Supporting Information

The Discovery of Novel Antimalarial Aminooxadiazoles as a Promising Non-endoperoxide Scaffold

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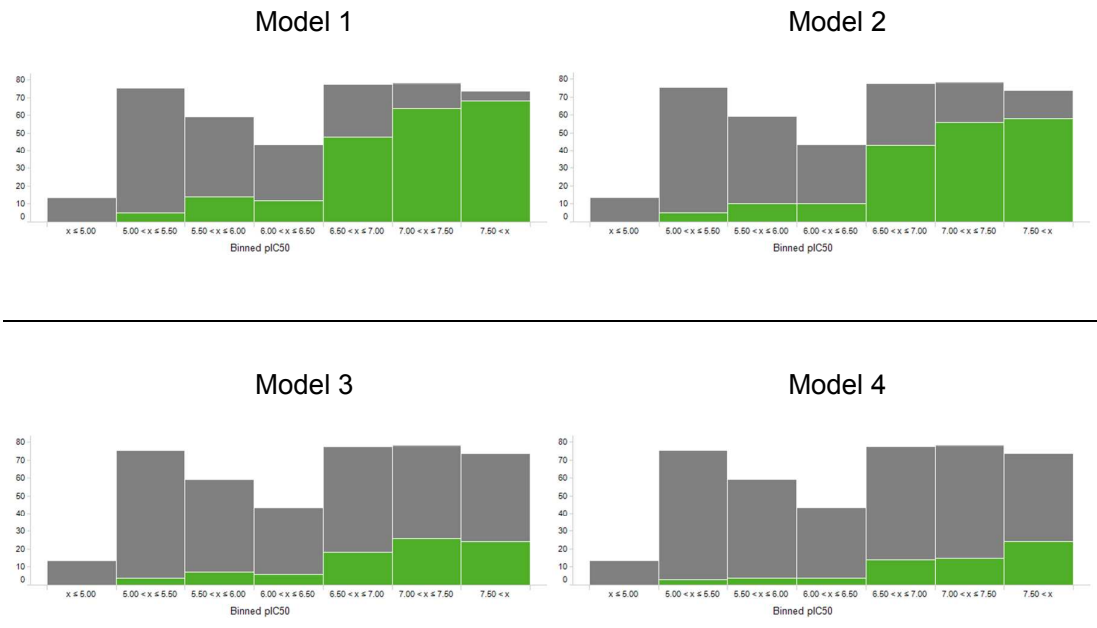
Pharmacophore modeling.

Table SX1. Number out of 418 project compounds hitting each of pharmacophore models 1-8, and the number overlapping between each. Models 1, 2, 5 and 8 had the highest hit-rates and the greatest overlap between them.

Pharmacophore #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1	211	167	76	61	200	111	64	198
2	x	182	60	50	166	90	56	177
3	x	x	85	54	79	55	58	80
4	x	x	x	64	63	38	53	62
5	x	x	x	x	206	112	65	201
6	x	x	x	x	x	120	43	64
7	x	x	x	x	x	x	69	68
8	x	x	x	x	x	x	x	217

Figure SX1. Enrichment performance of pharmacophore models 1-8 against 418 project compounds, binned by activity (pIC50). The proportion of compounds matching the model are colored green.



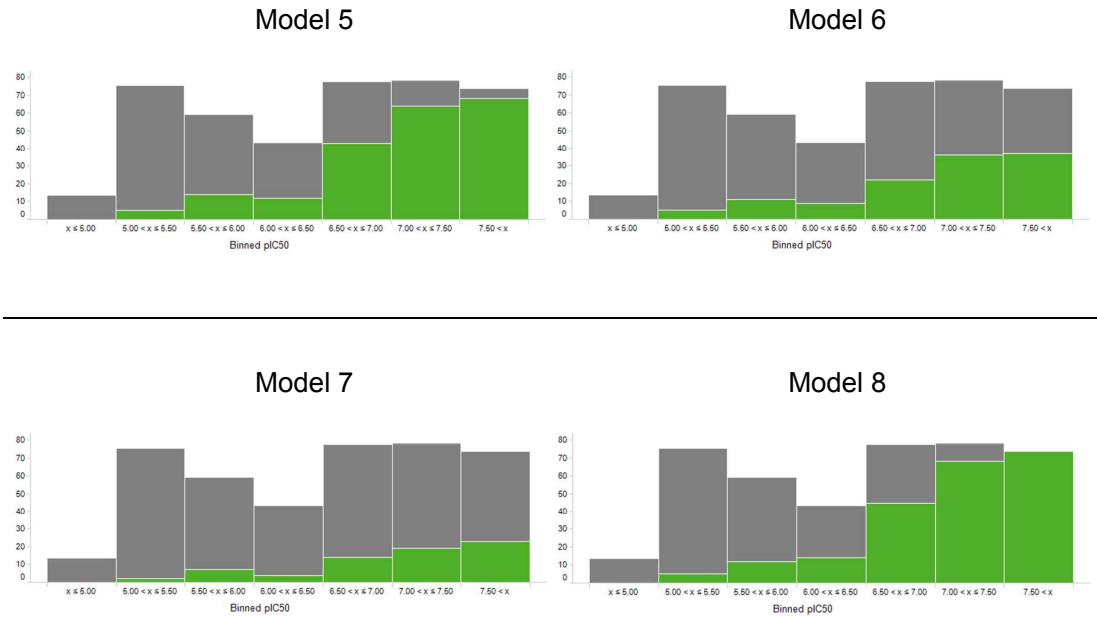
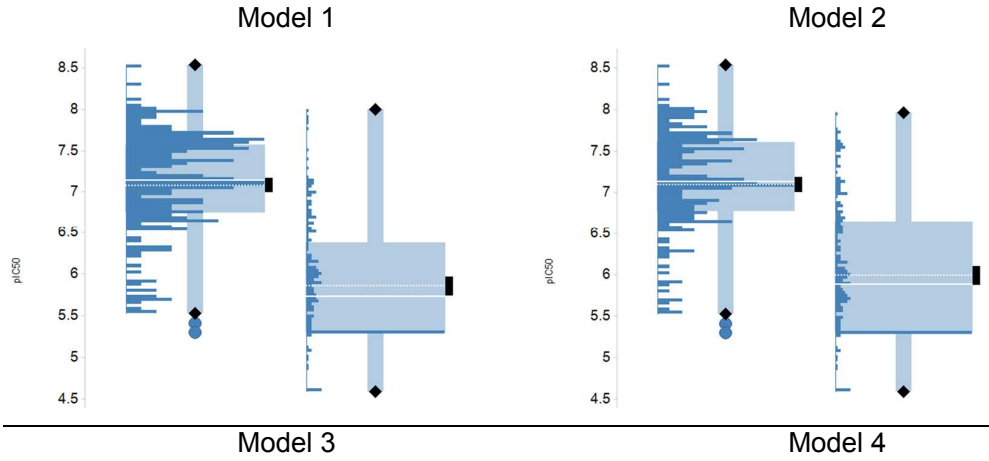
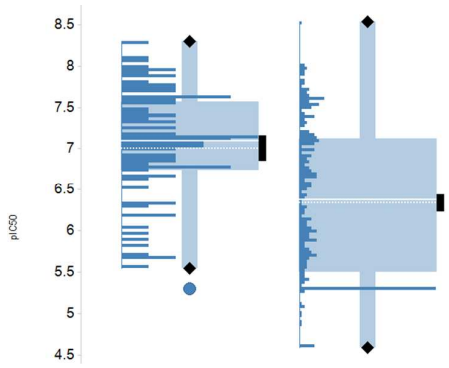
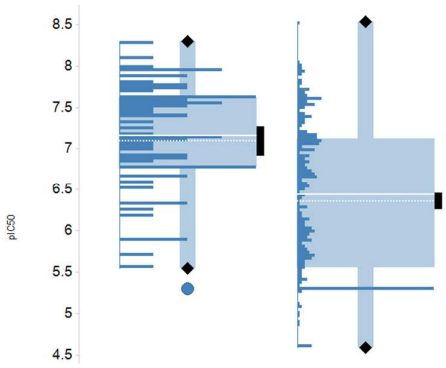


Figure SX2. Box plots showing enrichment performance of pharmacophore models 1-8 against 418 project compounds. For each, the pIC_{50} of compounds matching the pharmacophore (left) is compared to compounds not matching the pharmacophore (right). Interquartile ranges are shaded pale blue.

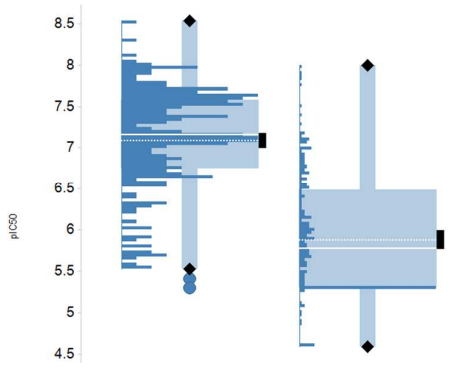


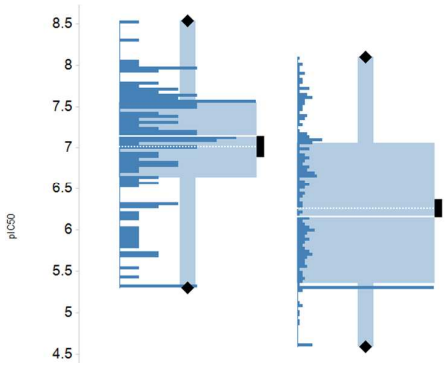




Model 5

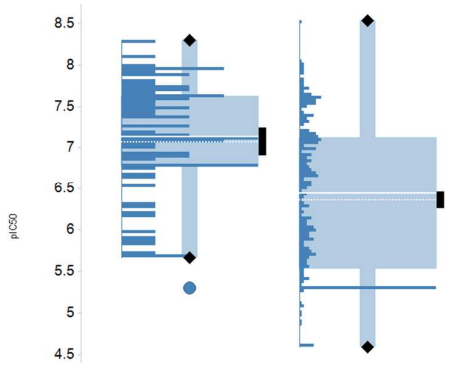
Model 6





Model 7

Model 8



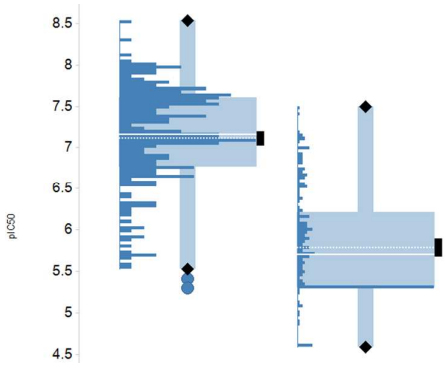
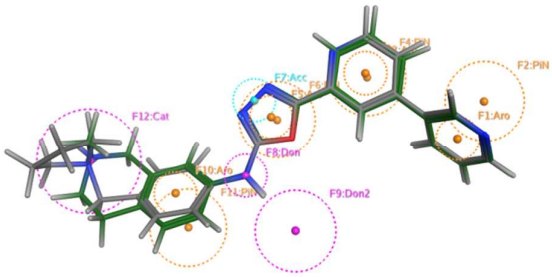
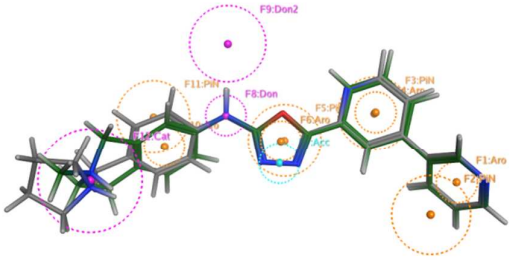


Figure SX3. Pharmacophore models 1, 2, 5 and 8.

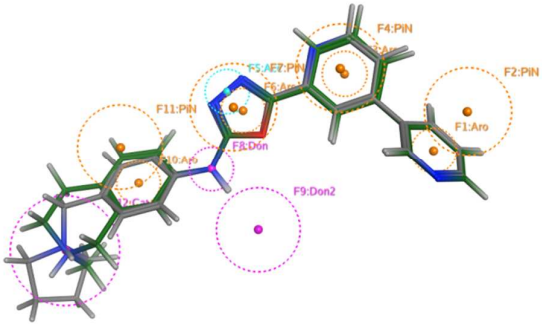
Model 1



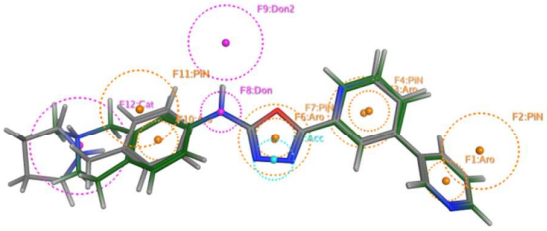
Model 2



Model 5



Model 8



SI Pharmacophore models 1, 2, 5 and 8.

This file contains four pharmacophore models. To import them into MOE (Chemical Computing Group) paste the sections below between the blank lines into four separate files with .ph4 extensions. Do not include the blank lines. Each .ph4 file should begin with #moe and end with #endpharmacophore

```
#moe:ph4que 2014.09
#pharmacophore 7 tag t value *
scheme t PCHD matchsize i 11 use_Hs i 1 abspos i 0 title t Model_1
useRval i 0 comment s $
#feature 12 expr tt color ix x r y r z r r r ebits ix gbits ix m ix
Aro ff8000 -3.11441666666667 7.18883333333333 3.91 0.8 0 400 a64cff PiN
ff8000 -2.51394917112188 9.1841397779672 3.65114507387556 1.5 0 400
a64cff Aro ff8000 -1.29975 6.15175 0.22825 0.8 0 400 a64cff PiN ff8000
0.557022818062353 6.84054870537678 0.926115552319014 1.5 0 400 a64cff
Aro ff8000 0.2063 2.5531 -0.2697 0.8 0 400 a64cff PiN ff8000
2.05826650191044 3.22468223353045 0.456513505762531 1.5 0 400 a64cff Acc
df2f2 0.5635 2.4625 -1.098 0.8 0 400 a64cff Don f20df2 0.439 0.497 1.024
0.8 0 400 a64cff Don2 f20df2 -1.03974804182032 0.547222840063573
3.47649309940549 1.5 0 400 a64cff Aro ff8000 1.5475 -1.91341666666667
0.18375 0.8 0 400 a64cff PiN ff8000 2.71459862778394 -2.0664825837996
1.90764889404963 1.5 0 400 a64cff Cat f20df2 3.4055 -3.6155 -2.444 2 0
400 a64cff
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```

```
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useRval i 0 comment s $
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PiN ff8000 -10.666421982215 -1.30649082344579 3.66709561716505 1.5 0 400
a64cff PiN ff8000 -8.25253698127028 3.53183180934657 5.58864364883265
1.5 0 400 a64cff Aro ff8000 -8.12183333333333 2.68608333333333
3.67366666666667 0.8 0 400 a64cff PiN ff8000 -4.74974380185889
2.12495240277575 6.53257477445661 1.5 0 400 a64cff Aro ff8000 -4.584
1.2528 4.63 0.8 0 400 a64cff Acc df2f2 -4.6575 0.433 4.994 0.8 0 400
a64cff Don f20df2 -2.252 1.96 4.498 0.8 0 400 a64cff Don2 f20df2
-1.92252579812214 4.38633845274774 3.01538385767363 1.5 0 400 a64cff Aro
ff8000 0.0101666666666667 0.5435 5.27666666666667 0.8 0 400 a64cff PiN
ff8000 0.783171999060945 2.19877592006825 6.28038373851213 1.5 0 400
a64cff Cat f20df2 2.4775 -1.7445 4.268 2 0 400 a64cff
#endpharmacophore

#moe:ph4que 2014.09
#pharmacophore 7 tag t value *
scheme t PCHD matchsize i 11 use_Hs i 1 abspos i 0 title t Model_5
useRval i 0 comment s $
#feature 12 expr tt color ix x r y r z r r r ebits ix gbits ix m ix
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a64cff PiN ff8000 4.2337084541711 -1.74709167318865 9.73792184585724 1.5
0 400 a64cff Aro ff8000 4.74183333333333 -3.5285 5.38291666666667 0.8 0
400 a64cff PiN ff8000 6.5039364164131 -2.43338594059297 5.70655762183108
1.5 0 400 a64cff Acc df2f2 5.1565 -2.94725 1.102 0.8 0 400 a64cff Aro
ff8000 4.6974 -2.3661 1.6241 0.8 0 400 a64cff PiN ff8000
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f20df2 3.78 -0.521 0.3135 0.8 0 400 a64cff Don2 f20df2 2.6642870902704
1.52889791494902 1.97379934735808 1.5 0 400 a64cff Aro ff8000
3.97458333333334 -0.024916666666667 -2.42308333333333 0.8 0 400 a64cff
PiN ff8000 6.05500531988829 -0.270752985706565 -2.33134676276119 1.5 0
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useRval i 0 comment s $
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1.5 0 400 a64cff Acc df2f2 -2.71775 -3.019 2.83925 0.8 0 400 a64cff Aro
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Don f20df2 -5.435 -2.166 2.299 0.8 0 400 a64cff Don2 f20df2
-7.83147820233506 -3.7334027943045 2.23340761512485 1.5 0 400 a64cff Aro
ff8000 -5.67416666666667 0.609249999999999 2.201 0.8 0 400 a64cff PiN
ff8000 -6.63700376670726 0.493521231492626 0.344482843200456 1.5 0 400
a64cff Cat f20df2 -7.418 3.6365 3.2025 2 0 400 a64cff
#endpharmacophore
```

***In vitro* whole cell drug susceptibility studies**

Plasmodium falciparum 3D7A, Dd2, V1/S, TM90C2A, TM90C2B, W2, HB3, K1, NF54, T9/94 strains (from the Malaria Research and Reference Reagent Resource Center MR4) were grown in complete medium (RPMI 1640 (Sigma) 25 mM HEPES and NaHCO3) supplemented with 2 gr/L D-sucrose, 0.3 gr/L L-glutamine and 0.150 mM hypoxanthine and with 5 gr/L AlbuMAX II.

Parasitized red blood cells (RBC) with 3D7A P. falciparum strain, (0.5% parasitemia, 2% hematocrit) in RPMI-1640, 5% AlbuMAX and 5 µM hypoxanthine was exposed to 3-fold serial dilutions of the compounds (9 serial dilutions 5 µM as maximal concentration). Plates were incubated 24 hours at 37ºC, 5% CO2, 5%O2, 90% N2. After 24 hours of incubation, 3H-hypoxanthine (0.2 µCi to each well, from a stock solution of 3H-hypoxanthine of 0.025 µCi/µL in RPMI-1640) was added and plates were incubated for another 24h period. After that, parasites are harvested on a glass fiber filter using a TOMTEC Cell harvester 96. Filters were dried and melt-on scintillator sheets are used to determine the incorporation of 3H-hypoxanthine. Radioactivity was measured using a microbeta counter. Data were normalized using the incorporation of the positive control (parasitized red blood cells without drug). IC50'swere determined using Grafit 7 program.

Assessment of* in vitro *frequency of spontaneous resistance and characterization of resistant mutants

A pool of parasitized RBC with W2/3D7A P. falciparum strains were grown in complete media supplemented with AlbuMAX and 150 µM hypoxanthine at 37ºC, 5% CO2, 5% O2, 90% N2 to reach a 4% parasitemia at 5% hematocrit. Then, different amounts of parasites were incubated in independent T-flasks at a concentration of 10 times the IC50 (10x IC50) of each compound, using four replicates. Two internal controls were used, heteroaryl-carboxamide GSK2645947A [**Error! Reference source not found.**, **Error! Reference source not found.**] and Atovaquone GR151218X.

In vivo *efficacy in* P. falciparum *mouse model*

Efficacy is assessed by administering one oral dose of different dose levels of compounds for four consecutive days and measuring their effect on blood parasitemia by flow cytometry. A qualitative analysis of the effect of treatment on P. falciparum $Pf3D7^{0087/N9}$ is assessed by microscopy and flow cytometry. Fresh samples of peripheral blood from P. falciparum-infected mice are stained with TER-119-Phycoerythrine (marker of murine erythrocytes) and SYTO-16 (nucleic acid dye) and then analyzed by flow cytometry (FACSCalibur, BD). Microscopy analysis is performed with Giemsa-stained blood smears from samples taken at days 5 and 7 (48 and 96 hours after starting treatment, respectively).

The parameters of efficacy estimated in the study are a) the dose in mg·kg-1 that reduces parasitemia at day 7 after infection by 90% (infective dose is 20x106 parasitized erythrocytes) with respect to vehicle-treated mice (parameter denoted as ED90) and b) the estimated average daily exposure in whole blood of the different compounds necessary to reduce *P. falciparum* parasitemia in peripheral blood at day 7 after infection by 90% with respect to vehicle-treated mice (parameter denoted as AUC_{ED90}).

The levels of compound in blood upon oral administration are measured in serial blood samples obtained during the 23 hours period after the first dose administration in all mice of the efficacy experiment. The area under the curve (AUC0-23h) of levels of compound obtained for each mice are used to estimate the potency of each compound.

hERG inhibition determination and cell cytotoxicity assays.

hERG Qpatch assay described in literature.[S1–S4] Cell cytotoxicity assays, described in literature.[S5]

(S1) Schmalhofer, W., et al., "A pharmacologically validated, high-capacity, functional Thallium flux assay for the human ether-a-go-go related gene potassium channel", Assay and Drug Development Technologies, December 2010, volume 8, number 6, pp. 714-726.

(S2) W.P. Janzen, P. Bernasconi (eds.), High Throughput Screening, Methods and Protocols, Second Edition,vol. 565, Humana Press, a part of Springer Science Business Media, LLC 2009, DOI 10.1007/978-1-60327-258-2_10, Springerprotocols.com.(see Chapter 10, "Automated Patch Clamping Using the QPatch", Kenneth A. Jones, Nicoletta Garbati,Hong Zhang, and Charles H. Large, pp. 209-223, authors formerly of GSK-Verona).

(S3) Mathes, C., et al., "QPatch: The Missing Link Between HTS and Ion Channel Drug Discovery", Combinatorial Chemistry & High Throughput Screening, 2009, 12, 78-95.

(S4) Kenneth A. Jones, Nicoletta Garbati, Hong Zhang, and Charles H. Large, "Automated Patch Clamping Using the QPatch", Chapter 10 of W.P. Janzen, P. Bernasconi (eds.), High Throughput Screening, Methods and Protocols, Second Edition, vol. 565, Humana Press, DOI 10.1007/978-1-60327-258-2_10, pp. 209 -223.

(S5) Crouch, S. P. M.; Kozlowski, R.; Slater, K. J.; Fletcher, J. The Use of ATP Bioluminescence as a Measure of Cell Proliferation and Cytotoxicity. J. Immunol. Methods 1993, 160, 81–88.

Tolerability assay.

The aim of this study is to establish a tolerable estimated systemic exposure after a single administration of compound to mice. Since this is a screening format assay, devised for compounds comparison purposes only, no formal conclusions about safety, neither about PK profile, should be drawn.

Modification of endpoints for calculation of maximal non lethal dose not only based in live/death (adverse signs) and additional plasma exposure levels form same animals (same animals are used for exposure levels, hematology and clinical chemistry).
Animals are dosed at one time according Up-&-Down Method (ref), starting at the best estimate of maximal non lethal dose (Limit dose). Irwin test (behavioral assessment, record adverse effects, bodyweight) during 4 first hours and 8 and 24h post-administration. The dose for each successive animal is adjusted up or down, depending on the outcome of the

previous animal. If no adverse effect is recorded, after 24h two additional animals are administered at same dose. After reaching adverse effect or death a decreasing dose is required until adverse effect disappears.

According to Up-&-Down statistical procedure, two dose levels are determined; a dose that causes signs of toxicity to 5% of animals (SD05) and a higher one that causes signs of toxicity at 95% of animals (SD95).

(S6) Serial vs Sparse sampling in toxicokinetic studies. Francis LS Tse; Jerry R Nedelman. Pharmaceutical research 13(7):1996
(S7) An Up-and-Down procedure for acute toxicity testing. Robert D Bruce. Toxicol. Sci. (1985) 5(1): 151-157

Blood samples for estimation of systemic exposure are taken from tail vein from treated groups following a sparse sampling schedule. Blood is sampled for clinical chemistry and hematology

Pharmacokinetic studies.

Female CD-1 mice were used for single dose PK studies. Dosing solutions were prepared in 20% Encapsin in saline solution and 1% methylcellulose, for intravenous and oral gavage administration, respectively. An IV dose of 1 mg/kg (dose volume of 10 ml/kg), and a PO dose of 10 mg/kg (dose volume of 20 ml/kg) were administered. After intravenous dosing, blood samples (25 mL) were collected at 5, 10, 15, 30 minutes, 1, 3, 6, 8 and 24 h post dose and after oral dosing, sampling schedule was the following 15, and 30 minutes, 1, 2, 4, 6, 8 and 24 h post dose. All the blood samples were diluted with 25 µL of an aqueous solution of saponine 1% (w:v), and stored at -80ºC until analysis.

Mice blood samples were analyzed for each compound using a method upon protein precipitation followed by LC-MS/MS analysis. Pharmacokinetic analysis of the concentration time profiles were performed by noncompartmental methods by using WinNonLin Phoenix Version 6.3.

Intrinsic Clearance (Cli) Assay

Intrinsic clearance (CLi) values were determined in mouse human liver microsomes. Test compounds (final concentration 0.5 µM) were incubated at 37 °C for 45 min in 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 mg microsomal protein/mL. The reaction

was started by addition of cofactor NADPH. The final concentration of solvent was 1% of the final volume. At 0, 5, 15, 30, and 45 min, an aliquot (100 µL) was taken, quenched with acetonitrile containing an appropriate internal standard, and analyzed by HPLC-MS/MS. The intrinsic clearance (CLi) was determined from the first-order elimination constant by nonlinear regression, corrected for the volume of the incubation and assuming 48.0 and 39.7 mg microsomal protein/g liver for mouse and human, respectively. Values for CLi were expressed as mL/min/g liver.

Fasted State Simulated Fluid (FaSSIF)

Determination of FaSSIF solubility was performed at pH 6.5 using 1-5 mg of substance, adding 1-5 mL of the appropiate solvent, and stopping the tube/vial. The sample was equilibrated at ambient temperature (~21–23 °C) or in a thermostatic water bath at 25 °C, unless a different temperature was desired or required, *e.g.* 37 °C. At each sample pull, the following was recorded: elapsed time, measured pH, and visual observations (*e.g.* colour changes, absence of solid). The sample was measured using HPLC. Typically, for this stage of development a generic chromatographic method was used.

ChromlogD assay

The Chromatographic Hydrophobicity Index (CHI)[S8] values are measured using reversed phase HPLC column (50 x 2 mm 3 µM Gemini NX C18, Phenomenex, UK) with fast acetonitrile gradient at starting mobile phase of pHs 2, 7.4 and 10.5. CHI values are derived directly from the gradient retention times by using a calibration line obtained for standard compounds. The CHI value approximates to the volume % organic concentration when the compound elutes. CHI is linearly transformed into ChromlogD[S9] by least-square fitting of experimental CHI values to calculated ClogP values for over 20K research compounds using the following formula: ChromlogD = 0.0857CHI-2.00. The average error of the assay is ±3 CHI unit or ±0.25 ChromlogD.

(S8) Valko K.; Bevan C.; Reynolds D. Chromatographic hydrophobicity index by fast-gradient RP-HPLC: A high-throughput alternative to logP/logD. Anal. Chem., 69, 1997, 2022-2029.

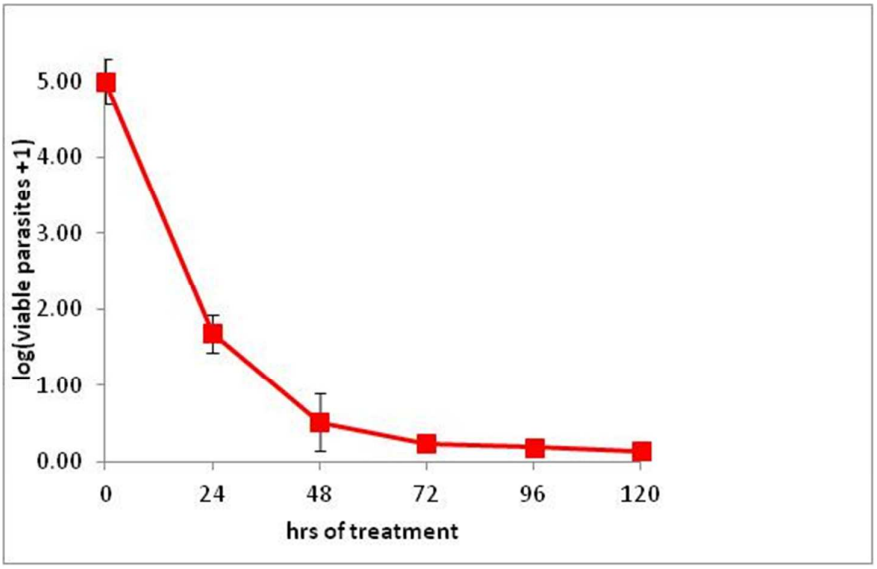
(S9) Robert J. Young, Darren V.S. Green, Christopher N. Luscombe, Alan P. Hill; Getting physical in drug discovery II: the impact of chromatographic hydrophobicity measurements and aromaticity Drug Discovery Today, Volume 16, Issues 17–18, September 2011, Pages 822–830

IC50 Standard desviation and controls

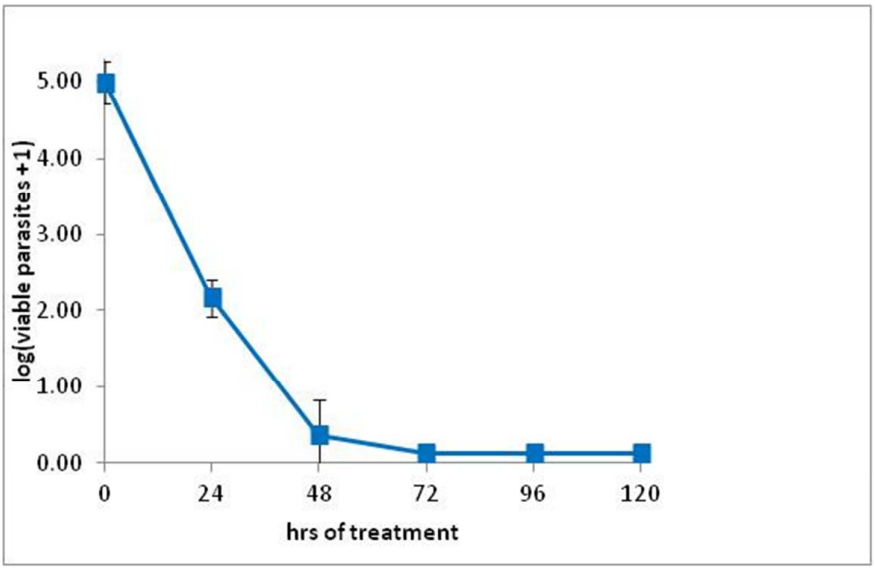
Compound number	PfIC50 (uM)	STV
2	0.07	0.036
4	0.66	0.007
5	0.10	0.01
6	0.07	0.021
7	1.70	0.227
8	0.69	0.32
9	5.53	4.6
10	0.05	0.01
11	0.05	0.007
12	0.48	0.15
13	0.23	0.1
14	0.02	0.005
15a	0.13	0.03
15b	0.02	0.002
15c	0.03	0.001
16a	0.04	0.01
16b	0.03	0.004
17a	0.03	
17b	0.09	0.005
18a	0.04	0.003
18b	0.03	0.007
18c	0.07	0.025
18d	1.9	0.15

Chloroquine 0.018±0.002

Parasite reduction rate figure for compound 2



Parasite reduction rate figure for compound 14



In vivo controls

Parameters of efficacy against *P falciparum Pf*3D7$^{0087/N9}$ in a standard '4-day-test' for **Artesunate**

Method of estimation	Goodness of fit	Parameter	Mean	Interval of confidence of the mean at 95% (IC_{95})	Unit of parameter
log fit	0.95	ED_{90}	10	9-12	$mg \cdot kg^{-1}$
log fit	0.96	AUC_{ED90}	0.018	0.011-0.03	$\mu g \cdot h \cdot ml^{-1} \cdot day^{-1}$

[1] AUC considered for artesunate plus dehydroartesunate in whole blood

Parameters of efficacy against *P falciparum Pf*3D7$^{0087/N9}$ in a standard '4-day-test' for **Chloroquine**

Method of estimation	Goodness of fit	Parameter	Mean	Interval of confidence of the mean at 95% (IC_{95})	Unit of parameter
log fit	0.97	ED_{90}	4.3	4-4.6	$mg \cdot kg^{-1}$
log fit	0.9	AUC_{ED90}	1	0.9-1.2	$\mu g \cdot h \cdot ml^{-1} \cdot day^{-1}$

Parameters of efficacy against *P falciparum Pf*3D7$^{0087/N9}$ in a standard '4-day-test' for **Piperaquine**

Method of estimation	Goodness of fit	Parameter	Mean	Interval of confidence of the mean at 95% (IC_{95})	Unit of parameter
log fit	0.98	ED_{90}	3.7	3.4-4.2	$mg \cdot kg^{-1}$
log fit	0.95	AUC_{ED90}	0.2	0.17-0.23	$\mu g \cdot h \cdot ml^{-1} \cdot day^{-1}$

Parameters of efficacy against *P falciparum Pf*3D7$^{0087/N9}$ in a standard '4-day-test' for **Mefloquine**

Method of estimation	Goodness of fit	Parameter	Mean	Interval of confidence of the mean at 95% (IC_{95})	Unit of parameter
log fit	0.97	ED_{90}	7.7	6.6-8.8	$mg \cdot kg^{-1}$

log fit	0.96	AUC_{ED90}	8.8	8-10	$\mu g \cdot h \cdot ml^{-1} \cdot day^{-1}$

Parameters of efficacy against *P falciparum Pf*3D7$^{0087/N9}$ in a standard '4-day-test' for **Primaquine**

Method of estimation	**Goodness of fit**	**Parameter**	**Mean**	**Interval of confidence of the mean at 95% (IC_{95})**	**Unit of parameter**
log fit	0.99	ED_{90}	3.75	3.1-4.3	$mg \cdot kg^{-1}$
log fit	0.98	AUC_{ED90}	0.03	0.023-0.031	$\mu g \cdot h \cdot ml^{-1} \cdot day^{-1}$