EFFECT OF RIFAMPICIN AND OMEPRAZOLE IN COMBINATION WITH CHLOROQUINE ON CHLOROQUINE RESISTANT PLASMODIA IN VITRO AND IN VIVO

By

LOW JEN HOU

Thesis submitted in fulfillment of the requirements for the degree of

Master of Science

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette transporter superfamily
ATP	adenosine triphosphate
CDC	Center for Disease Control
CFTR	cystic fibrosis transmembrane conductance regulator
CQ	chloroquine
CQR	chloroquine resistant
CQS	chloroquine sensitive
CYP	cytochrome P-450 mixed function oxidases
dhfr	dihydrofolate reductase gene
dhps	dihydropteroate synthase gene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dpm	disintegrations per minute
dsDNA	double-stranded deoxyribonucleic acid
DV	digestive vacuole
FIC	fractional inhibitory concentration
h	hours
IC_{50}	median inhibitory concentration
IRBC	infected red blood cells
MDR	multi drug resistance
min	minutes
MRP	multi resistant protein
NAD	nicotinamide adenine dinucleotide
OMP	omeprazole
PCR	polymerase chain reaction
PCV	packed cell volume
Pfcrt	plasmodium falciparum chloroquine resistance transporter
Pfmdr1	plasmodium falciparum multidrug resistance gene
Pgh1	p-glycoprotein homologue 1
P-gp	p-glycoprotein
pLDH	parasite lactate dehydrogenase
RBC	red blood cells
RIF	rifampicin
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
s	seconds
SNP	single-nucleotide polymorphism
TCM	tissue culture medium
VPL	verapamil
WHO	World Health Organization

ABSTRAK

Kekebalan terhadap chloroquine telah mengakibatkan peningkatan kematian dan morbiditi daripada penyakit malaria *P. falciparum*. Ketiadaan vaksin yang berkesan mengakibatkan terapi malaria bergantung pada kombinasi ubat alternatif seperti Mefloquine dan Artesunate dan tahap kekebalan terhadap ubat-ubat alternatif tersebut kerap meningkat. Adalah diketahui bahawa agen perencat glycoprotein P berupaya menghalang kekebalan sel barah terhadap pelbagai ubat melalui pencegahan effluks ubat anti-barah oleh glycoprotein P. P. falciparum juga didapati membentuk homolog glycoprotein P (pgh1) pada membrannya dan Verapamil dan pengujakimia lain (contohnya: antihistamin) boleh mengekang kekebalan Chloroquine. Oleh demikian, adalah dicadangkan bahawa agen perencat glycoprotein P boleh berfungsi sebagai pengujakimia yang berkesan kerana pengangkut dalam kelas "ABC superfamili" kerap mempunyai substrat dan agen perencat yang serupa.

Dua agen perencat p-gp (rifampicin dan omeprazole) diuji dalam kajian ini secara in vitro dan in vivo dengan matlamat menghalang kekebalan terhadap Chloroquine.

P. falciparum yang sensitif terhadap chloroquine and yang kebal terhadap chloroquine (strain HB3, TM90C2B, Dd2) dicairkan daripada keadaan sejukbeku dan dikultur dalam media RPMI 1640, sel darah merah 'O' dan serum pada 37˚C b◌̊erdasarkan protocol Trager-Jansen yang diubahsuai. Chloroquine dicairkan secara berperingkat dan diuji bersama Rifampicin/Omeprazole dalam nisbah

kepekatan yang berbeza-beza dalam plat micro ELISA steril yang mengandungi kultur parasit. Selepas tempoh inkubasi yang tertentu, plat ujian dikeluarkan and keputusan dicapai melalui kaedah kematangan schizont atau melalui pergabungan hipozantina radioaktif dengan DNA parasit.

P. berghei (strain NK65, ANKA) juga dicairkan dan disuntik ke dalam ruang peritoneal tikus Swiss Albino yang berumur 6-7 minggu. Bila virulen menjadi stabil, pelbagai amaun Chloroquine diberikan bersama Rifampicin atau Omeprazole mengikut regim Peter's selama 4 hari berturut-turut. Peratusan parasitemia diketahui melalui calitan nipis darah tikus pada sisip kaca. Hari parasit muncul kembali dalam darah dan hari kematian tikus juga dicatat.

Kajian in vitro menunjukkan bahawa Rifampicin bersifat amat antagonis terhadap chloroquine untuk strain HB3 dan TM90C2B (FIC purata >2). Takat antagonis berkurangan apabila tempoh inkubasi drug bersama parasit dipanjangkan ke 72 jam. Keputusan ini disokong dalam kajian in vivo di mana tikus yang diberi rifampicin serta chloroquine menunjukkan parasit muncul kembali dengan lebih awal dan kematian tikus meningkat.

Kajian in vitro Omeprazole menunjukkan ia juga bersifat antagonis terhadap Chloroquine tetapi tidak seteruk berbanding interaksi Rifampicin-Chloroquine. Pada dos harian 5-20 mg/kg, kajian in vivo mendedahkan bahawa Omeprazole mengurangkan kematian tikus dalam tempoh pemerhatian sebulan dan melambatkan kemunculan semula parasit dalam darah, tetapi keputusan tersebut tidak mencapai tahap signifikan bila diuji dengan statistik. Tiada kesan yang nyata pada kadar penurunan parasitemia.

Rifampicin bersifat amat antagonis terhadap chloroquine secara in vitro and in vivo. Maka, kombinasi Rifampicin-Chloroquine patut dielakkan dalam amalan klinikal. Kemungkinan bahawa penggunaan Rifampicin dalam pesakit TB yang juga menghidap malaria boleh menyebarluaskan kekebalan Chloroquine kerana pemanjangan tempoh untuk membasmi parasit.

Kedua-dua inhibitor p-glycoprotein tidak merupakan pengujakimia yang berkesan buat gejala kekebalan chloroquine. Maka mekanisme kekebalan parasit malaria terhadap Chloroquine perlu diselidik semula untuk menjumpai agen yang lebih sesuai untuk mengekang kekebalan Chloroquine.

ABSTRACT

Chloroquine resistance has resulted in a resurgence of morbidity and mortality from *P. falciparum* malaria. The lack of an effective vaccine forces dependence on alternative drug combination therapies based on mefloquine or artesunate, of which the incidence of multidrug resistance is steadily rising. P-glycoprotein inhibitors reverse multidrug resistance in tumour cells by preventing efflux of anti-neoplastic drugs by p-glycoprotein, an ATP-binding cassette transporter (ABC). *P. falciparum* was also found to possess a p-gp homologue (pgh1) and that verapamil and other chemosensitizers (antihistamines, antidepressants) are able to reverse chloroquine resistance. It was postulated that potent p-gp inhibitors would be effective chemosensitizers as transporters of the ABC superfamily frequently share similar substrates and inhibitors. 2 known p-gp inhibitors (rifampicin and omeprazole) are assayed in this study, *in vitro* and *in vivo*, aiming to achieve chemoreversal of chloroquine resistance.

Chloroquine-sensitive and chloroquine-resistant *P. falciparum* (HB3, TM90C2B, Dd2 strains) were thawed and maintained in continuous culture using RPMI 1640 media, 'O' RBCs and pooled serum at 37°C using a modified Trager-Jansen candle jar protocol. Serial two fold dilutions of chloroquine and assayed chemosensitizers were performed on sterile ELISA microplates in checkerboard pattern to achieve different drug ratios in respective culture wells.

After a fixed incubation period, test plates were harvested and results obtained by assessing schizont maturation or incorporation of radioactive hypoxanthine.

P. berghei (NK65, ANKA strains) were thawed and injected intraperitoneally into naïve Swiss Albino mice (6-7 weeks old). Upon stabilization of virulence, various chloroquine doses combined with rifampicin or omeprazole were administered intraperitoneally following a standard Peters' consecutive 4-day treatment regime. Percentage parasitemia was assessed by thin smears of tail blood. Day of recrudescence and survival within a month were also noted.

In vitro rifampicin showed marked antagonism when combined with chloroquine against *P. falciparum* strains HB3 and TM90C2B (average FIC >2). Antagonism was less marked with a longer incubation period (72 hours). These results were supported in the *in vivo* study where mice receiving rifampicin and chloroquine showed earlier recrudescence and a drop in overall survival.

In vitro tests showed that omeprazole is mildly antagonistic when combined with chloroquine. The in vivo study revealed that for ANKA and NK65 strains, omeprazole at doses of 5, 10 and 20 mg/kg/day, slightly improves day of recrudescence and overall survival figures at the end of a month without reaching significance. Effect on rate of decline of parasitemia is very minimal.

As rifampicin is strongly antagonistic to chloroquine both *in vitro* and *in vivo*, such combinations should be avoided in clinical practice. It is possible that concomitant use of rifampicin in TB patients who also contracted malaria, may have helped propagate chloroquine resistance due to the lengthier clearance times of the parasite. As neither p-glycoprotein inhibitor is a suitable chemosensitizer for chloroquine resistance perhaps a reevaluation of resistance mechanisms would expedite the search for a suitable chemoreversal agent.

CHAPTER 1

INTRODUCTION

1.1 Malaria overview

Malaria, a parasitic infection transmitted by certain anopheline mosquitoes, is one of the most prevalent and pernicious diseases of humans, estimated to kill between 700,000 – 2.7 million people (mainly children) worldwide each year as shown in Table 1.1. It affects about 5% of the world's population. (Breman, 2001)

While Malaysia has one of the oldest malaria control programs in the world and large scale eradication programs and vector control measures that have resulted in a steep drop in incidence in the nineties, from 2.99/1000 population in 1994 to 0.56/1000 population in 2000. However despite the general success in controlling malaria, it remains an important health issue in remote areas. A good majority of cases occur in Sabah where emerging chloroquine resistance has been noted.

The widespread availability of cheap and effective anti-malarials, particularly chloroquine and sulphadoxine-pyrimethamine has curbed the extent of mortality and morbidity, but it has also encouraged the development and spread of resistance. While many strategies have been offered to counter chloroquine resistance, the lack of a truly effective vaccine has spurred investigators to intensify the search for cheap and potent new drugs/drug combinations to address this need.

Table 1.1 Deaths and malaria-related deaths (1,000s)

Area	**Population**	All deaths (%)	Malaria deaths (%)	Malaria/total (%)
World	6,122,210	56,554	1,124	2.0
Africa	655,476	10,681	963 (85.7)	9.0
America	837,967	(18.9)	1 (-)	0.02
Eastern	493,091	5,911 (10.5)	55 (4.9)	1.3
Mediterranean	874,178	4,156 (7.3)	0	-
Europe	1,559,810	9,703 (17.2)	95 (8.5)	0.7
Southeast Asia	1,701,689	14,467	10 (0.9)	0
Western Pacific		(25.6)		
		11,636		
		(20.6)		

Adapted from the World Health Organization World Health Report, 2002

1.2 Malaria lifecycle and clinical manifestations

There are four species of Plasmodia which infect humans, but *Plasmodium falciparum* accounts for the majority of instances of morbidity and mortality. Its virulence and adaptability are important factors which make it a deadly threat. While drug resistance for *P. vivax* has also been noted, this phenomenon is nowhere near as widespread as that of *P. falciparum*.

The lifecycle of a malarial parasite is a complex one as shown in Figure 1.1. An infected mosquito injects sporozoites into the blood of the host. Sporozoites enter liver cells, multiply and release merozoites into the blood stream. Merozoites invade red blood cells and start a phase of multiplication where they mature from ring forms to early trophozoites, late trophozoites, immature schizonts and mature schizonts which burst to release another batch of merozoites; a cycle which can be repeated indefinitely causing malarial symptoms. A small portion of merozoites develop sexually into gametocytes which are then taken up by mosquitoes when they feed, thus completing the cycle. A summary of various clinical manifestations of malaria is given in Figure 1.2.

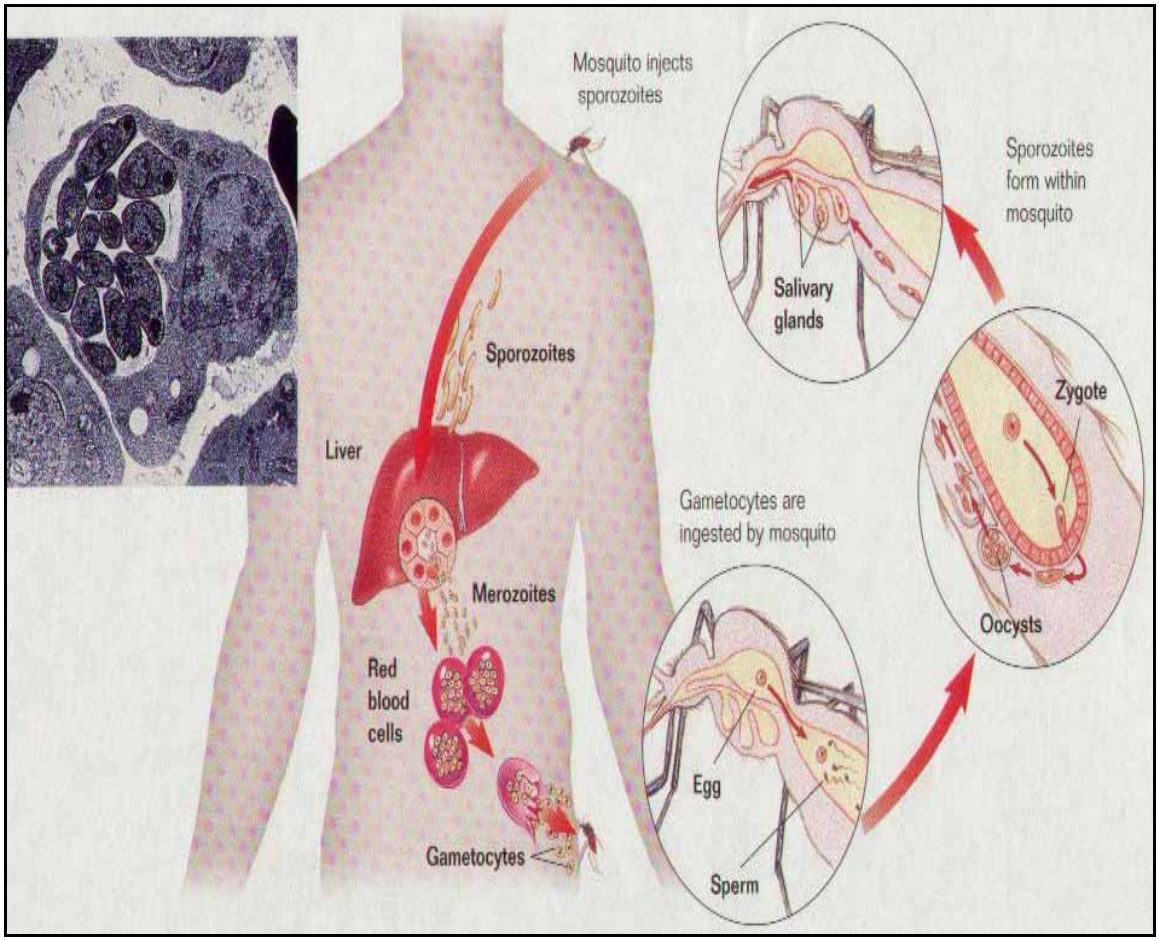

Figure 1.1 Lifecycle of Plasmodia