EFFECTS OF HYDROXYPYRIDINONE IRON CHELATORS IN COMBINATION WITH ANTIMALARIAL DRUGS ON THE *IN VITRO* GROWTH OF *PLASMODIUM FALCIPARUM*

Kovit Pattanapanyasat[1], Kitchaporn Kotipun[2], Kosol Yongvanitchit[3], Robert C Hider[4], Dennis E Kyle[3], D Gray Heppner[3] and Douglas S Walsh[3]

[1]Center of Excellence for Flow Cytometry, Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand; [2]Central Laboratory, Charoenkrung Pracharak Hospital, Bangkok Metropolitan Administration, Bangkok, Thailand; [3]Department of Immunology and Medicine, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; [4]Department of Pharmacy, King's College, University of London, Stamford Road, London, UK

Abstract. Using standard *in vitro* drug susceptibility methods, we assessed the antimalarial activity of 3 orally administered iron chelators (hydroxypyridinones) alone and in combination with conventional antimalarials drugs (quinine, mefloquine, artesunate, tetracycline, atovaquone) against a chloroquine-resistant *Plasmodium falciparum* isolate. When tested alone, all iron chelators and antimalarial compounds inhibited the growth of the parasites. IC_{50} values for iron chelators were 60-70 μM, whereas the IC_{50} values for antimalarial drugs were in nM ranges, with artesunate being the most potent. The derived isobolograms for the interaction of hydroxypyridinones and antimalarial drugs showed addition or mild antagonism, similar to desferroxamine (Sum of Fractional Inhibitory Concentration, Σ FIC < 0.5 or > 4.0). Despite the absence of synergy with conventional drugs, intrinsic antimalarial activity of hydroxypyridinones supports the continued assessment of these iron chelators as treatment adjuncts.

INTRODUCTION

The global emergence of multiple drug-resistant (MDR) *Plasmodium falciparum* underscores the need for new antimalarial drugs, especially combination therapies. Clinical studies have shown that iron supplementation in iron deficient individuals exacerbates malaria, implying that iron is important to parasite growth (Murray *et al*, 1983; Oppenheimer *et al*, 1986). Thus, it has been suggested that iron chelators, especially those with a high affinity for iron III, may be useful adjunctive antimalarial agents. Indeed, the iron chelator desferroxamine (DFO) has been shown to affect parasitemia in preclinical and clinical trials (Raventos-Suarez *et al*, 1982; Bunnag *et al*, 1992; Gordeuk *et al*,1992a,b; 1993) and, as an adjunctive therapy, it appeared to shorten recovery from cerebral malaria (Gordeuk *et al*, 1992a). However, DFO is parenterally administered, has a short half-life, and is expensive. In contrast, another group of iron chelators called α-keto hydroxypyridinones (KHPs) are orally administered, relatively inexpensive, and suppress the growth of *P. falciparum in vitro* (Heppner *et al*, 1988; Hershko *et al*, 1991; Mastrardrea *et al*, 1992; Pattanapanyasat *et al*, 1997). Here, we evaluated KHPs alone and in combination with conventional antimalarial drugs (quinine, mefloquine, artesunate, tetracycline and atovaquone) against *in vitro* growth of *P. falciparum.*

Correspondence: Dr Kovit Pattanapanyasat, Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Bangkok 10700, Thailand.
Tel: (66-2) 4117000 ext 6644; Fax: (66-2) 4110155
E-mail: grkpy@mahidol.ac.th

MATERIALS AND METHODS

Culture of *Plasmodium falciparum*

A chloroquine-resistant clone of *P. falciparum* (TM267TR) was obtained from a stock of a continuous line maintained in human red blood cells in RPMI 1640 medium (Gibco) containing 10% human serum, 25 mM HEPES (Sigma), and 25 mM $NaHCO_3$. Parasite growth was synchronized by a sorbitol lysis method (Lambros and Vanderberg, 1979). Before use, the parasites were washed twice with warm RPMI 1640 medium, and diluted with normal red blood cells to a final hematocrit and parasitemia of 1% and 0.5%, respectively. Tests for drug susceptibility were performed by an established method (Webster *et al*, 1985) whereby 200 μl of a parasitized red blood cell suspension was incubated with 50 μl of the iron chelator alone or in combination with an antimalarial drug at various concentrations in 96 well microtiter plates. The plates were incubated at 37°C in 5% O_2, 5% CO_2 and 90% N_2. After 24 hours, the cultures were pulsed with ^{3}H-hypoxanthine (specific activity 1Ci/ml) by adding 0.6 μCi of isotope to each well. Microtiter plates were returned to the incubation chamber for an additional 18 hours. Then, each plate was harvested onto glass fiber discs using a TOMTEC MASH II cell harvester. Scintillation cocktail (Omnifluor, New England Nuclear Research Products, Boston) was added and radioactivity was determined by a Betaplate liquid scintillation counter (Wallac, Finland).

Preparation of iron chelators and antimalarial drugs

1,2-dimethyl-3-hydroxypyridin-4-one (deferiprone, L1, CP20), 1-(2′ carboxyethyl)-2-methyl-3-hydroxypyridin-4-one (CP38) and 1-(2′ carboxyethyl)-2-ethyl-3-hydroxypyridin-4-one (CP110) were prepared as previously described (Dobbin *et al*, 1993). The CP20 stock solution was dissolved in RPMI 1640 medium at 10 mg/ml. Stock solutions of CP38 and CP110 were made in DMSO at a concentration of 10 mg/ml. Stock solutions of the antimalarial drugs quinine, mefloquine, and artesunate (a succinate ester derivative of dihydroartemisinin) were prepared in 95% ethanol to give concentrations of 1, 4, and 4 mg/ml, respectively. Tetracycline and atovaquone (2-[trans-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1, 4-naphthoquinone) were solubilized in DMSO with subsequent dilution in RPMI 1640 medium to give concentrations of 20 and 1 mg/ml, respectively. All stock solutions were then diluted with RPMI 1640 culture medium to initial concentrations 10 to 50 times the estimated 50% inhibition concentrations (IC_{50}). When studying drug combinations, solutions of these initial concentrations were combined in various ratios of iron chelator to antimalarial, 4:1, 2:1, 1:2 and 1:4. Single and combination test solutions were then added into 96-well microtiter plates to give triplicate wells of iron chelators alone, an antimalarial drug alone, or a combination of two agents. Seven serial dilutions of the agents with media were made to fill the plate using a 12-channel pipetter.

In separate experiments, preformed fully-saturated chelator-iron complexes were prepared by adding a newly neutralized acid solution of ferric chloride (Merck) with each chelator using a chelator (KHP) to iron molar ratio of 3:1. Chelator-iron complexes were added to the parasite cultures at a final concentration of 300 μM, a 4-fold higher concentration than their respective IC_{50}s.

Data analysis

The IC_{50} values of individually tested agents were obtained from dose response curves generated from serial dilutions conducted in triplicate by a computerized, non-linear regression analysis. Drug combinations comprised of iron chelators and antimalarial drugs were expressed as the sum of the fractional inhibitory concentrations (Σ FIC), according to the method of Berenbaum (1978):

$$\Sigma\ FIC = \frac{IC_{50} \text{ of agent A in mixture}}{IC_{50} \text{ of agent A alone}} + \frac{IC_{50} \text{ of agent B in mixture}}{IC_{50} \text{ of agent B alone}}$$

Σ FIC values were defined as synergism (< 0.5), antagonism (> 4.0), and additive (unity). Isobolograms were constructed from the resulting IC_{50}s. A convex isobole indicated antagonism, a straight line addition, and a concave line synergism.

RESULTS

Table 1 shows comparative IC_{50} data for

Table 1
IC_{50} for iron chelators and conventional antimalarial drugs on the growth of *P. falciparum*.

Agents	No. of experiments	IC_{50}
CP20	18	67.5 μM
CP38	18	56.1 μM
CP110	20	53.1 μM
Quinine	9	215.3 nM
Mefloquine	9	40.7 nM
Artesunate	9	2.6 nM
Tetracycline	9	80.3 nM
Atovaquone	12	8.6 nM

Table 2
Effect of KHPs, iron, or preformed chelator-iron complexes (chelates) on *P. falciparum* growth.

Reagents	% of control[a]
Medium alone	100
Iron alone	98.1 ± 7.6
CP20	4.3 ± 2.1
CP20 + Iron	89.2 ± 9.4
CP38	6.1 ± 3.3
CP38 + Iron	92.5 ± 7.6
CP110	5.5 ± 2.7
CP110 + Iron	95.1 ± 10.2

[a]Data represent mean values from at least 4 independent experiments.

Table 3
Σ FIC values for combinations of KHPs and conventional antimalarial drugs against *P. falciparum*[a].

Drug	CP20	CP38	CP110
Quinine	1.28 - 1.58	1.16 - 1.59	0.9 - 1.56
Mefloquine	1.16 - 1.66	1.03 - 1.53	1.02 - 1.54
Artesunate	1.19 - 1.60	1.20 - 1.86	1.30 - 2.14
Tetracycline	0.82 - 1.28	1.09 - 1.70	0.86 - 1.66
Atovaquone	0.93 - 1.25	0.92 - 1.26	0.88 - 1.38

[a]S FIC values < 0.5 ~ syngerism, 1 ~ addition, > 4.0 ~ antagonism.

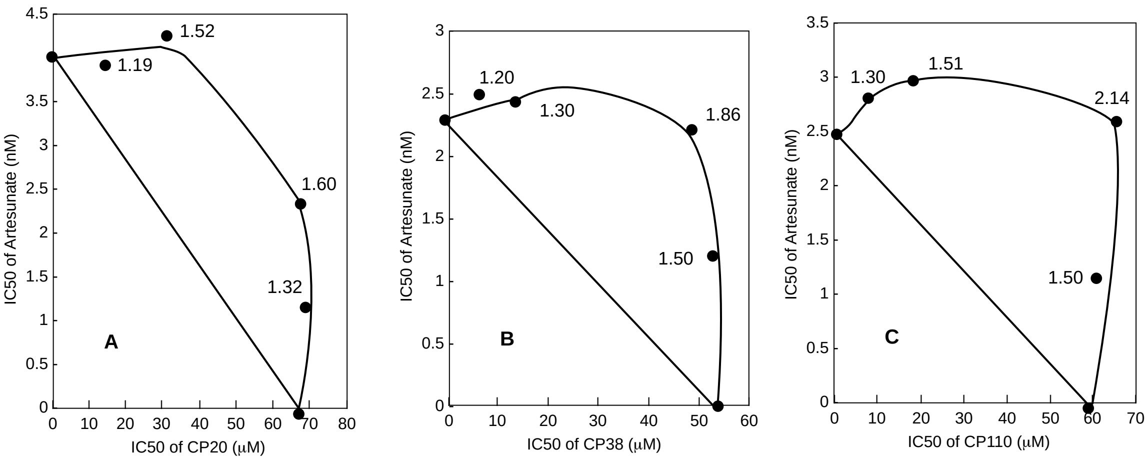

Fig 1– Isobolograms depicting interactions of artesunate with iron chelators CP20 **(A)**, CP38 **(B)** and CP110 **(C)**. Numbers in the figure indicate Σ FIC values from each combination.

KHPs and standard antimalarial drugs. The mean IC_{50} ± SEM for KHPs was 50-70 μM. Among the antimalarial drugs, artesunate had a mean IC_{50} of 2 nM whereas the mean IC_{50} of quinine was 215 nM. When chelators were mixed with ferric iron and before addition to parasite cultures, the antimalarial activity of the chelators at 300 μM, which resulted in more than 90% parasite growth inhibition, was totally abolished (Table 2). Ferric chloride alone, at the concentration tested, had no effect on parasite growth.

The combined effects of KHPs and antimalarial drugs at various concentrations as indicated by the Σ FIC are summarized in Table 3. The KHPs exhibited similar activities. Typical isobolograms determined by IC_{50} from

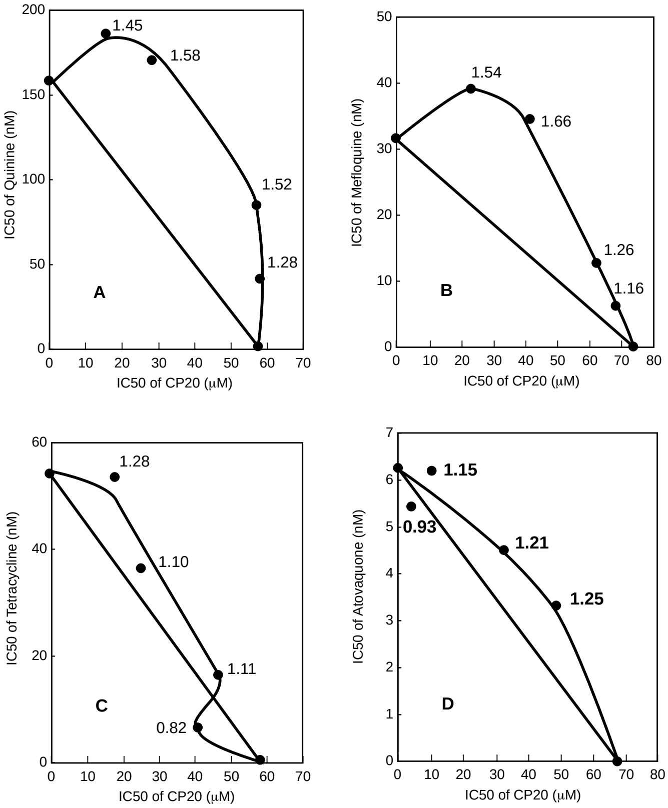

Fig 2–Isobolograms depicting interactions of iron chelator CP20 with antimalarial drugs quinine **(A)**, mefloquine **(B)**, tetracycline **(C)** and atovaquone **(D)**. Numbers in the figure indicate Σ FIC values from each combination.

each KHP in combination with artesunate showed slight antagonism, as depicted by a moderately upward convex curve (Fig 1). Combination of KHPs and other antimalarial drugs resulted in mild antagonistic or additive effects. Representative isobolograms of CP20 and antimalarial drugs are shown in Fig 2.

DISCUSSION

Clinical and laboratory observations suggest that iron metabolism and malaria infections are closely inter-related (Murray *et al*, 1983; Oppenheimer *et al*, 1986; Harvey *et al*, 1989). Depriving parasites of iron with iron chelators results in suppression of growth (Raventos-Suarez *et al*, 1982; Heppner *et al*, 1988; Hershko *et al*, 1991; Gordeuk *et al*, 1992, 1993; Bunnag *et al*, 1992). Our assessment of the susceptibility of a chloroquine-resistant clone of *P. falciparum* to several synthetic orally active KHPs is consistent with previous findings. KHPs exerted a significant growth inhibitory effect, perhaps by sequestrating endogenous iron as pre-saturation of the chelator with ferric iron resulted in neutralization of the chelator-alone anti-malarial activity. Inhibition of parasite growth by iron chelators may be attributed to suppression of ribonucleotide reductase activity, an iron-containing enzyme necessary for DNA synthesis that is inhibited by DFO and KHPs (Lederman *et al*, 1984; Pattanapanyasat *et al*, 1992). The effect on the enzyme is probably due to iron deprivation, affecting *de novo* enzyme synthesis, as well as the availability of metabolically active iron (Nyholm *et al*, 1993).

Despite *in vitro* data, clinical studies with DFO show that iron sequestration alone is insufficient to eliminate the malaria parasite. Thus, some recommend that iron chelators be used in combination with other antimalarial agents (Traore *et al*, 1991; Gordeuk *et al*, 1992b; 1993). However, the effect of antimalarial drugs on the parasite can be partially reversed by iron chelators (Kamchonwongpaisan *et al*, 1992; Posner *et al*, 1992; Zhang *et al*, 1992). Here, a modest antagonistic effect was observed, particularly for the KHP-artesunate combinations. This may be related to the iron requirement of artesunate whereby iron-mediated cleavage of the drug's endoperoxide bridge generates oxygen radicals that are toxic to the parasite (Meshnick *et al*, 1989; 1993; Posner *et al*, 1995). Additive or slight antagonistic effects when KHPs were combined with mefloquine, quinine, tetracycline and atovaquone are in general agreement with DFO studies whereby DFO-quinine and DFO-chloroquine were additive (van Zyl *et al*, 1992; Basco and Le Bras, 1993). An additive effect suggests an independent inhibition of parasite growth by each compound.

Although KHP-antimalarial drug cocktails were at best additive in suppressing *P. falciparum*, the concept of iron chelation by a KHP, in relation to DFO, warrants consideration: 1) both inhibit parasite growth by iron deprivation; 2) both form non-toxic iron chelators; 3) both exhibit additive or mildly antogonistic effects with leading antimalarial drugs; and 4) both inhibit the ability of iron to generate hydroxy radical-mediated tissue damage. We predict KHPs will exhibit *in vivo* antimalarial activity similar to DFO and encourage their evaluation as affordable iron chelators of potential benefit in *P. falciparum* malaria.

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