CHAPTER III

Results

3.1 *In vitro* antimalarial drug-susceptibility testing of *P. falciparum* strains

In this study, 3D7 was used as PYR- and CQ-sensitive *P. falciparum* strain. Similarly, K1, CSL-2, and V1/S were used as PYR- and CQ-resistant *P. falciparum* strains. All of these strains were used as control *P. falciparum* for determination of *in vitro* antimalarial drug susceptibility testing using malaria SYBR Green I-based fluorescence (MSF) assay as described in section 2.5. Briefly, antimalarial drugs were incubated with strains of *P. falciparum* for 48 hr. The cells were then lysed with lysis buffer and the DNA was stained with SYBR green I for 1 hr before the fluorescence signal was measured by a fluorescence plate reader. The dose response curve, describes the relationship between response to drug treatment and drug concentration. It was plotted using a linear scale of percentage of parasite growth against a logarithmic scale of dose (concentration) and fitted by non-linear regression to provide IC_{50} values.

Malaria SYBR Green I-based fluorescence (MSF) assay was developed and validated by Smilkstein, M. *et al*. The relationship between the level parasitemia and the level of fluorescence was assessed at the beginning of this study to confirm there was not technical problems in our settings. **Figure 3-1** illustrates the agreement of increased level of SYBR Green I fluorescence and the increased numbers of parasitemia with linear relationship ($r^2 = 0.9763$).

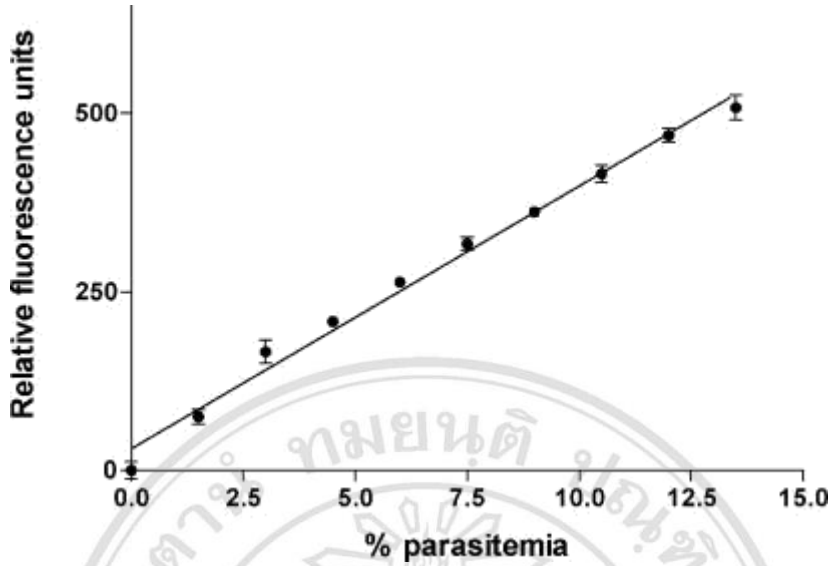

Figure 3-1 Relationship between parasitemia and measured fluorescence. Values are plotted as the means ± standard errors of the means for triplicate wells after subtraction of the background fluorescence for nonparasitized erythrocytes (93).

3.1.1 Drug-susceptibility of *P. falciparum* strains against Pyrimethamine (PYR)

This part of experiments was to investigate and confirm the effect of antimalarial drug; pyrimethamine (PYR), on growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S. In the assay, 200, 100, 50, 10, 1, 0.1, 0.01, and 0.001 μM concentration of PYR were incubated with strains of *P. falciparum* for 48 hr.

As shown in **Figure 3-2** and summarized in **Table 3-1**, PYR has different efficiencies on growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S with IC_{50} values at 0.05, 19.7, 27.3, and 78.5 µM, respectively. This study confirmed that 3D7; PYR- and CQ- sensitive strain, has higher sensitive to PYR than the other strains of *P. falciparum*. Whereas K1, CSL-2, and V1/S; PYR- and CQ- resistant strains, have low sensitivity to PYR.

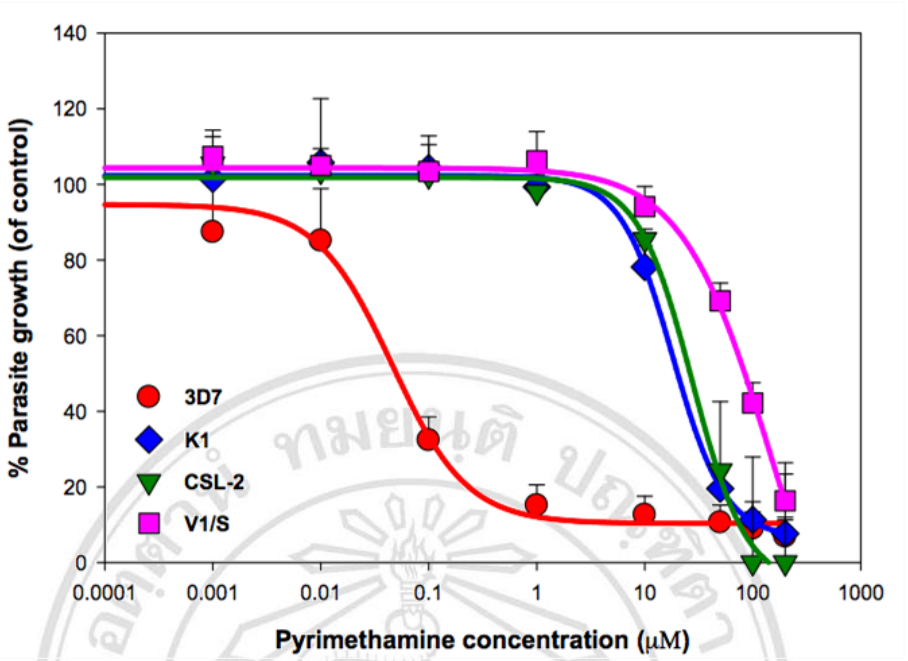

Figure 3-2 Effect of Pyrimethamine (PYR) on the growth of *P. falciparum* strains. The *P. falciparum* strains 3D7, K1, CSL-2, and V1/S were treated with PYR at the range of 0-200 μM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

Table 3-1 *In vitro* susceptibility of *P. falciparum* strains against Pyrimethamine (PYR).

Strains of *Plasmodium falciparum*	PYR (μM)
	IC_{50} (± SD)
3D7 (PYR- and CQ- sensitive strain)	0.05 (±0.02)
K1 (PYR- and CQ- resistant strain)	19.7 (±3.8)
CSL-2 (PYR- and CQ- resistant strain)	27.3 (±7.1)
V1/S (PYR- and CQ- resistant strain)	78.5 (±4.0)

Abbreviation: IC_{50} = 50% inhibitory drug concentration.

3.1.2 Drug-susceptibility of *P. falciparum* strains against Chloroquine (CQ)

This study was to investigate and confirm the effect of antimalarial drug; Chloroquine (CQ), on growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S. In the assay, 720, 360, 180, 90, 30, 10, 3, and 1 nM concentration of CQ were incubated with strains of *P. falciparum* for 48 hr.

As shown in **Figure 3-3** and summarized in **Table 3-2**, CQ has different efficiencies on growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S with IC_{50} values of 15.4, 181.7, 136.5, and 444.3 nM, respectively. This study confirmed that 3D7 is sensitive to CQ, while K1, CSL-2, and V1/S are resistant to CQ. In our experiments, we confirmed that V1/S is more resistant to CQ than K1 and CSL-2.

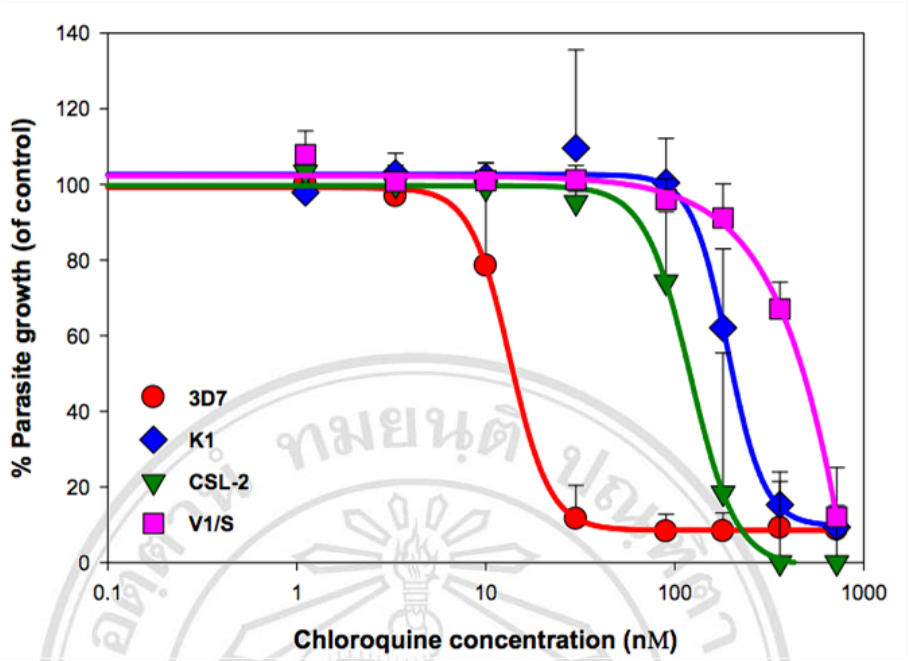

Figure 3-3 Effect of Chloroquine (CQ) on the growth of *P. falciparum* strains. The *P. falciparum* strains 3D7, K1, CSL-2, and V1/S were treated with CQ in the range of 0-720 nM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

Table 3-2 *In vitro* susceptibility of *P. falciparum* strains against Chloroquine (CQ).

Strains of *Plasmodium falciparum*	CQ (nM)
	IC_{50} (± SD)
3D7 (PYR- and CQ- sensitive strain)	15.4 (±1.1)
K1 (PYR- and CQ- resistant strain)	181.7 (±50.6)
CSL-2 (PYR- and CQ- resistant strain)	136.5 (±48.4)
V1/S (PYR- and CQ- resistant strain)	444.3 (±31.9)

Abbreviation: IC_{50} = 50% inhibitory drug concentration.

3.1.3 Drug-susceptibility of *P. falciparum* strains against Mefloquine (MQ)

This study was to investigate the effect of antimalarial drug; Mefloquine (MQ), on growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S. In the assay, 270, 90, 30, 10, 3, and 1 nM concentration of MQ were incubated with strains of *P. falciparum* for 48 hr.

As shown in **Figure 3-4**, MQ has different efficiencies on the growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S with IC_{50} value at 51.8, 18.1, 14.6, and 27.2 nM, respectively, as summarized in **Table 3-3**. This study showed that 3D7; PYR- and CQ- sensitive strain, has lower sensitive to MQ than the other strains of *P. falciparum*. Whereas CSL-2, K1, and V1/S, which are PYR- and CQ- resistant strains, have higher sensitivity to MQ. This may summarize that MQ; the first-line drug used

routinely in the field, is still highly effective against the resistant *P. falciparum* strains, of which CSL-2 is the most sensitive.

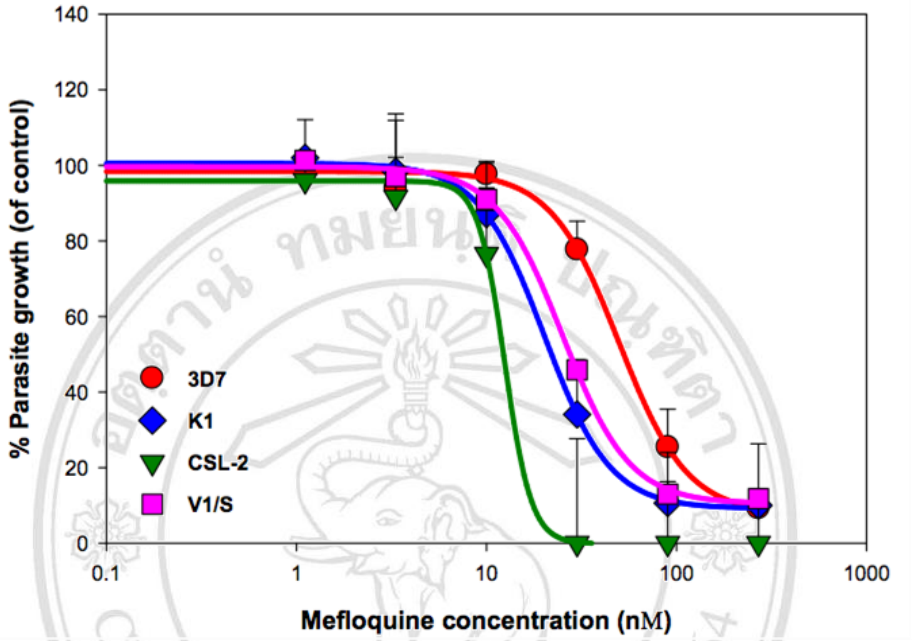

Figure 3-4 Effect of Mefloquine (MQ) on the growth of *P. falciparum* culture. The *P. falciparum* strains 3D7, K1, CSL-2, and V1/S were treated with MQ in the range of 0-270 nM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

Table 3-3 *In vitro* susceptibility of *P. falciparum* strains against Mefloquine (MQ).

Strains of *Plasmodium falciparum*	MQ (nM)
	IC_{50} (± SD)
3D7 (PYR- and CQ- sensitive strain)	51.8 (±4.9)
K1 (PYR- and CQ- resistant strain)	18.1 (±3.8)
CSL-2 (PYR- and CQ- resistant strain)	14.6 (±2.7)
V1/S (PYR- and CQ- resistant strain)	27.2 (±1.6)

Abbreviation: IC_{50} = 50% inhibitory drug concentration.

3.1.4 Drug-susceptibility of *P. falciparum* strains against Dihydroartemisinin (DHA)

This study was to investigate the effect of antimalarial drug; Dihydroartemisinin (DHA), on growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S. In assay, 27, 9, 3, 1, 0.3, and 0.1 nM concentration of DHA were incubated with strains of *P. falciparum* for 48 hr.

As shown in **Figure 3-5** and summarized in **Table 3-4**, DHA had different efficiencies on the growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S with the IC_{50} values of 2.4, 1.7, 1.2, and 1.6 nM, respectively. This study confirmed that all of the tested *P. falciparum* strains are sensitive to DHA. In this study, 3D7; PYR- and CQ- sensitive strain, is the least sensitive to DHA as compared to the other strains of *P. falciparum*, CSL-2, V1/S, and K1 which are PYR- and CQ- resistant

strains. This study also confirmed that DHA; the first-line drug used in the field, is still highly effective to standard antimalarial-resistant *P. falciparum* strains.

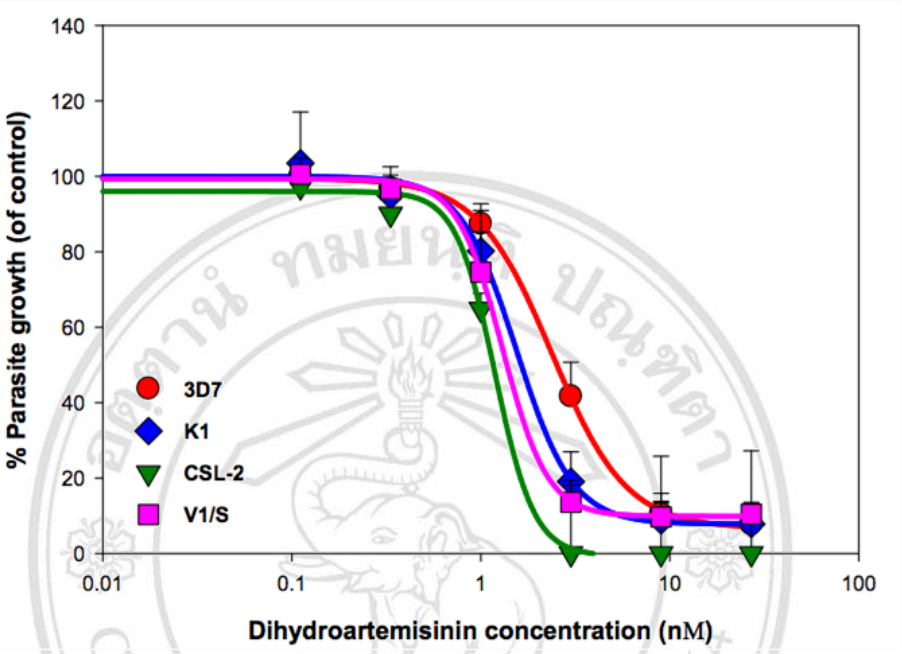

Figure 3-5 Effect of Dihydroartemisinin (DHA) on the growth of *P. falciparum* culture. The *P. falciparum* strains 3D7, K1, CSL-2, and V1/S were treated with DHA in the range of 0-27 nM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

Table 3-4 *In vitro* susceptibility of *P. falciparum* strains against Dihydroartemisinin (DHA).

Strains of *Plasmodium falciparum*	DHA (nM)
	IC_{50} (± SD)
3D7 (PYR- and CQ- sensitive strain)	2.4 (±0.6)
K1 (PYR- and CQ- resistant strain)	1.7 (±0.2)
CSL-2 (PYR- and CQ- resistant strain)	1.2 (±0.1)
V1/S (PYR- and CQ- resistant strain)	1.6 (±0.2)

Abbreviation: IC_{50} = 50% inhibitory drug concentration.

3.1.5 Drug-susceptibility of *P. falciparum* strains on the new antimalarial drug candidate 'P218'

This study was to investigate the effect of the new antimalarial drug candidate; P218, on growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S. In assay, 1000, 100, 10, 1, 0.1, and 0.01 μM concentration of P218 were incubated with strains of *P. falciparum* for 48 hr.

As shown in **Figure 3-6** and summarized in **Table 3-5**, P218 had different efficiencies on the growth of *P. falciparum* strains 3D7, K1, CSL-2, and V1/S with the IC_{50} values of 1.8, 1.7, 15.3, and 52.4 nM, respectively. In this study, 3D7; PYR- and CQ- sensitive strain, and K1; PYR- and CQ- resistant strain, are more sensitive to P218 than CSL-2 and V1/S, which are PYR- and CQ- resistant strains.

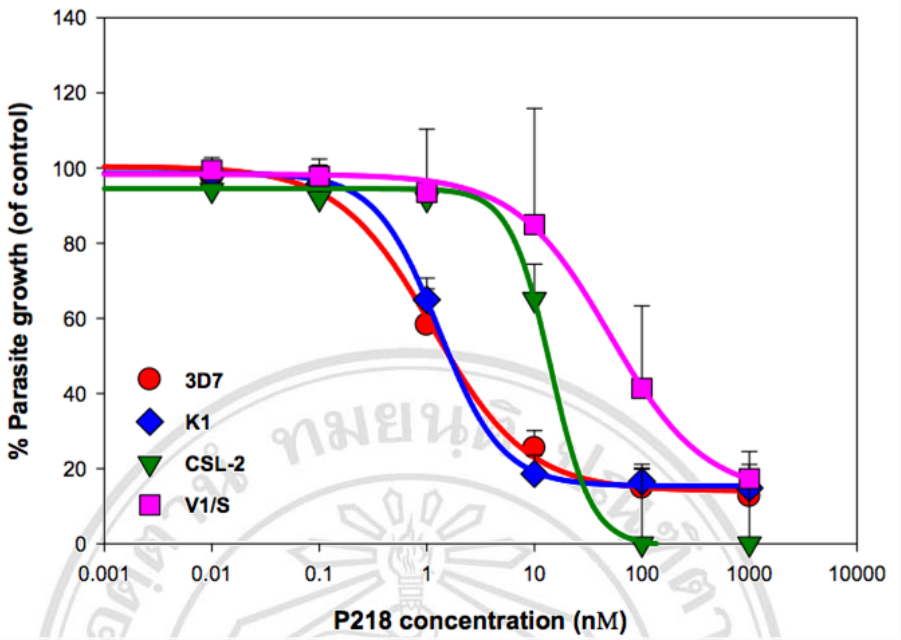

Figure 3-6 Effect of the new antimalarial drug candidate 'P218' on the growth of *P. falciparum* culture. The standard *P. falciparum* strains 3D7, K1, CSL-2, and V1/S were treated with P218 in the range of 0-1000 nM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

Table 3-5 *In vitro* susceptibility of *P. falciparum* strains against new antimalarial drug candidate 'P218'.

Strains of *Plasmodium falciparum*	P218 (nM)
	IC_{50} (± SD)
3D7 (PYR- and CQ- sensitive strain)	1.8 (±0.001)
K1 (PYR- and CQ- resistant strain)	1.7 (±0.001)
CSL-2 (PYR- and CQ- resistant strain)	15.3 (±0.002)
V1/S (PYR- and CQ- resistant strain)	52.4 (±0.010)

Abbreviation: IC_{50} = 50% inhibitory drug concentration.

3.2 Comparison the effect of antimalarial drug Pyrimethamine and the new antimalarial drug candidate 'P218' on growth of *P. falciparum* strains

The new antimalarial drug candidate 'P218' is a derivative of antifolate compound developed by research group at BIOTEC, NSTDA. Its structure was based on rigid antifolate PYR structure in order to increase the specificity and good affinity to *P. falciparum* DHFR enzyme. As summarized in **Table 3-6**, 3D7; PYR-sensitive strain, is more sensitive to PYR than P218. Instead, K1, CSL-2, and V1/S; PYR-resistant strain, are more sensitive to P218 than PYR.

Table 3-6 Comparison the effect of antimalarial drug Pyrimethamine (PYR) and the new antimalarial drug candidate 'P218' on growth of *P. falciparum* strains

Strains of *Plasmodium falciparum*		**PYR (*μ*M)**	**P218 (nM)**
		IC_{50} (± SD)	IC_{50} (± SD)
PYR- and CQ- sensitive strain	**3D7**	0.05 (±0.02)	1.8 (±0.001)
PYR- and CQ- resistant strains	**K1**	19.7 (±3.8)	1.7 (±0.001)
	CSL-2	27.3 (±7.1)	15.3 (±0.002)
	V1/S	78.5 (±4.0)	52.4 (±0.010)

Abbreviation: IC_{50} = 50% inhibitory drug concentration.

3.3 *In vitro* drug-susceptibility testing of *P. falciparum* isolates from malaria patients in Mae-Sariang district area, Mae Hong Son province.

In this study, 36 field *P. falciparum* isolates from Mae-Sariang district area, Mae Hong Son province were tested for their sensitivity to antimalarial drugs PYR, CQ, MQ, DHA, and P218 using malaria SYBR Green I-based fluorescence (MSF) assay as described in section 2.5.

3.3.1 Drug-susceptibility of *P. falciparum* isolates on Pyrimethamine (PYR)

To investigate the effect of antimalarial drug PYR on the growth of *P. falciparum* isolates, the isolates were cultured in the condition of 1% ring at 2% hematocrit in 200, 100, 50, 10, 1, 0.1, 0.01, and 0.001 μM concentration of PYR. The *P. falciparum* strains, 3D7 and V1/S, were used as control in all experiments.

As shown in **Figure 3-7**, all of the *P. falciparum* field isolates were clearly resistant to PYR when compared with 3D7 (PYR-sensitive) and V1/S (PYR-resistant) strains. The IC_{50} value of each *P. falciparum* isolate was summarized in **Table 3-7**.

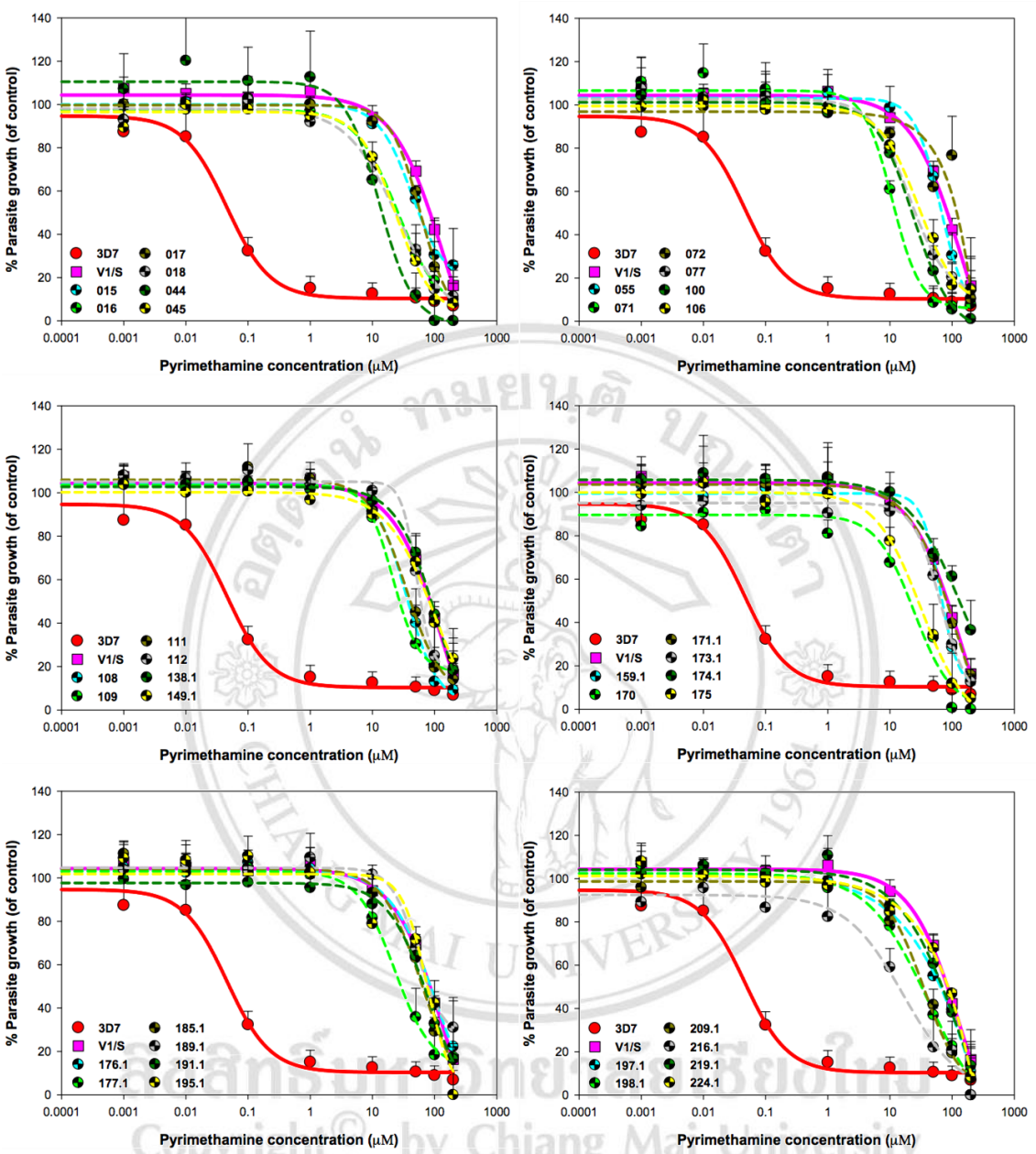

Figure 3-7 The effect of Pyrimethamine (PYR) on the growth of *P. falciparum* field isolates. The *P. falciparum* field isolates including *P. falciparum* control strains were treated with PYR in the range of 0-200 μM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

3.3.2 Drug-susceptibility of *P. falciparum* isolates on Chloroquine (CQ)

To investigate the effect of antimalarial drug; CQ, on growth of *P. falciparum* field isolates, the isolates were cultured in the condition of 1% ring at 2% hematocrit in 720, 360, 180, 90, 30, 10, 3, and 1 nM concentration of CQ for 48 hr. The *P. falciparum* strains, 3D7 and V1/S, were used as control in all experiments.

As shown in **Figure 3-8,** *P. falciparum* field isolates were, at different level, resistant to CQ; like PYR, when compared with 3D7 (CQ-sensitive) and V1/S (CQ-resistant) strains. The IC_{50} value of each *P. falciparum* isolate was summarized in **Table 3-7.**

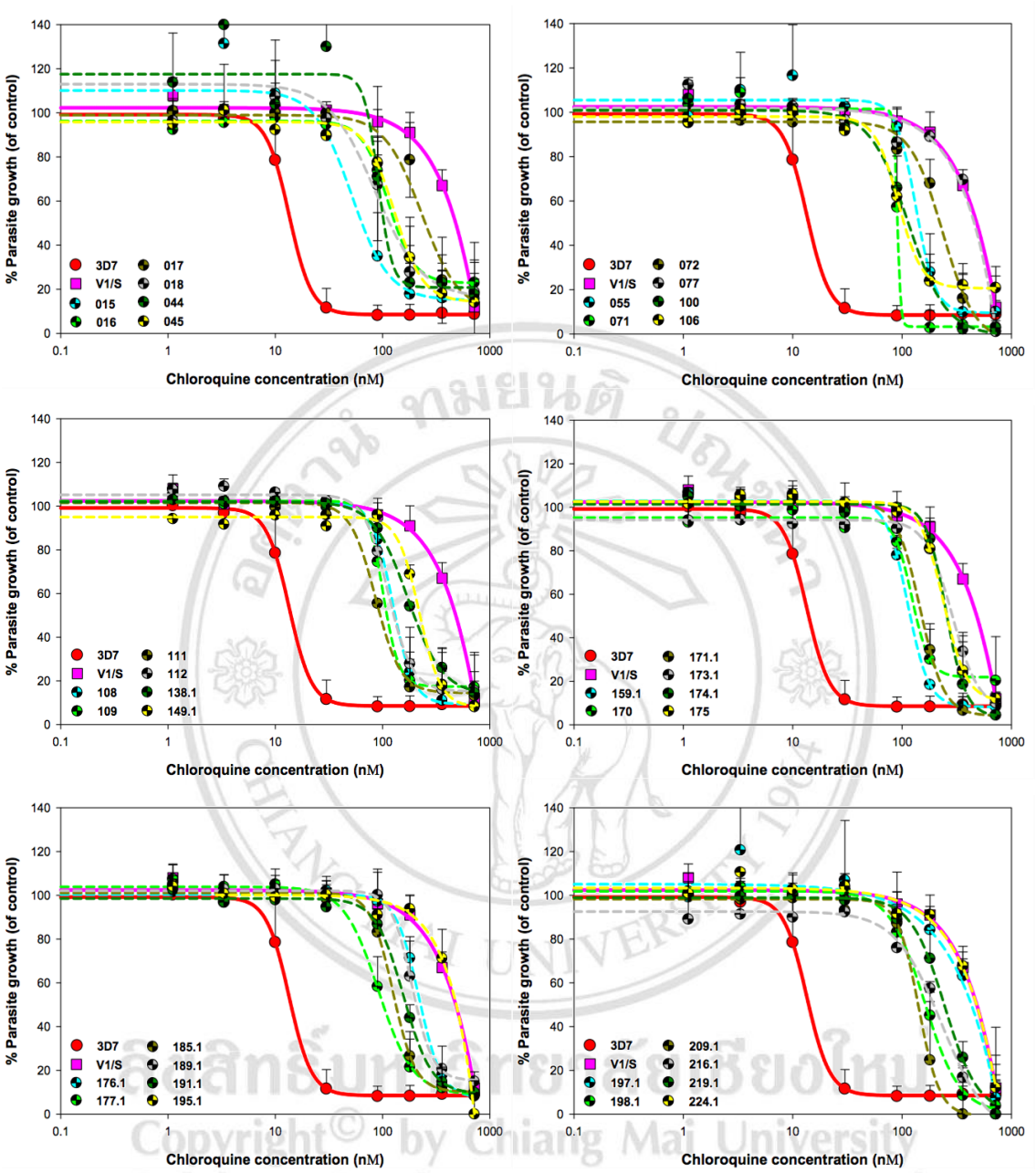

Figure 3-8 The effect of Chloroquine (CQ) on the growth of *P. falciparum* field isolates. The *P. falciparum* field isolates including *P. falciparum* control strains were treated with CQ in the range of 0-720 nM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was

obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

3.3.3 Drug-susceptibility of *P. falciparum* isolates on Mefloquine (MQ)

To investigate the effect of antimalarial drug; MQ, on growth of *P. falciparum* field isolates, the isolates were cultured in the condition of 1% ring at 2% hematocrit in 270, 90, 30, 10, 3, and 1 nM concentration of MQ for 48 hr.

As shown in **Figure 3-9,** some, but not all, *P. falciparum* field isolates were resistant to MQ, when compared with control 3D7 and V1/S strains. The IC_{50} value of each *P. falciparum* isolate was summarized in **Table 3-7.**

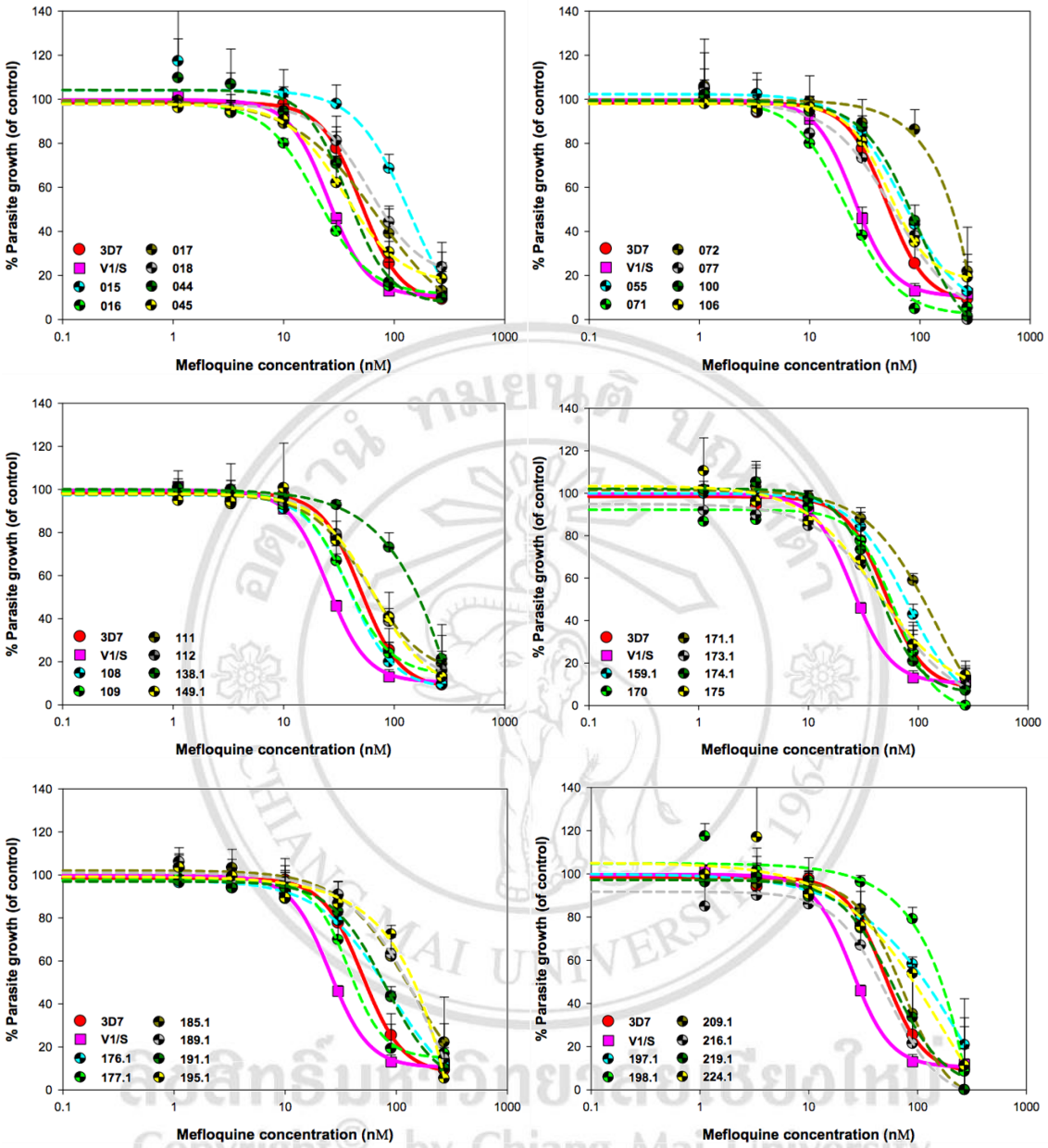

Figure 3-9 The effect of Mefloquine (MQ) on the growth of *P. falciparum* field isolates. The *P. falciparum* isolates including *P. falciparum* control strains were treated with MQ in the range of 0-270 nM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

3.3.4 Drug-susceptibility of *P. falciparum* field isolates on Dihydroartemisinin (DHA)

To investigate the effect of antimalarial drug; DHA, on growth of *P. falciparum* field isolates, the isolates were cultured in the condition of 1% ring at 2% hematocrit in 27, 9, 3, 1, 0.3, and 0.1 nM concentration of DHA for 48 hr.

As shown in **Figure 3-10**, almost all of the *P. falciparum* field isolates were still sensitive to DHA with only some isolates showed slight resistant to DHA. The IC_{50} value of each *P. falciparum* isolate was summarized in **Table 3-7.**

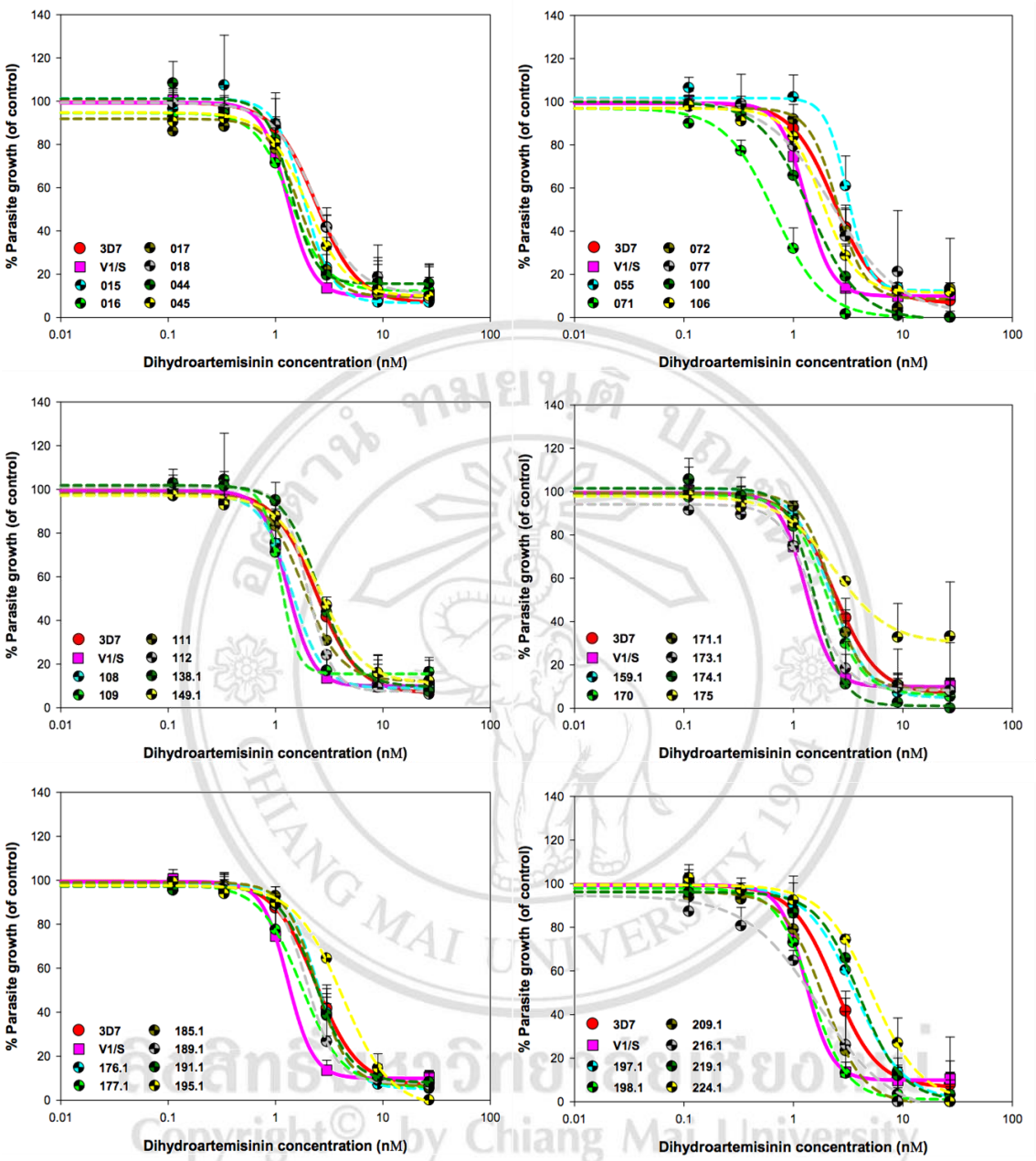

Figure 3-10 The effect of Dihydroartemisinin (DHA) on the growth of *P. falciparum* isolates. The *P. falciparum* isolates including *P. falciparum* control strains were treated with DHA in the range of 0-27 nM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

3.3.5 Drug-susceptibility of *P. falciparum* field isolates on the new antimalarial drug candidate 'P218'

To investigate the effect of antimalarial drug; P218, on growth of *P. falciparum* field isolates, the isolates were cultured in the condition of 1% ring at 2% hematocrit in 1000, 100, 10, 1, 0.1, and 0.01 nM concentration of P218 for 48 hr. The *P. falciparum* strains, 3D7 and V1/S, were used as control in all experiments.

As shown in **Figure 3-11**, some *P. falciparum* field isolates showed sensitivity to P218 at the level of PYR-sensitive control 3D7 strain, while some field isolates showed sensitivity to P218 at the level of PYR-resistant control V1/S strain. The IC_{50} value of each *P. falciparum* isolate was summarized in **Table 3-7**.

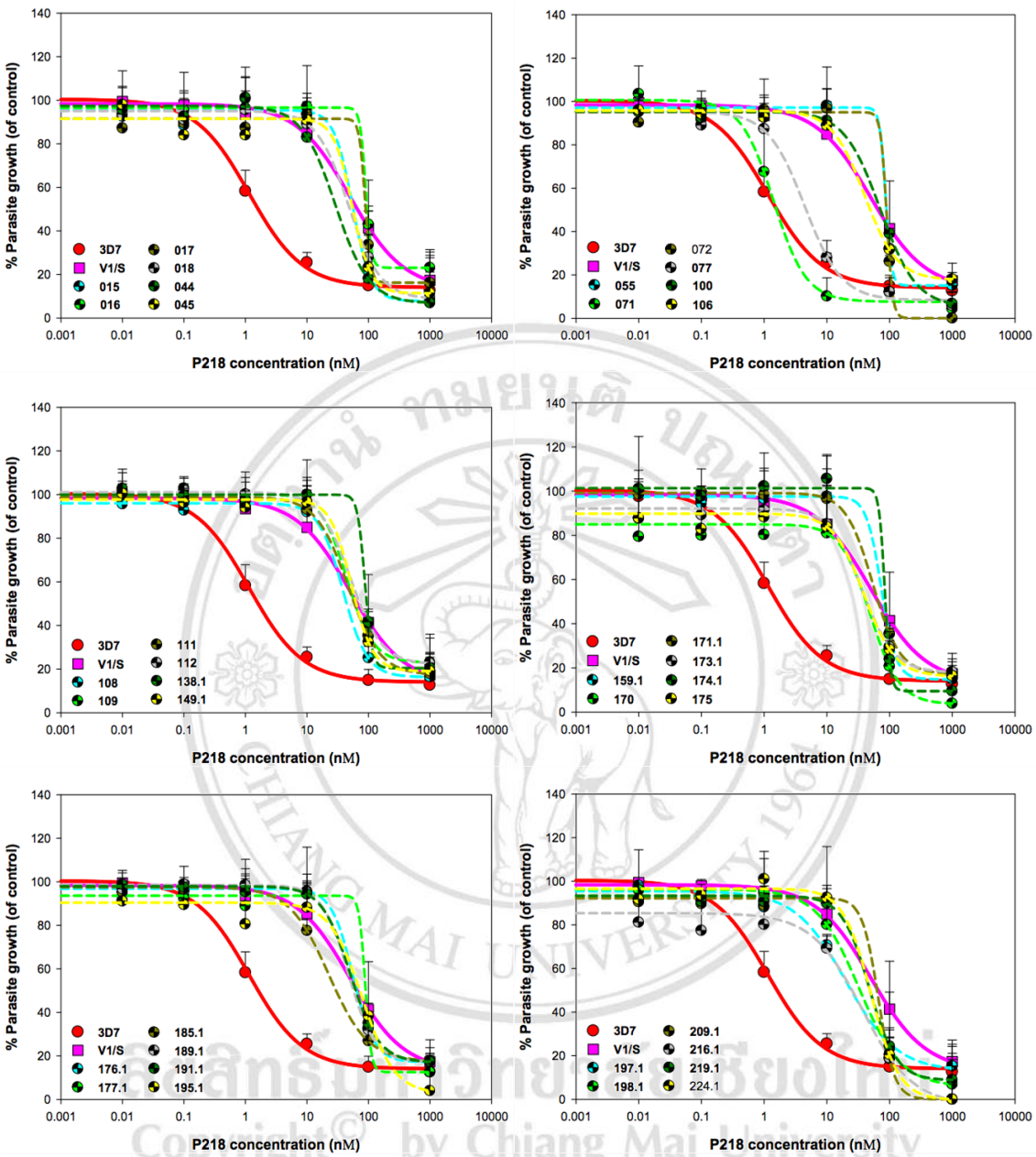

Figure 3-11 The effect of P218 on the growth of *P. falciparum* field isolates. The *P. falciparum* isolates including *P. falciparum* control strains were treated with P218 in the range of 0-1000 nM for 48 hr. The percentage of parasite growth comparing to untreated control was calculated by measuring the level of DNA-stained SYBR Green I fluorescence signal using a fluorescence microplate reader. Data was obtained from three independent experiments performed in triplicate and expressed as mean ± SD.

Table 3-7 IC_{50} values of Pyrimethamine (PYR), Chloroquine (CQ), Mefloquine (MQ), Dihydroartemisinin (DHA), and the new antimalarial drug candidate 'P218' against *P. falciparum* from field isolates and reference strains.

Pf. strains	PYR (μM)	CQ (nM)	MQ (nM)	DHA (nM)	P218 (nM)
3D7	0.04 (±0.02)	15.4 (±1.1)	51.7 (±4.9)	2.4 (±0.6)	1.8 (±0.00)
K1	19.7 (±3.8)	181.7 (±50.6)	18.1 (±3.8)	1.7 (±0.2)	1.7 (±0.00)
CSL-2	27.3 (±7.1)	136.5 (±48.4)	14.6 (±2.7)	1.2 (±0.1)	15.3 (±0.00)
V1/S	78.5 (±4.0)	444.3 (±31.9)	27.2 (±1.6)	1.6 (±0.2)	52.4 (±0.01)

Pf. isolates	PYR (μM)	CQ (nM)	MQ (nM)	DHA (nM)	P218 (nM)
15	31.8 (±16.2)	49.5 (±28.6)	94.2 (±44.9)	1.5 (±0.9)	52.0 (±0.02)
16	25.4 (±2.1)	98.9 (±24.7)	21.8 (±1.4)	1.6 (±0.0)	76.0 (±0.02)
17	62.9 (±8.1)	223.8 (±61.3)	67.0 (±17.9)	2.0 (±0.5)	73.0 (±0.02)
18	31.5 (±11.9)	96.9 (±44.7)	100.5 (±38.7)	2.2 (±0.5)	46.0 (±0.01)
44	13.6 (±2.8)	64.9 (±22.5)	31.1 (±4.6)	0.7 (±0.1)	32.0 (±0.01)
45	30.3 (±16.5)	165.0 (±80.7)	40.9 (±15.9)	2.3 (±0.6)	64.0 (±0.03)
55	66.3 (±5.6)	145.2 (±19.3)	74.4 (±2.7)	3.2 (±1.7)	58.0 (±0.01)
71	14.0 (±1.5)	93.8 (±11.2)	26.0 (±7.5)	0.8 (±0.3)	2.0 (±0.00)
72	132.9 (±1.8)	226.5 (±10.4)	152.0 (±7.6)	2.5 (±0.5)	46.0 (±0.01)
77	28.7 (±1.7)	439.2 (±8.8)	63.1 (±8.7)	2.3 (±0.6)	49.0 (±0.00)
100	17.3 (±2.5)	101.0 (±23.3)	79.8 (±13.1)	1.3 (±0.1)	64.0 (±0.01)
106	27.7 (±6.9)	108.7 (±5.5)	58.4 (±8.1)	1.9 (±0.1)	49.0 (±0.01)

Pf. isolates	PYR (μM)	CQ (nM)	MQ (nM)	DHA (nM)	P218 (nM)
108	37.8 (±6.8)	133.7 (±16.9)	41.4 (±3.5)	1.5 (±0.2)	42.0 (±0.01)
109	30.1 (±6.7)	122.2 (±22.1)	46.2 (±0.5)	1.5 (±0.2)	60.0 (±0.01)
111	46.1 (±18.9)	93.4 (±14.3)	96.4 (±44.2)	2.1 (±0.4)	55.0 (±0.01)
112	69.6 (±15.8)	137.4 (±26.1)	67.0 (±2.7)	2.8 (±1.4)	71.0 (±0.01)
138.1	89.8 (±4.8)	199.8 (±10.7)	152.2 (±5.9)	3.3 (±0.9)	68.0 (±0.01)
149.1	76.7 (±5.1)	233.9 (±18.9)	85.2 (±31.4)	3.1 (±0.5)	54.0 (±0.01)
159.1	71.6 (±0.97)	155.9 (±3.2)	74.1 (±7.9)	2.2 (±0.3)	58.0 (±0.01)
170	92.4 (±13.2)	150.9 (±18.9)	115.6 (±46.4)	3.1 (±1.5)	153.0 (±0.10)
171.1	80.8 (±6.5)	151.2 (±15.9)	111.2 (±8.7)	2.3 (±0.2)	58.0 (±0.00)
173.1	73.5 (±22.2)	283.2 (±24.9)	48.2 (±9.6)	1.6 (±0.1)	43.0 (±0.01)
174.1	144.9 (±19.9)	259.2 (±14.1)	51.2 (±10.6)	1.7 (±0.3)	48.0 (±0.00)
175	38.1 (±15.7)	266.3 (±41.9)	49.8 (±1.9)	2.9 (±1.1)	54.0 (±0.00)
176.1	92.4 (±17.8)	236.3 (±21.4)	74.0 (±4.9)	2.5 (±0.4)	53.0 (±0.00)
177.1	46.2 (±5.9)	99.0 (±6.1)	50.8 (±8.3)	1.9 (±0.6)	55.0 (±0.01)
185.1	65.6 (±9.7)	149.4 (±28.8)	126.9 (±12.2)	2.6 (±0.5)	36.0 (±0.01)
189.1	82.0 (±3.5)	194.3 (±60.8)	107.5 (±23.8)	2.4 (±0.7)	53.0 (±0.01)
191.1	70.7 (±11.5)	173.6 (±18.4)	90.1 (±18.6)	2.6 (±0.6)	57.0 (±0.01)
195.1	93.9 (±4.9)	429.2 (±27.8)	131.1 (±6.7)	4.1 (±0.1)	59.0 (±0.02)
197.1	56.1 (±2.4)	375.1 (±17.2)	98.2 (±12.0)	3.1 (±0.7)	29.0 (±0.01)
198.1	30.1 (±6.5)	173.4 (±22.6)	141.3 (±8.3)	1.5 (±0.3)	34.0 (±0.01)
209.1	36.9 (±7.6)	143.3 (±34.4)	68.3 (±20.4)	1.9 (±0.4)	38.0 (±0.01)

Pf. isolates	PYR (μM)	CQ (nM)	MQ (nM)	DHA (nM)	P218 (nM)
216.1	16.0 (±7.6)	168.6 (±53.1)	44.8 (±3.9)	1.23 (±0.3)	17.0 (±0.01)
219.1	61.8 (±21.4)	247.5 (±34.2)	93.6 (±20.7)	2.71 (±0.8)	42.0 (±0.01)
224.1	89.6 (±6.8)	461.9 (±44.3)	102.9 (±13.9)	1.39 (±0.5)	39.0 (±0.00)

3.4 Identification of mutation(s) in drug resistant genes in *P. falciparum* field isolates.

In order to identify mutations in *Pfcrt* and *Pfdhfr* genes that cause resistance to CQ and PYR, respectively, the total genomic DNA from *P. falciparum* field isolates was extracted and used as DNA template for PCR to generate PCR products that are specific for *Pfdhfr* and *Pfcrt*; the genes involve in PYR and CQ resistance. The PCR products were then subjected to DNA sequencing and the sequencing results were analysed by sequence comparison software to identify mutation(s) in *Pfdhfr* and *Pfcrt*, as representative results shown in **Figure 3-12** and **Figure 3-13**, respectively. As summarized in **Table 3-8, Table 3-9** and **Table 3-12,** all PYR-resistant isolates contain mutations at DHFR enzyme. Most of all isolates contain quadruple mutations (N51I, C59R, S108N, and I164L), the same mutations with PYR-resistant V1/S strain, whereas 3 isolates contain triple mutations (C59R, S108N, and I164L), the same mutations with PYR-resistant CSL-2 strain, and 1 isolate contains double mutations (C59R and S108N), the same mutations with PYR-resistant K1.

It has been known that CQ resistance is related to mutation of Chloroquine resistance-related transporter or CRT at position 76 from lysine (K) to threonine (T). As shown in **Table 3-10, Table 3-11** and **Table 3-12,** our results showed that all of *P. falciparum* field isolates possess mutations in CRT at amino acid positions 74, 75, 76 from MNK to IET, respectively, which is the CQ resistance profile the same as K1, CSL-2, and V1/S which are CQ-resistant *P. falciparum*. Summary of *in vitro* antimalarial drug sensitivity testing result and mutations at *Pf*CRT and *Pf*DHFR from *P. falciparum* isolates is described in **Table 3-13**.

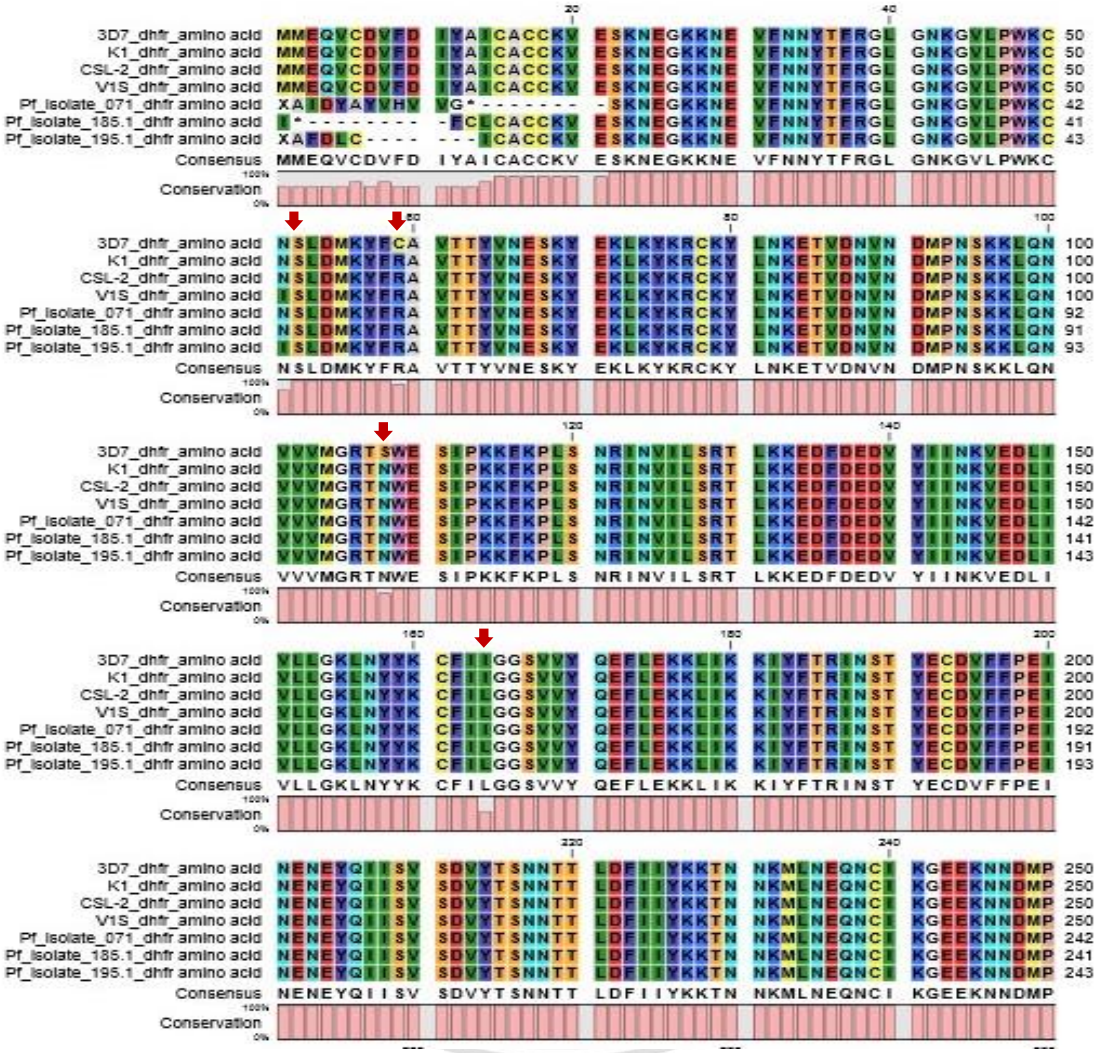

Figure 3-12 Representative sequence alignment results of DHFR from *P. falciparum* field isolates and *P. falciparum* strains. The total genomic DNA from *P. falciparum* isolates were extracted and used as DNA template for PCR to generate PCR product that is specific for *dhfr* gene, which mutations are responsible for PYR resistance, and subjected to DNA sequencing. Isolate number 071 was representative of DHFR double mutation (C59R, S108N), isolate number 185.1 was representative of DHFR triple mutation (C59R, S108N, and I164L), isolate number 195.1 was representative of DHFR quadruple mutation (N51I, C59R, S108N, and I164L). The sequence alignment analysis was performed using the CLC sequencing viewer program version 6.8.1.

Figure 3-13 Representative sequence alignment results of CRT from *P. falciparum* field isolates and *P. falciparum* strains. The total genomic DNA from *P. falciparum* isolates were extracted and used as DNA template for PCR to generate PCR product that is specific for *crt* gene, which mutations are responsible for CQ resistance, and subjected to DNA sequencing. The sequence alignment analysis was performed using the CLC sequencing viewer program version 6.8.1.

Table 3-8 Mutations of DHFR enzyme that involves in PYR-resistant *P. falciparum* strains.

Strain of *Plasmodium falciparum*	DHFR mutation (N51, C59, S108, I164)	
3D7 (PYR^S)	NCSI	WT
K1 (PYR^R)	NRNI	2x
CSL-2 (PYR^R)	NRNL	3x
V1/S (PYR^R)	IRNL	4x

Abbreviation: WT = Wild type, 2x = double mutation, 3x = triple mutation, 4x = quadruple mutation, PYR^S = PYR-sensitive, PYR^R = PYR-resistant.

Table 3-9 Mutations of DHFR enzyme that involves in PYR-resistant *P. falciparum* field isolates.

Isolates	DHFR mutation (N51, C59, S108, I164)		Isolates	DHFR mutation (N51, C59, S108, I164)	
015	IRNL	4x	**159.1**	IRNL	4x
016	IRNL	4x	**170**	IRNL	4x
017	IRNL	4x	**171.1**	IRNL	4x
018	IRNL	4x	**173.1**	IRNL	4x
044	IRNL	4x	**174.1**	IRNL	4x
045	IRNL	4x	**175**	IRNL	4x
055	IRNL	4x	**176.1**	IRNL	4x
071	NRNI	2x	**177.1**	IRNL	4x
072	NRNL	3x	**185.1**	NRNL	3x
077	NRNL	3x	**189.1**	IRNL	4x
100	IRNL	4x	**191.1**	IRNL	4x
106	IRNL	4x	**195.1**	IRNL	4x
108	IRNL	4x	**197.1**	IRNL	4x
109	IRNL	4x	**198.1**	IRNL	4x
111	IRNL	4x	**209.1**	IRNL	4x
112	IRNL	4x	**216.1**	IRNL	4x
138.1	IRNL	4x	**219.1**	IRNL	4x
149.1	IRNL	4x	**224.1**	IRNL	4x

Abbreviation: WT = Wild type, 2x = double mutation, 3x = triple mutation, 4x = quadruple mutation, PYR^S = PYR-sensitive, PYR^R = PYR-resistant.

Table 3-10 Mutations at CRT enzyme that involves in CQ-resistant *P. falciparum* strains.

Strain of *Plasmodium falciparum*	CRT mutation (M74, N75, K76)
3D7 (CQ^S)	MNK (WT)
K1 (CQ^R)	IET
CSL-2 (CQ^R)	IET
V1/S (CQ^R)	IET

Abbreviation: WT = Wild type, CQ^S = CQ-sensitive, CQ^R = CQ-resistant.

Table 3-11 Mutations at CRT enzyme that involves in CQ-resistant *P. falciparum* field isolates.

Isolates	CRT mutation (M74, N75, K76)	Isolates	CRT mutation (M74, N75, K76)
015	IET	**159.1**	IET
016	IET	**170**	IET
017	IET	**171.1**	IET
018	IET	**173.1**	IET
044	IET	**174.1**	IET
045	IET	**175**	IET
055	IET	**176.1**	IET
071	IET	**177.1**	IET
072	IET	**185.1**	IET
077	IET	**189.1**	IET
100	IET	**191.1**	IET
106	IET	**195.1**	IET
108	IET	**197.1**	IET
109	IET	**198.1**	IET
111	IET	**209.1**	IET
112	IET	**216.1**	IET
138.1	IET	**219.1**	IET
149.1	IET	**224.1**	IET

Abbreviation: WT = Wild type, CQ^S = CQ-sensitive, CQ^R = CQ-resistant.

Table 3-12 Summary of tested *P. falciparum* numbers and percentage of *Pf*CRT and *Pf*DHFR mutations.

Resistance genes		*Pf*CRT	*Pf*DHFR		
		K76T	C59R, S108N (x2)	C59R, S108N and I164L (x3)	N51I, C59R, S108N and I164L (x4)
Pf Isolates	N = 36	36	1	3	32
	%	100	2.78	8.33	88.89

Table 3-13 Summary of *in vitro* antimalarial drug sensitivity testing result and mutations at *Pf*CRT and *Pf*DHFR from *P. falciparum* isolates.

Antimalarial drugs	*P. falciparum* Isolates (N = 36)			
	Sensitive	Resistant	*Pf*CRT mutations	*Pf*DHFR mutations
PYR	0	36	36	36
CQ	0	36	36	36
MQ	9	27	36	36
DHA	22	14	36	36
P218	36	0	36	36