APPENDIX A

Chemical and reagents for short term *in vitro* drug sensitivity assay

1. The test medium:

The test medium used for *in vitro* drug sensitivity test of *P. vivax* was a mixture of RPMI 1640 with L-glutamine and without sodium bicarbonate (GIBCO BRL, Cat. No 31800-002) and McCoy's 5A medium with L-glutamine and without sodium bicarbonate [modified] (SIGMA-ALDRICH, Cat. No M4892) containing 25% AB^+ human serum.

1.1 Preparation of RPMI 1640 medium with L-glutamine and without sodium bicarbonate (GIBCO BRL)

The medium was prepared by dissolving 10.4 g RPMI 1640 with 960 ml of double distilled water. 5.94 g of N'-12 hydroxyethyl piperazine-N'-2-ethanesulfonic (HEPES) buffer (Sigma) and 1 ml gentamycin sulfate were added before sterilization by filtration through a Millipore filter of 0.45 microns porosity. One hundred ml of the sterile medium were transferred into sterile glass bottles as a stock medium and stored up to 6 months at 4^oC until use. Prior to use, 100 ml of the stock medium was supplemented with 4 ml of 5% (w/v) sodium bicarbonate solution.

1.2 Preparation of McCoy's 5A medium with L-glutamine and without sodium bicarbonate (SIGMA-ALDRICH)

The medium was prepared by dissolving McCoy's 5A powdered with double distilled water. 2.2 g sodium bicarbonate and 5.94 g of HEPES were added for each liter of final volume of medium and adjust pH to 7.4 with 1N NaOH before sterilization by filtration using a membrane with a porosity of 0.22 microns. The medium was stored at 2-8^oC in the dark until use. Prior to use, 100 ml of stock medium was supplemented with 25% AB^+ human serum.

2. Preparation of pre-dosed drug plate

All antifolate drugs were obtained from Jacobus Pharmaceutical (Princeton, NJ). A stock solution of each drug was prepared in 1% DMSO. Dilution of each drug were subsequently made in RPMI 1640 medium to obtained the drug concentration desired for testing. Fifty microliters of the final drug solution was added to each well of a 96 well microculture plate (Nunc).

The molecular weight of tested drugs

Drug	Molecular Weight
Pyrimethamine	248.7
Chlorcycloguanil	322.63
WR99210	431.16
Sulfadoxine	310.34
Dapsone	248.3

Drug plate lay out

Drug		Well A	Well B	Well C	Well D	Well E	Well F	Well G	Well H
Pyrimethamine	pmol/50ul	0	0.125	0.375	1.25	3.75	12.5	37.5	125
	nM		2.5	7.5	25	75	250	750	2,500
Chlorcycloguanil	pmol/50ul	0	10	30	100	300	1,000	3,000	10,000
	nM		200	600	2,000	6,000	20,000	60,000	200,000
WR99210	pmol/50ul	0	2	4	8	16	32	64	128
	nM		40	80	160	320	640	1,280	2,560
Sulfadoxine	pmol/50ul	0	10	30	100	300	1,000	3,000	10,000
	nM		200	600	2,000	6,000	20,000	60,000	200,000
Dapsone	pmol/50ul	0	10	30	100	300	1,000	3,000	10,000
	nM		200	600	2,000	6,000	20,000	60,000	200,000

Stock concentration of tested drugs

Drug	Stock concentration (in 1% DMSO)
Pyrimethamine	2,500 x 2 time of total volume = 5,000 nM
Chlorcycloguanil	200,000 x 2 time of total volume = 400,000 nM
WR99210	2,560 x 2 time of total volume = 5120 nM
Sulfadoxine	200,000 x 2 time of total volume = 400,000 nM
dapsone	200,000 x 2 time of total volume = 400,000 nM

Drug calculation:

2.1 Pyrimethamine

Well	Final concentration of pyrimethamine (2X)	
Well H	5,000 nM	
Well G	1,500 nM	[33 ml of 5,000 nM stock + 67 ml RPMI 1640 media]
Well F	500 nM	[30ml of 1,500 nM stock + 70 ml RPMI 1640 media]
Well E	150 nM	[33 ml of 500 nM stock + 67 ml RPMI 1640 media]
Well D	50 nM	[30ml of 150 nM stock + 70 ml RPMI 1640 media]
Well C	15 nM	[33 ml of 50 nM stock + 67 ml RPMI 1640 media]
Well B	5 nM	[30ml of 15 nM stock + 70 ml RPMI 1640 media]
Well A	0 nM	[No drug]
Well	**Final concentration of pyrimethamine in well (1X)**	
Well H	2,500 nM	[50 µl of drug + 50 µl blood medium mixture]
Well G	750 nM	[50 µl of drug + 50 µl blood medium mixture]
Well F	250 nM	[50 µl of drug + 50 µl blood medium mixture]
Well E	75 nM	[50 µl of drug + 50 µl blood medium mixture]
Well D	25 nM	[50 µl of drug + 50 µl blood medium mixture]
Well C	7.5 nM	[50 µl of drug + 50 µl blood medium mixture]
Well B	2.5 nM	[50 µl of drug + 50 µl blood medium mixture]
Well A	0 nM	[50 µl of RPMI + 50 µl blood medium mixture]

2.2 chlorcycloguanil, sulfadoxine and dapsone

Well	Final concentration of chlorcycloguanil, sulfadoxine and dapsone (2X)
Well H	400,000 nM
Well G	120,000 nM [33 ml of 400,000 nM stock + 67 ml RPMI 1640 media]
Well F	40,000 nM [30ml of 120,000 nM stock + 70 ml RPMI 1640 media]
Well E	12,000 nM [33 ml of 40,000 nM stock + 67 ml RPMI 1640 media]
Well D	4,000 nM [30ml of 12,000 nM stock + 70 ml RPMI 1640 media]
Well C	1,200 nM [33 ml of 4,000 nM stock + 67 ml RPMI 1640 media]
Well B	400 nM [30ml of 1,200 nM stock + 70 ml RPMI 1640 media]
Well A	0 nM [No drug]
Well	**Final concentration of chlorcycloguanil, sulfadoxine and dapsone in well (1X)**
Well H	200,000 nM [50 µl of drug + 50 µl blood medium mixture]
Well G	60,000 nM [50 µl of drug + 50 µl blood medium mixture]
Well F	20,000 nM [50 µl of drug + 50 µl blood medium mixture]
Well E	6,000 nM [50 µl of drug + 50 µl blood medium mixture]
Well D	2,000 nM [50 µl of drug + 50 µl blood medium mixture]
Well C	600 nM [50 µl of drug + 50 µl blood medium mixture]
Well B	200 nM [50 µl of drug + 50 µl blood medium mixture]
Well A	0 nM [50 µl of RPMI + 50 µl blood medium mixture]

2.3 WR99210

Well	Final concentration of WR99210 (2X)
Well H	5,120 nM
Well G	2,560 nM [50 ml of 5,120 nM stock + 50 ml RPMI 1640 media]
Well F	1,280 nM [50ml of 2,560 nM stock + 50 ml RPMI 1640 media]
Well E	640 nM [50 ml of 1,280 nM stock + 50 ml RPMI 1640 media]
Well D	320 nM [50ml of 640 nM stock + 50 ml RPMI 1640 media]
Well C	160 nM [50 ml of 320 nM stock + 50 ml RPMI 1640 media]
Well B	80 nM [50ml of 160 nM stock + 50 ml RPMI 1640 media]
Well A	0 nM [No drug]
Well	**Final concentration of WR99210 in well (1X)**
Well H	2,560 nM [50 µl of drug + 50 µl blood medium mixture]
Well G	1,280 nM [50 µl of drug + 50 µl blood medium mixture]
Well F	640 nM [50 µl of drug + 50 µl blood medium mixture]
Well E	320 nM [50 µl of drug + 50 µl blood medium mixture]
Well D	160 nM [50 µl of drug + 50 µl blood medium mixture]
Well C	80 nM [50 µl of drug + 50 µl blood medium mixture]
Well B	40 nM [50 µl of drug + 50 µl blood medium mixture]
Well A	0 nM [50 µl of RPMI + 50 µl blood medium mixture]

3. Preparation of blood medium mixture

Five ml of infected blood in lithium heparin collection tube was centrifuge at 200 x g for 5 min. The supernatant was removed, and the pellet was washed 2 times with RPMI medium. The pellet was resuspended to a hematocrit of 20% in RPMI medium.

Prior to start the drug assay, a 10 ml syringe tipped with a small quantity of glass wool was filled with 5 ml of CF11 cellulose powder (Whatman), covered with foil, and then autoclaved. When ready to use, the CF11 column was wetted with 5 ml of a phosphate-buffered saline (PBS) solution. The blood medium mixture was then added slowly to the CF11 column. The blood was washed through the column by the addition of approximately 5 ml of PBS until the column started to turn clear. The collection tube was centrifuge at 200 x g for 5 min. The supernatant was removed, and the pellet was resuspended to a hematocrit of 40% in AB^+ human serum. One microliter of this blood–serum mixture was added to 9 ml of McCoy's 5A medium containing 25% AB^+ human serum. Fifty microliters of this blood medium mixture was added to each well of the predosed drug plates.

4. Short term *in vitro* drug sensitivity assay

The short term *in vitro* sensitivity assay base on schizont maturation was performed with *P. vivax* field isolates using a modified method of Russell (Russell *et al*., 2003).

Drug plates containing the blood-medium mixture were incubated in a candle jar and incubated at 37^oC, until more than 50% of the ring stage parasites had matured to schizonts (36-48 h). Following the incubation time, the supernatant was removed and a thick blood film was made from the content of each well. The thick blood film were stained with Giemsa and examined by microscopy. The number of normal schizonts per 200 asexual stage parasites was determined in each blood film. The number of schizonts in each well that contained drug was compared with that in the control well and expressed as a percentage of the control. The dose-response curve was analyzed by nonlinear regression analysis to obtain the IC_{50} value, the concentration that inhibits schizont maturation by 50% compared with the no drug control.