutchinson L, Harradine PJ, Martin IJ and Littlewood P. Control and Measurement of Plasma pH in Equilibrium Dialysis: Influence on Drug Plasma Protein Binding. *Drug Metab. Dispos.* **2011**, *39*, 551-557.

S7 Katneni K, Pham T, Saunders J, Chen G, Patil R, White KL, Abla N, Chiu FCK, Shackleford DM and Charman SA. Using Human Plasma as an Assay Medium in Caco-2 Studies Improves Mass Balance for Lipophilic Compounds. *Pharm. Res.* **2018**, *35*, 210-223.

S8 Walsky RL and Obach RS. Validated assays for human cytochrome P450 activities. *Drug Metab. Dispos.* **2004**, *32*, 647-660.

S9 El-Kattan AF, Poe J, Buchholz L, Thomas HV, Brodfuehrer J and Clark A. The use of 1-Aminobenzotriazole in Differentiating the Role of CYP-Mediated First Pass Metabolism and Absorption in Limiting Drug Oral Bioavailability: A Case Study. *Drug Metab. Letters* **2008**, *2*, 120-124