รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษาคุณสมบัติของ pyronaridine ที่มีฤทธิ์ต้านเชื้อมาลาเรีย

โดย นางสาวสรัญญา อุปรักขิตานนท์

มิถุนายน 2549

สัญญาเลขที่ TRG4780005

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หน่วยพิษวิทยา ภาควิชาพยาธิวิทยา คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี
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บทคัดย่อ

รหัสโครงการ	**TRG4780005**
ชื่อโครงการ	โครงการการศึกษาคุณสมบัติของ **pyronaridine** ที่มีฤทธิ์ต้านเชื้อมาลาเรีย
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สาร **pyronaridine** เป็นสารชนิด **Mannich base** มีโครงสร้างคล้ายกับยา **quinacrine** ซึ่งเป็นสารประเภท **aminoacridine** **pyronaridine** ถูกสังเคราะห์ขึ้นครั้งแรกที่ประเทศจีน ทดสอบฤทธิ์ต่อเชื้อมาลาเรีย ***Plasmodium falciparum*** ชนิด **K1** พบว่า **pyronaridine** มีฤทธิ์ต้านเชื้อมาลาเรีย โดยมีค่า IC_{50} เท่ากับ **3** นาโนโมลาร์ และทดสอบความสามารถในการยับยั้งการเกิด **β-hematin formation** พบว่า **pyronaridine** มีฤทธิ์ยับยั้งการเกิด **β-hematin formation** มีค่า IC_{50} เท่ากับ **125** ไมโครโมลาร์ โดย **pyronaridine** จับกับ **hematin** มีค่า **stoichiometry** เท่ากับ **1:2** การที่ **pyronaridine** จับกับ **hematin** ทำให้ **pyronaridine** เสริมฤทธิ์กับ **hematin** ในการทำให้เม็ดเลือดแดงแตกมากกว่าในการทดลองที่มี **hematin** เพียงอย่างเดียวและพบว่า **glutathione** กระตุ้นการสลายของ **hematin** เมื่อทดสอบฤทธิ์ของ **pyronaridine** กับยาต้านมาลาเรียตัวอื่น ๆ ที่มีความสามารถยับยั้งการเกิด **β-hematin formation** ได้แก่ **chloroquine** **mefloquine** และ **quinine** ที่เป็นสัดส่วนกันพบว่าผลที่ได้เป็นไปในทาง **antagonism effect** แต่กับ **concanamycin A** ซึ่งเป็นสารยับยั้งเอนไซม์ **vacuolar H^+-ATPase** และมีฤทธิ์ต้านเชื้อมาลาเรีย ***Plasmodium falciparum*** ชนิด **K1** IC_{50} ที่ต่ำมากคือ **0.2** นาโนโมลาร์ พบว่า **pyronaridine** มีฤทธิ์กับ **concanamycin A** เป็นแบบ **additive effect**

คำหลัก **Pyronaridine, มาลาเรีย, *Plasmodium falciparum*, β-hematin formation**

ABSTRACT

Project Code: TRG4780005

Project Title: Studies on the mechanism of action of the antimalarial drug pyronaridine

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Project Period: July 2004 – June 2006

Pyronaridine, 2-methoxy-7-chloro-10[3',5'-bis(pyrrolidinyl-1-methyl)4'hydroxyphenyl] amino-benzyl-(b)-1,5-naphthyridine, a new Mannich base schizontocide originally developed in China and structurally related to the aminoacridine drug quinacrine, is currently undergoing clinical testing. We now show that pyronaridine targets hematin, as demonstrated by its ability to inhibit in vitro ß-hematin formation (at a concentration equal to that of chloroquine), to form a complex with hematin with a stoichiometry of 1:2, to enhance hematin-induced red blood cell lysis (but at 1/100 of the chloroquine concentration), and to inhibit glutathione-dependent degradation of hematin. Our observations that pyronaridine exerted this mechanism of action *in situ*, based on growth studies of *Plasmodium falciparum* K1 in culture showing antagonism of pyronaridine in combination with antimalarials (chloroquine, mefloquine, and quinine) that inhibit ß-hematin formation, were equivocal. Pyronaridine also exhibited an additive effect with concanamycin A, a macrolide antibiotic inhibitor of vacuolar H^+-ATPase derived from *Streptomyces* sp., which had a surprisingly low IC_{50} value of 0.2 nM.

Keywords: Pyronaridine, Malaria, *Plasmodium falciparum*, β-hematin formation

กิตติกรรมประกาศ

การศึกษาวิจัยนี้บรรลุผลสำเร็จได้โดยการสนับสนุนของคณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี และภาควิชาชีวเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ที่ให้ใช้เครื่องมือและสถานที่ และสำนักงานกองทุนสนับสนุนการวิจัย ที่ให้ทุนวิจัย

ผู้วิจัย
มิถุนายน 2549

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INTRODUCTION

Malaria still presents an important health problem in tropical regions of the world, with over 275 million new cases annually and mortality reaching 2 million, particularly among children in Africa (1). Control of this debilitating disease has been severely compromised by the development in malaria parasites, particularly *Plasmodium falciparum*, the most virulent of the four species infecting man, of resistance to nearly all currently employed antimalarial drugs used for prophylaxis and treatment. Thus, there is a compelling and urgent need for new antimalarials to replace those that are becoming obsolete, and also to identify the mechanism of action of existing antimalarial drugs so that appropriate modifications can be made to them for future use in drug-resistant parasites.

Antimalarial drugs may be broadly classified into two groups: lysosomotropic quinoline-containing drugs and antimetabolites. The former compounds include chloroquine, quinine, quinacrine, amodiaquine, mefloquine and related drugs (2). All of these drugs act exclusively on the blood stages of *P. falciparum*. The quinolines, mainstays of treatment and prophylaxis, are excellent parasiticidal agents for susceptible organisms but are becoming increasingly less efficacious. Chloroquine was introduced in 1940s with great optimism for the control of malaria because the drug was cheap, highly effective, and well tolerated. Due to its specificity, stability and safety, chloroquine has been one of the most successful and widely used antimalarial drugs. However, the mechanism of action of these drugs that contain a quinoline nucleus has, until recently, remained obscure.

In *P. falciparum*, hemoglobin is degraded in an acidic vacuolar compartment to yield amino acids for parasite protein synthesis, and this occurs predominantly at the trophozoite and early schizont stages (3). In higher eukaryotes, heme oxygenase catabolizes the porphyrin moiety to carbon monoxide, ferric ion and biliverdin (4). Although the malaria parasite contains heme oxygenase (5), this metabolic pathway is not utilized (6) and, instead, heme is detoxified by a number of other mechanisms. These include sequestration into an insoluble material termed hemozoin or malaria pigment (7), and degradation by glutathione-dependent (8) and peroxidative processes (9), but the major mechanism of heme detoxification appears to be the hemozoin sequestration pathway (10).

Chloroquine has recently been shown to inhibit hemozoin formation within the parasite food vacuole (11). Hemozoin was originally considered to be formed by the polymerization of heme (12), but it has now been demonstrated to be a crystalline cyclic dimer of ferriprotoporphyrin IX (13). Antimalarials such as chloroquine can be considered as

crystallization inhibitors or as acting to divert heme from participating in the crystallization process (13), leading to accumulation of free heme that is potentially toxic. This mechanism has also been attributed to the action of antimalarial acridines such as quinacrine (14). Thus hemozoin synthesis, a process unique to the malaria parasite, offers a logical and valuable potential target for new antimalarial drug development.

Pyronaridine, (2-methoxy-7-chloro-10[3',5'-bis(pyrrolidinyl-1-methyl)4'-hydroxyphenyl] amino-benzo-(b)1,5-naphthyridine), a new Mannich base and highly active blood schizonticidal antimalarial drug developed in China, is structurally related to the aminoacridine drug, quinacrine, and is a potential cheap substitute for chloroquine (15). The Chinese have had over ten years of successful experience from it, particularly against the chloroquine-resistant malaria. However, the mechanism of action remains unclear. The current Chinese oral formulation is reportedly effective and well tolerated, but its oral bioavailability is low, and this contributes to an unacceptably high treatment cost. WHO is now developing a capsule formulation, which appears to be more bioavailable, but there is currently no industrial partner (16).

In *P. falciparum* infections in monkey (*Aotus trivirgatus*), pyronaridine appears to interfere with the parasitic digestive system (17). Chavalitshewinkoon *et al* reported in 1993 that *P. falciparum* DNA topoisomerase II is inhibited by pyronaridine *in vitro* (18). Topoisomerase poisons act by stabilizing the covalent topoisomerase-DNA complexes with an enzyme-linked DNA break on a single strand or both strands. The trapping of such cleavable complexes prevents enzyme turnover and hinders the reclosure of DNA breaks. Drug-promoted DNA-protein adducts have proven extremely valuable because they provide a simple method to detect the existence of inhibitor-sensitive topoisomerase activity within cells and a means for assaying inhibitor potency *in vivo* (19). However, we have recently shown that pyronaridine does not generate protein-DNA complexes *in situ* and thus does not target malaria parasite DNA topoisomerase II (20).

OBJECTIVE

Based on its similarity to chloroquine (see Figure1), I hypothesize that pyronaridine acts in a similar way, namely, inhibits β-hematin formation *in vitro* (a process which closely parallels hemozoin synthesis within the parasite food vacuole), forms drug-hematin complex, and enhances hematin-induced lysis of red blood cells.

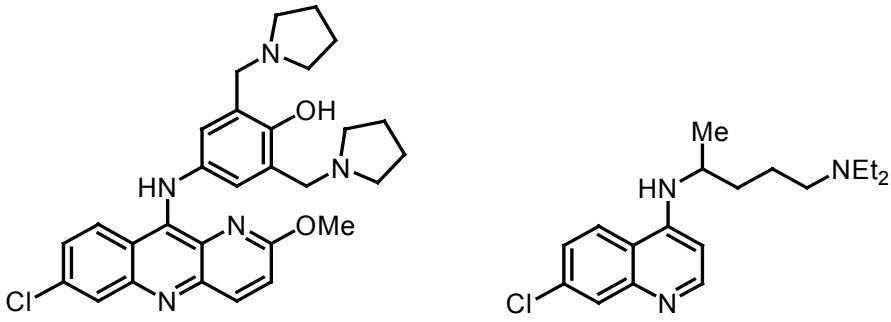

Figure1. Structure of pyronaridine and chloroquine

MATERIALS AND METHODS

Materials

All chemicals and reagents used in this study were reagent grade.

Malaria parasite

Plasmodium falciparum K1 (21) and T9/94 (22) were maintained under continuous culture *in vitro* using the candle-jar method of Trager and Jensen (23).

Materials for cultivation of *P. falciparum*

1. RPMI 1640 powder, Formula No. 430-1800, was product of GIBCO Laboratories.

2. Human erythrocytes (O, Rh^+) from local blood donors were kept at 4°C in blood bags containing acid citrate dextrose (ACD).

3. Human serum (group A, B, AB, or O) was stored at -20°C.

4. Human plasma (group A, B, AB, or O) was collected from ACD blood, platelets were removed and stored at -20°C.

5. Tissue culture plastic petri dish (60×15 mm and 100×15 mm) was purchased from Nunc.

Materials for drug susceptibility testing

1. Pyronaridine was kindly provided by Chang Sik Shin, Shin Poong Pharmaceutical Co., Ltd., Korea.

2. Chloroquine, concanamycin A, mefloquine and quinine were obtained commercially.

3. [^{3}H]-hypoxanthine (specific activity of 28.0 Ci/ mmol) was purchased from Amersham.

4. 96-Wells microculture plastic plates were purchased from Nunc.

Materials for inhibition of β-hematin formation assay, hematin binding assay and compound-induced red cell lysis.

1. Hematin was purchased from Sigma.

2. Sodium hydroxide, glacial acetic acid and dimethyl sulfoxide (DMSO) were purchased from Merck.

Instruments

Centrifuge	SORVAL RC 2 B Plus
Spectrophotometer	SHIMADZU UV- 250 IPC
Microcentrifuge	Hettich, MEQO 24-48R
Minicell harvester	Nunc
Liquid scintillation counter	Beckman, model LS-1801

***In vitro* assessment of antimalarial activity**. The protocol is a modification of the [^{3}H]-hypoxanthine incorporation method of Desjardins *et al.* (24). Sorbitol-synchronized parasite cultures (25) with mostly ring stages of *P. falciparum* K1 (chloroquine- and pyrimethamine-resistant) strain (21) and T9/94 (drug-sensitive) clone (22) are used. Packed infected red cells are adjusted to approximately 5.0% by the addition of 50% (v/v) uninfected erythrocytes. An aliquot of 0.3 mL of this suspension (50% hematocrit, 5.0% parasitemia) is added to 9.7 mL of complete medium (10% serum in RPMI 1640 culture medium supplemented with 32 mM $NaHCO_3$ and 25 mM HEPES buffer, pH 6.5). Pyronaridine is initially dissolved in dimethylsulfoxide (DMSO) and diluted with the above supplemented RPMI 1640 culture medium to the required concentrations. Aliquots of 200 µL of the above cell suspension (1.0% parasitemia of early ring stage) are incubated with 25 µL of the drug-containing medium in 96-well microtiter plates under “candle jar” condition for 24 h at 37°C prior to the addition of 25 µL of 0.5 µCi [^{3}H]-hypoxanthine (23). After a further incubation for 18 h, parasites are harvested from each well onto glass fiber filters, and lysed with distilled water. The filter discs are then placed in 1.5 mL microcentrifuge tubes and dried at 60°C for 1 h. An aliquot of 0.6 mL of liquid scintillation cocktail (0.35% (w/v) 2,4-diphenyloxazole and 0.005% (w/v) 1,4-bis[2-(5-phenyloxazoyl)]benzene in toluene) is added to each tube. The microcentrifuge tubes are placed in scintillation vials and [^{3}H]-hypoxanthine incorporation into parasite is determined in a liquid scintillation counter. The IC_{50} values (50% inhibition of radioactivity incorporation compared to control) are obtained from the drug dose-response curves.

Determination of drug combination

IC_{50} values of one drug (A) in the presence of a series of fixed concentrations of the other drug (B) were measured as described above. Results were expressed as the mean sums of the fractional inhibitory concentrations (FIC), defined as (IC_{50} of drug A in mixture/ IC_{50} of drug A alone) + (IC_{50} of drug B in mixture/ IC_{50} of drug B alone) for each fixed concentration. The IC_{50} value (nM) for *P. falciparum* K1 strain was as follows: chloroquine 590, concanaymycin A 0.2, mefloquine 22, pyronaridine 3, quinine 380. Three types of drug interaction were defined as follows: synergism, FIC < 0.5; antagonism, FIC > 4; and additive, FIC = 1 (26).

Inhibition assay of β-hematin formation. Inhibition of β-hematin formation is based on the method of Baelmans *et al*. (27) A 100 μL aliquot of freshly prepared hematin solution (41.175 mg of hematin in 10 mL of 0.2 M NaOH) is mixed with 50 μL of drug solution, or 50 μL of water for control, prior to the addition of 200 μL of 3 M sodium acetate and 50 μL of 17.4 M acetic acid (final pH was 4.8). After 24 h of incubation at 37°C the tubes are centrifuged at 4000*g* for 15 min and the supernatants removed. Pellets are resuspended five times in 200 μL of DMSO to remove unreacted hematin from β-hematin, which is insoluble in this solvent. The pellets are then dissolved in 0.1 M NaOH for spectroscopic quantification at 405 nm. Results of drug testing are expressed as 50% inhibitory concentration (IC_{50}) of β-hematin formation obtained from non-linear regression analysis of the drug dose-response curves.

Drug-hematin interaction assay. An aqueous DMSO (40% v/v) solution of 10 μM hematin, pH 7.4, is freshly prepared by mixing 25 μL of 4 mM hematin in 0.1 M NaOH solution with 4 mL of DMSO and 1 mL of 0.02 M sodium phosphate buffer, pH 6.0, and making up to 10 mL with doubly distilled deionized water (under these conditions hematin is monomeric) (28). Solutions of pyronaridine are similarly prepared. To examine hematin-drug interaction a continuous variation technique (Job's plot) is performed to determine the spectral changes (29). For each compound, solutions containing the following fourteen drug:heme (molar) combinations are prepared: 0:1, 1:9, 1:4, 3:7, 2:3, 1:1, 3:2, 5:3, 13:7, 27:13, 7:3, 4:1, 9:1, 1:0. The final combined concentration of hematin plus drug in the mixtures is 10 μM. Spectra are recorded in a Shimadzu UV-250 IPC spectrophotometer between 240-700 nm at a speed of 0.5 nm/min. Baselines are routinely subtracted from the spectra.

Drug-hematin induced red blood cell lysis.

Experiments on lysis of human red blood cells by hematin and drug-hematin complexes are conducted by incubating 0.03% (v/v) cell suspensions in phosphate buffer saline, pH 7.4, at 37°C for 1 h with varying concentrations of hematin in the absence or presence of pyronaridine and measuring the decrease in absorbance at 700 nm.

RESULTS

Chloroquine is believed to act within the malaria food vacuole by binding with heme and thereby interfering with the formation of crystalline hemozoin (11). This can be demonstrated in vitro by showing the capability of chloroquine to inhibit the formation of β-hematin, a process that closely parallels hemozoin synthesis within the parasite food vacuole (reviewed in reference 30). Pyronaridine inhibited β-hematin production with the same IC50 as chloroquine, 0.125 mM (31). In addition, pyronaridine formed a complex with hematin with a stoichiometry of 1:2 (Fig. 2), as did chloroquine under the same conditions (data not shown) (31).

Although the main site of action of chloroquine is within the malaria parasite acidic food vacuole, it has been reported that chloroquine can interfere with a glutathionedependent heme degradation process (8). Figure 3 shows that pyronaridine exhibited this property, in agreement with a previous report (32).

In addition to inhibiting hemozoin synthesis, the heme-chloroquine complex is capable of enhancing heme-induced destabilization of biological membranes (33, 34). Fifty percent hemolysis of 0.03% human blood cells, obtained with 4 μM hematin, is enhanced to completion in the presence of at least 1.0 μM chloroquine (31). Pyronaridine was also capable of enhancing hematin-induced lysis of human red blood cells (Fig. 4), but surprisingly, in the presence of 4 μM hematin, the minimum concentration of pyronaridine needed for complete hemolysis was 10 nM, about 1/100 of the concentration seen with chloroquine.

The data obtained thus far on pyronaridine-hematin interactions were obtained from in vitro studies. If pyronaridine interferes with hemozoin formation in situ, it should antagonize the inhibitory effect on *P. falciparum* K1 growth in culture of antimalarials known to interfere with hemozoin production by binding to heme (30). Combination studies of pyronaridine with choroquine, mefloquine, and quinine showed mild antagonistic effects (sum of FIC values ranging from 1.07 to 1.67). Ringwald et al. (35), using a fixed drug combination of 1:1, have demonstrated similar results between pyronaridine and chloroquine (FIC = 1.09), mefloquine (FIC = 1.54), and quinine (FIC = 1.55).

As it is unlikely that any new antimalarial drug will be used clinically as a monotherapy, we also investigated the combination of concanamycin A, a macrolide antibiotic inhibitor of vacuolar H^+-ATPase derived from *Streptomyces* sp., and pyronaridine. Sum of FIC of concanamycin A and pyronaridine in a drug combination study of their antimalarial activity *in vitro* demonstrated an additive effect (sum of FIC in the range 1.15 to 1.65).

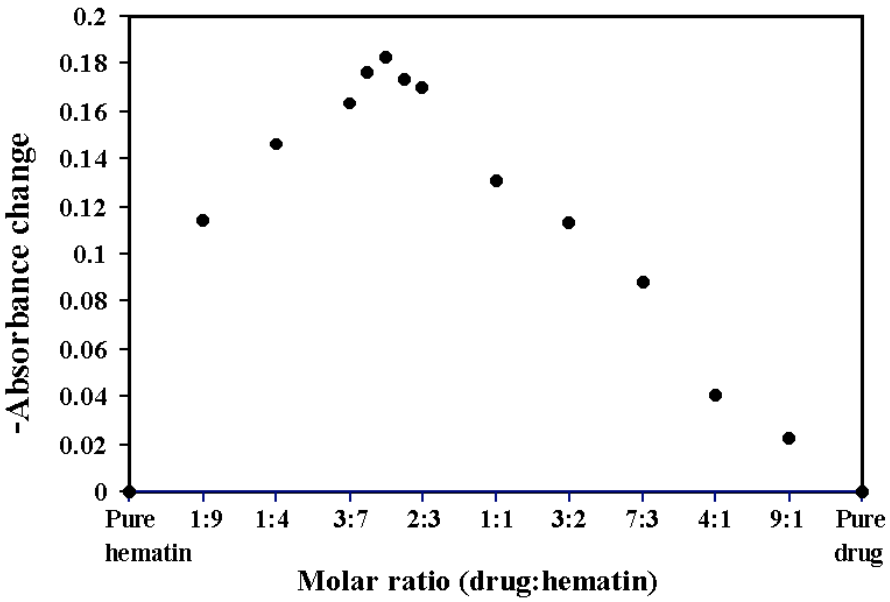

Figure 2 Job's plot of pyronaridine binding to hematin. The total concentration of the two components was 10 μM in 40% aqueous DMSO with mole fractions varying from 0 to 1. Absorbance was measured at 400 nm at 25ºC.

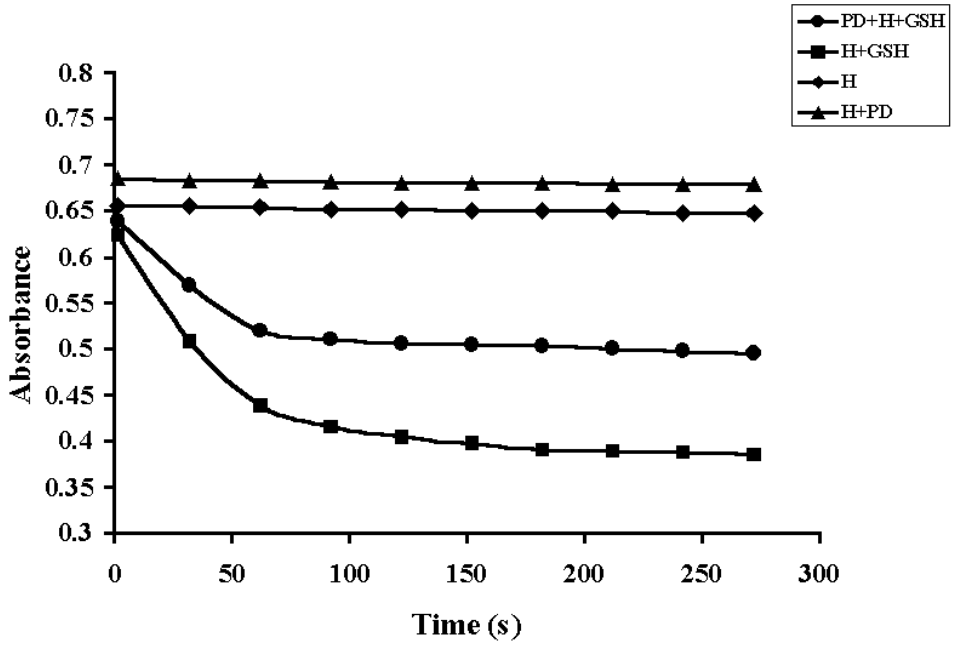

Figure 3 Effect of pyronaridine on GSH-mediated degradation of hematin. Hematin (5 μM) was incubated with GSH (2.5 mM) in the presence of 2.5 μM pyronaridine, and hematin degradation was followed spectrophotometrically at 400 nm.

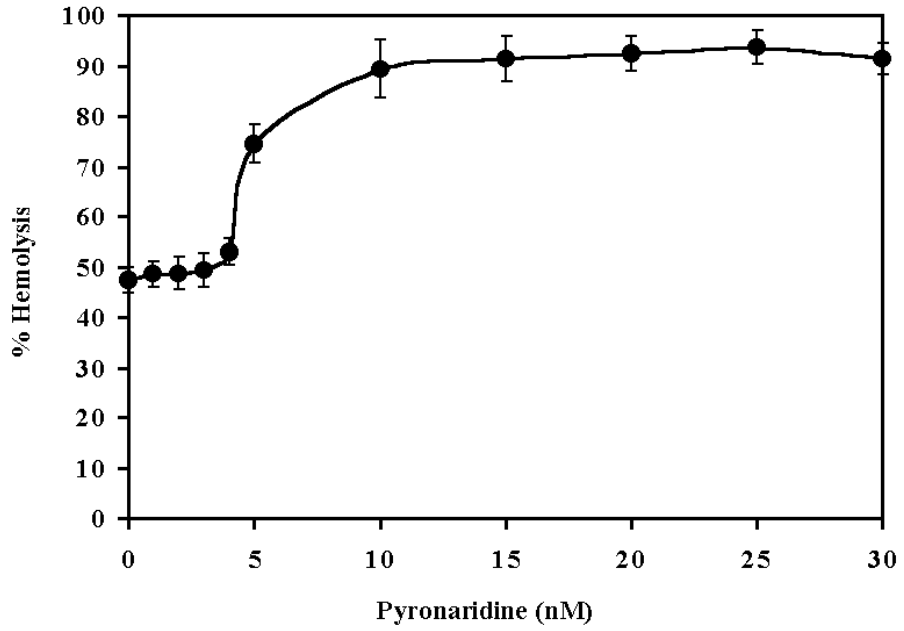

Figure 4 Effect of pyronaridine on hematin-induced hemolysis. Aliquots of 0.03% suspension of human red blood cells were incubated at 37°C and pH 7.4 for 1 h in the presence of 4 μM hematin and varying concentrations of pyronaridine. Hemolysis was determined by measuring the decrease in absorbance at 700 nm. Four μM hematin alone resulted in 50% hemolysis, and pyronaridine in the absence of hematin did not cause any hemolysis.

DISCUSSION

We have provided experimental evidences showing that pyronaridine acts as an antimalarial with a mechanism of action similar to the well-known 4-aminoquinoline, chloroquine, namely, it inhibits β-hematin formation *in vitro* (a process which closely parallels hemozoin formation within the parasite food vacuole), forms drug-hematin complex, inhibits glutathione-dependent degradation of hematin and enhances hematin-induced lysis of red blood cells, but at 1/100 the concentration seen with chloroquine. An interaction of pyronaridine with hematin had earlier been noted (36). In the case of the 4-aminoquinolines, the 7-Cl group has been shown to be absolutely required for inhibition of β-hematin formation (37); pyronaridine also contains the related Cl group. Translocation of free heme from the food vacuole to the cytosol has yet to be established and the major mechanism of heme detoxification appears to be the hemozoin sequestration pathway (10).

The unexpectedly low concentrations of pyronaridine in enhancing heme-induced red blood cell lysis result may account for the low IC_{50} of pyronaridine of 3 nM when compared with that of 590 nM for chloroquine. It has been suggested that the destabilizing effect of hematin on the red blood cell membrane results from direct binding or incorporation, which may affect the reciprocal interactions between membrane and cytoskeleton proteins (38). The mechanism by which chloroquine enhances hematin lysis of red blood cells has not been studied in detail but it has been demonstrated that hematin-chloroquine complex allows more efficient transfer of the hematin in solution to the phospholipids bilayer membrane (39). The ability of pyronaridine at surprisingly low concentrations to enhance the membrane perturbing property of free hematin suggests that this assay should be included in any future protocols designed to search for novel antimalarial pharmacophores interacting with hematin, a validated target of the malaria parasite. There has been an earlier report of ultrastructural changes caused by pyronaridine in intra-erythrocytic forms of *P. falciparum* occurring first in the food vacuoles (40). Results of experiments to show that pyronaridine targeted hematin *in situ* by showing in *P. falciparum* K1 growth in culture antagonism of pyronaridine in combination with antimalarials (chloroquine, mefloquine, quinine) that inhibit β-hematin formation was equivocal.

Although it is not possible to determine from the current study which of the various *P. falciparum* V-type H^+-ATPases is the target of concanamycin A, the very high sensitivity of *P. falciparum* to inhibition by this macrolide antibiotic suggests that it may be acting on the

parasite-encoded enzyme located at the host red blood cell plasma membrane, which is believed to play a key role in maintaining intracellular pH of the infected erythrocyte (40). However, the other parasite V-type H^+-ATPases cannot be ruled out as possible targets. Alkalinization of isolated *P. falciparum* digestive food vacuole has been shown to be achieved with 75 nM concanamycin A (41).

WHO is currently developing a combination of pyronaridine and artesunate for treatment of malaria (www.who.int/mediacentre/news/notes/np8/en/). Examples of the therapeutic use of combinations of antimalarial with an antibiotic include quinine-tetracycline or doxycycline, quinine-clindamycin and chlorproguanil-dapsone (Lapdap) (42).

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OUTPUT
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Publication

1. Saranya Auparakkitanon, Soebsakul Chapoomram, Kannika Kuaha, Thamrong Chirachariyavej and Prapon Wilairat. Targeting of hematin by the antimalarial pyronaridine. Antimicrob. Agents Chemother 2006; 50:2197-2200.

Manuscript

2. Saranya Auparakkitanon and PraponWilairat. Antimalarial activity of concanamycin a alone and in combination with pyronaridine. SE Asian J Trop Med Publ Hlth (Accepted).

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APPENDIX

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2006, p. 2197–2200 Vol. 50, No. 6
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Targeting of Hematin by the Antimalarial Pyronaridine

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Pyronaridine, 2-methoxy-7-chloro-10[3′,5′-bis(pyrrolidinyl-1-methyl-)4′hydroxyphenyl]aminobenzyl-(b)-1,5-naphthyridine, a new Mannich base schizontocide originally developed in China and structurally related to the aminoacridine drug quinacrine, is currently undergoing clinical testing. We now show that pyronaridine targets hematin, as demonstrated by its ability to inhibit in vitro β-hematin formation (at a concentration equal to that of chloroquine), to form a complex with hematin with a stoichiometry of 1:2, to enhance hematin-induced red blood cell lysis (but at 1/100 of the chloroquine concentration), and to inhibit glutathione-dependent degradation of hematin. Our observations that pyronaridine exerted this mechanism of action in situ, based on growth studies of *Plasmodium falciparum* K1 in culture showing antagonism of pyronaridine in combination with antimalarials (chloroquine, mefloquine, and quinine) that inhibit β-hematin formation, were equivocal.

Malaria constitutes a major health problem in tropical regions of the world, with 200 to 350 million cases annually and mortality reaching 3 million, particularly among children in sub-Saharan Africa (17). *Plasmodium falciparum*, the most virulent of the four species infecting humans, has become resistant to nearly all currently employed antimalarial drugs used for prophylaxis and treatment, with the exception of the artemisinins. Thus, there is an urgent need to identify new antimalarial drugs and to understand their mechanisms of action so that appropriate measures can be taken in their use to delay possible eventual ineffectiveness.

Pyronaridine, 2-methoxy-7-chloro-10[3′,5′-bis(pyrrolidinyl-1-methyl-)4′hydroxyphenyl]aminobenzyl-(b)-1,5-naphthyridine, is a new highly active blood schizonticidal Mannich base antimalarial drug developed in China, effective in treating malaria-infected patients in regions of chloroquine resistance (6, 18, 19, 21, 22). However, its mechanism of action remains unclear. Due to pyronaridine's similarity in structure to anilinoacridine (pyronaridine can be considered an aza-acridine), Chavalitshewinkoon et al. (5) reported that *P. falciparum* DNA topoisomerase II is inhibited in vitro by pyronaridine and anilinoacridine analogs. However, more recent studies have shown that pyronaridine does not cause the formation of a protein-DNA complex in situ and thus does not appear to target the malaria parasite DNA topoisomerase II (1).

However, certain antimalarial 9-anilinoacridines, in addition to inhibiting parasite DNA topoisomerase II, interact with hematin in a fashion similar to that of chloroquine (2). Based on these findings and on pyronaridine's similarity in structure to chloroquine (Fig. 1), we now demonstrate that pyronaridine acted similarly to chloroquine with regard to inhibition of β-hematin formation in vitro, formation of a drug-hematin complex, inhibition of glutathione (GSH)-dependent degradation of hematin, and enhancement of hematin-induced lysis of red blood cells. To prove that pyronaridine exerts this mechanism of action in situ, growth studies of *P. falciparum* in culture were conducted with pyronaridine in the presence of antimalarials that inhibit β-hematin formation (chloroquine, mefloquine, and quinine).

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MATERIALS AND METHODS

Parasite culture. *P. falciparum* strain K1 (chloroquine resistant) (26) was maintained in culture under the "candle jar" condition described by Trager and Jensen (27).

In vitro assessment of antimalarial activity. The protocol was a modification of the [^{3}H]hypoxanthine incorporation method of Desjardins et al. (9) and has been described previously (2).

Drug combination studies. Fifty percent inhibitory concentration (IC_{50}) values of one drug (A) in the presence of a series of fixed concentrations of the other drug (B) were measured as described above. Results were expressed as the mean sum of the fractional inhibitory concentrations (FIC) for each fixed concentration, defined as follows: (IC_{50} of drug A in mixture/IC_{50} of drug A alone) + (IC_{50} of drug B in mixture/IC_{50} of drug B alone). The IC_{50} values (nM) for *P. falciparum* K1 strain were as follows: chloroquine, 590; mefloquine, 22; pyronaridine, 3; and quinine, 380. Three types of drug interaction were defined as follows: synergistic, FIC < 0.5; antagonistic, FIC > 4; and additive, FIC = 1 (4).

Inhibition assay of β-hematin formation. Inhibition of β-hematin formation was based on the method of Baelmans et al. (3) as described previously (2). Results of the inhibition assay were expressed as IC_{50} values of β-hematin formation obtained from nonlinear regression analysis of the drug dose-response curves. Chloroquine was included as a control in these experiments as well as in all the following experimental protocols.

Pyronaridine-hematin interaction assay. To examine pyronaridine-hematin interaction, a continuous variation technique (Job's plot) was performed to determine the spectral changes (16) as described previously (2). Solutions containing the following 14 pyronaridine/hematin (molar) combinations were prepared: 0:1, 1:9, 1:4, 3:7, 2:3, 1:1, 3:2, 5:3, 13:7, 27:13, 7:3, 4:1, 9:1, and 1:0. The final combined concentration of hematin plus pyronaridine in the mixtures was 10 μM. Spectra were recorded in a Shimadzu UV-250 IPC spectrophotometer between 240 and 700 nm at a speed of 0.5 nm/min.

Pyronaridine-hematin-induced red blood cell lysis. Experiments on lysis of human red blood cells by hematin and pyronaridine-hematin complexes were conducted by incubating 0.03% (vol/vol) cell suspensions in phosphate-buffered

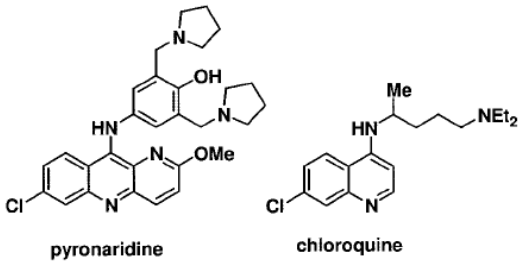

FIG. 1. Structures of pyronaridine and chloroquine.

saline, pH 7.4, at 37°C for 1 h and measuring the decrease in absorbance at 700 nm in the presence of increasing concentrations of hematin alone, to determine the concentration required for 50% hemolysis (4 μM), and then in the presence of 4 μM hematin with various concentrations of pyronaridine.

GSH-dependent hematin degradation. An aliquot of 500 μl of hematin (10 μM in 100% dimethyl sulfoxide [DMSO]) was mixed with 250 μl of pyronaridine (10 μM in 100% DMSO), and the absorbance of the solution was monitored at 405 nm every 15 s at room temperature (Shimadzu UV-250 IPC spectrophotometer), immediately following the addition of 250 μl of 10 mM GSH in HEPES buffer, pH 7.0.

RESULTS

Chloroquine is believed to act within the malaria food vacuole by binding with heme and thereby interfering with the formation of crystalline hemozoin (24). This can be demonstrated in vitro by showing the capability of chloroquine to inhibit the formation of β-hematin, a process that closely parallels hemozoin synthesis within the parasite food vacuole (reviewed in reference 25). Pyronaridine inhibited β-hematin production with the same IC_{50} as chloroquine, 0.125 mM (2). In addition, pyronaridine formed a complex with hematin with a stoichiometry of 1:2 (Fig. 2), as did chloroquine under the same conditions (data not shown) (2).

Although the main site of action of chloroquine is within the malaria parasite acidic food vacuole, it has been reported that chloroquine can interfere with a glutathione-dependent heme degradation process (15). Figure 3 shows that pyronaridine exhibited this property, in agreement with a previous report (13).

In addition to inhibiting hemozoin synthesis, the heme-chloroquine complex is capable of enhancing heme-induced destabilization of biological membranes (7, 8). Fifty percent hemolysis of 0.03% human blood cells, obtained with 4 μM hematin, is enhanced to completion in the presence of at least 1.0 μM chloroquine (2). Pyronaridine was also capable of enhancing hematin-induced lysis of human red blood cells (Fig. 4), but surprisingly, in the presence of 4 μM hematin, the minimum concentration of pyronaridine needed for complete hemolysis was 10 nM, about 1/100 of the concentration seen with chloroquine.

The data obtained thus far on pyronaridine-hematin interactions were obtained from in vitro studies. If pyronaridine interferes with hemozoin formation in situ, it should antagonize the inhibitory effect on *P. falciparum* K1 growth in culture of antimalarials known to interfere with hemozoin production by binding to heme (25). Combination studies of pyronaridine with choroquine, mefloquine, and quinine showed mild antagonistic effects (sum of FIC values ranging from 1.07 to 1.67). Ringwald et al. (23), using a fixed drug combination of 1:1, have demonstrated similar results between pyronaridine and chloroquine (FIC = 1.09), mefloquine (FIC = 1.54), and quinine (FIC = 1.55).

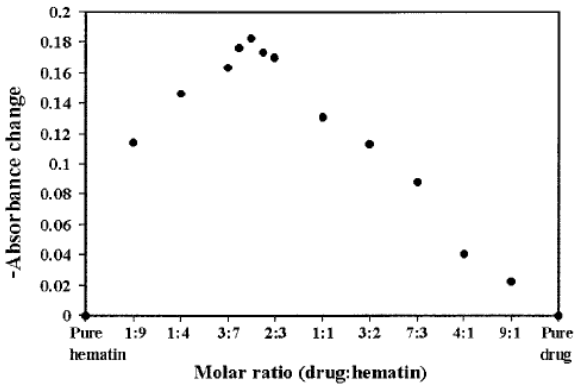

FIG. 2. Job's plot of pyronaridine binding to hematin. The total concentration of the two components was 10 μM in 40% aqueous DMSO, with mole fractions ranging from 0 to 1. Absorbance was measured at 400 nm at 25°C.

DISCUSSION

We have provided experimental evidences showing that pyronaridine acts as an antimalarial with a mechanism of action similar to that of the well-known 4-aminoquinoline chloroquine, namely, it inhibits β-hematin formation in vitro (a process which closely parallels hemozoin formation within the parasite food vacuole), forms a drug-hematin complex, inhibits glutathione-dependent degradation of hematin, and enhances hematin-induced lysis of red blood cells, but at 1/100 of the concentration seen with chloroquine. An interaction of pyronaridine with hematin had earlier been noted (10). In the case of the 4-aminoquinolines, the 7-chloro group has been shown to be absolutely required for inhibition of β-hematin formation (11); pyronaridine also contains the related chloro group. Translocation of free heme from the food vacuole to the cytosol has yet to be established, and the major mechanism of heme detoxification appears to be the hemozoin sequestration pathway (12).

The unexpectedly low concentrations of pyronaridine in enhancing heme-induced red blood cell lysis may account for the low IC_{50} of pyronaridine of 3 nM compared with 590 nM for chloroquine. It has been suggested that the destabilizing effect of hematin on the red blood cell membrane results from direct binding or incorporation, which may affect the reciprocal interactions between membrane and cytoskeleton proteins (20). The mechanism by which chloroquine enhances hematin lysis of red blood cells has not been studied in detail, but it has been demonstrated that the hematin-chloroquine complex allows more efficient transfer of the hematin in solution to the phospholipid bilayer membrane (14). The ability of pyronaridine at surprisingly low concentrations to enhance the membrane-perturbing property of free hematin suggests that this assay should

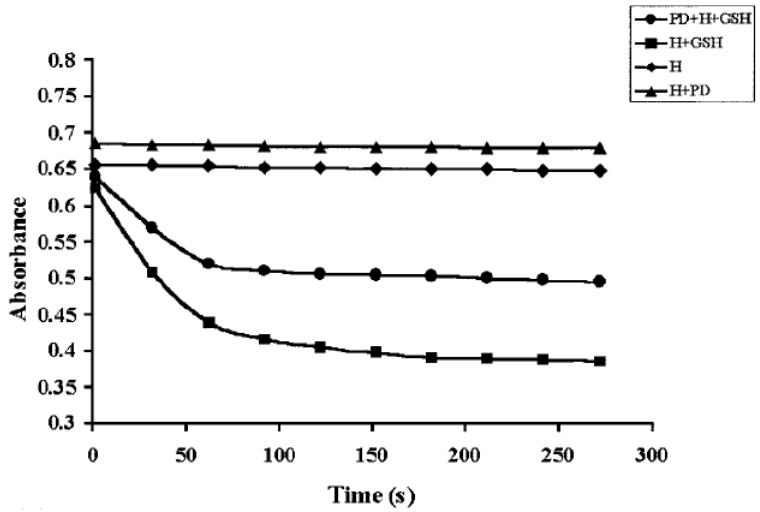

FIG. 3. Effect of pyronaridine on GSH-mediated degradation of hematin. Hematin (H) (5 μM) was incubated with GSH (2.5 mM) in the presence of 2.5 μM pyronaridine (PD), and hematin degradation was followed spectrophotometrically at 400 nm.

be included in any future protocols designed to search for novel antimalarial pharmacophores interacting with hematin, a validated target of the malaria parasite.

There has been an earlier report of ultrastructural changes, caused by pyronaridine in intraerythrocytic forms of *P. falciparum*, occurring first in the food vacuoles (28). Results of experiments in which pyronaridine targeted hematin in situ, with *P. falciparum* K1 growth in culture showing antagonism of pyronaridine in combination with antimalarials (chloroquine, mefloquine, and quinine) that inhibit β-hematin formation, were equivocal.

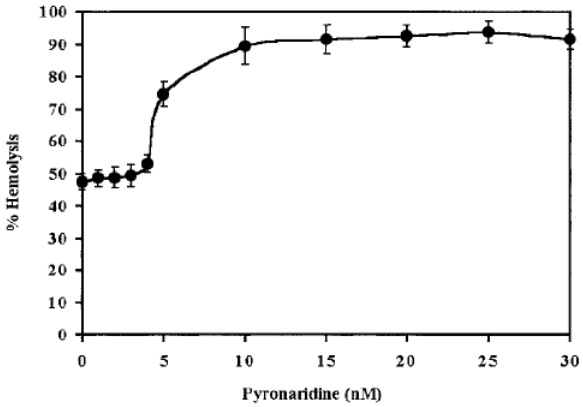

FIG. 4. Effect of pyronaridine on hematin-induced hemolysis. Aliquots of 0.03% suspension of human red blood cells were incubated at 37°C and pH 7.4 for 1 h in the presence of 4 μM hematin and various concentrations of pyronaridine. Hemolysis was determined by measuring the decrease in absorbance at 700 nm. Hematin alone (4 μM) resulted in 50% hemolysis, and pyronaridine in the absence of hematin did not cause any hemolysis.

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Dear Dr. Saranya,

Your paper "Research note antimalaria activity of concanamycin a alone and in combination with pyronaridine" has been reviewed and accepted for publication with editorial amendements. You will receive proofs in due course.

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ANTIMALARIAL ACTIVITY OF CONCANAMYCIN A ALONE AND IN COMBINATION WITH PYRONARIDINE

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Running title: Antimalarial activity of concanamycin A

Key words: antimalarial, concanamycin A, *Plasmodium falciparum*, pyronaridine

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ABSTRACT

Concanamycin A, a macrolide antibiotic inhibitor of vacuolar H^+-ATPase derived from *Streptomyces* sp., inhibited *Plasmodium falciparum* K1 growth in culture with an IC_{50} value of 0.2 nM. It exhibited an additive effect when tested together with the antimalarial pyronaridine.

INTRODUCTION

It is estimated that there are about 500 million clinical attacks of malaria every year in tropical regions of the world, with over one million deaths, especially in children in sub-Saharan Africa (Greenwood *et al*, 2005). Control of this disease is handicapped by the existence of resistance in the most virulent of the four species of human malaria parasites, *Plasmodium falciparum*, to nearly all antimalarial drugs currently used in chemotherapy. Thus there is a need to find new drugs and to understand the mechanisms of their actions, so that rationale changes can be made to their structures to circumvent resistance once it appears in the parasite.

Malaria parasites develop in the red blood cells by digesting host hemoglobin within the parasite acidic food vacuole (Sherman, 1979). The low pH of this organelle is maintained by the activity of a vacuolar (V) H^+-ATPase pump (Saliba *et al*, 2003). V-type H^+-ATPase is also found on the parasite plasma membrane (Saliba and Kirk, 1999) and it has recently been shown that the malaria parasite exports this enzyme to the host red blood cell plasma membrane (Marchesini *et al*, 2005). Thus we have examined the inhibitory effects on *P. falciparum* growth in culture of concanamycin A, a macrolide antibiotic inhibitor of vacuolar H^+-ATPase derived from *Streptomyces* sp. (Drose *et al*, 1993). As it is unlikely that any new antimalarial drug will be used clinically as a monotherapy, we also investigated the combination of concanamycin A and pyronaridine (2-methoxy-7-chloro-10[3',5'-bis(pyrrolidinyl-1-methyl-)4'hydroxyphenyl] amino-benzyl(b)-1,5-naphthyridine), a new highly active blood schizonticidal Mannich base antimalarial drug developed in China, effective in treating chloroquine-resistant malaria (Looareesuwan *et al*, 1996).

MATERIALS AND METHODS

Parasite culture

P. falciparum strain K1 (chloroquine-resistant) (Thaithong and Beale, 1981) was maintained in culture under Trager and Jensen 'candle jar' condition (Trager and Jensen, 1976).

Assessment of *in vitro* antimalarial activity

The protocol of the [^{3}H]-hypoxanthine incorporation method has been described previously (Auparakkitanon *et al*, 2003). In brief, aliquots of 200 µL of the parasite cell suspension (1.0% parasitemia of early ring stage) were incubated with 25 µL of the drug-containing medium in 96-well microtiter plates under candle jar condition for 24 h at 37°C prior to the addition of 25 µL of 0.5 µCi [^{3}H]-hypoxanthine (specific activity, 28.0 Ci/mmol; Amersham, Little Chalfont, United Kingdom). After a further incubation for 18 h, parasites were harvested from each well onto glass fiber filters, and lysed with distilled water. Radioactivity on the filter discs was determined in a liquid scintillation counter. The IC_{50} values (50% inhibition of radioactivity incorporation compared to control) were obtained from drug dose-response curves.

Determination of drug combination

IC_{50} values of one drug in the presence of a series of fixed concentrations of the other drug were measured as described above. Results were expressed as the mean sums of the fractional inhibitory concentrations (FIC), defined as (IC_{50} of concanamycin A in mixture/ IC_{50} of concanamycin A alone) + (IC_{50} of pyronaridine in mixture/ IC_{50} of pyronaridine alone) for each fixed concentration. Three types of drug interaction were defined as follows: additive, sum of FIC = 1; synergism, sum of FIC < 0.5; antagonism, sum of FIC > 4. Results were also plotted as an isobologram.

RESULTS

Assessment of the inhibitory effects of concanamycin A and pyronaridine on growth of *P. falciparum* K1 strain in culture yielded IC_{50} value of 0.2 nM and 3nM respectively. Fig 1 shows an isobologram obtained from a plot of FIC of concanamycin A versus FIC of pyronaridine in a drug combination study of their antimalarial activity *in vitro*, demonstrating an additive effect (sum of FIC in the range 1.15 to 1.65).

DISCUSSION

Although it is not possible to determine from the current study which of the various *P. falciparum* V-type H^+-ATPases is the target of concanamycin A, the very high sensitivity of *P. falciparum* to inhibition by this macrolide antibiotic suggests that it may be acting on the parasite-encoded enzyme located at the host red blood cell plasma membrane, which is believed to play a key role in maintaining intracellular pH of the infected erythrocyte (Marchesini *et al*, 2005). However, the other parasite V-type H^+-ATPases cannot be ruled out as possible targets. Alkalinization of isolated *P. falciparum* digestive food vacuole has been shown to be achieved with 75 nM concanamycin A (Saliba *et al*, 2003).

We have recently shown that *in vitro* pyronaridine binds hematin, inhibits β-hematin (hemozoin) formation and enhances hematin-induced lysis of red blood cells (Auparakkitanon *et al*, 2006). Although localization of pyronaridine within the parasite acidic food vacuole has yet to be shown, ultrastructural changes caused by this drug to food vacuoles in intra-erythrocytic forms of *P. falciparum* have been reported (Wu *et al*, 1988). WHO is currently developing a combination of pyronaridine and artesunate for treatment of malaria (www.who.int/mediacentre/news/notes/np8/en/). Examples of the therapeutic use of combinations of antimalarial with an antibiotic include quinine-tetracycline or doxycycline, quinine-clindamycin and chlorproguanil-dapsone (Lapdap) (Rosenthal, 2001).

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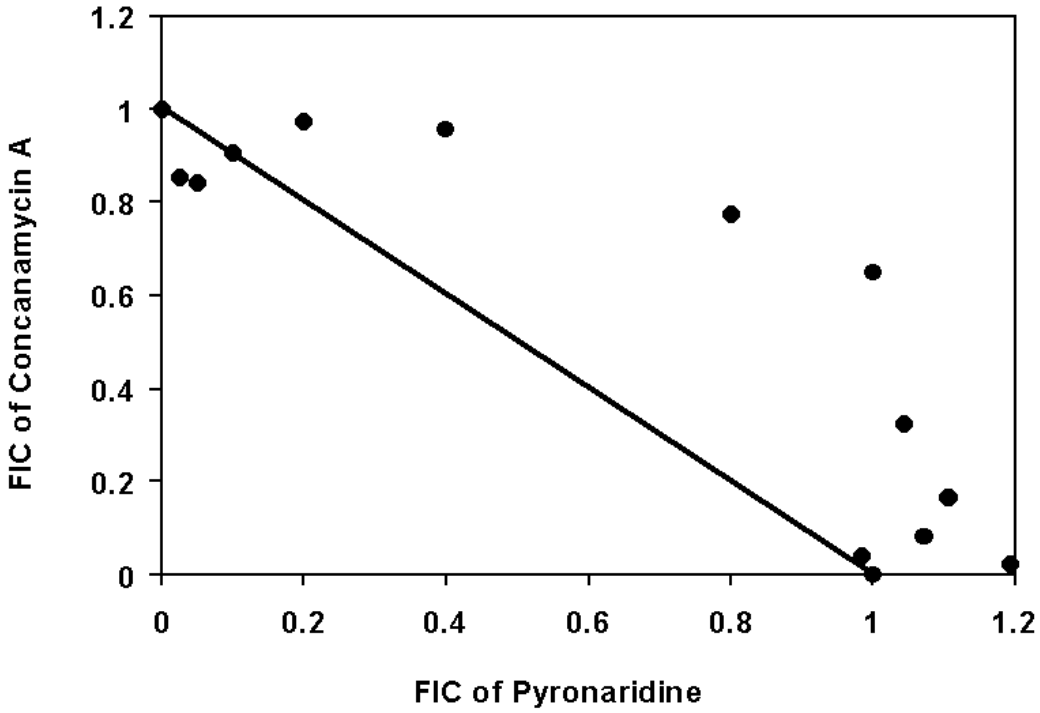
Figure legend

Fig 1. Isobologram of concanamycin A and pyronaridine. The solid line indicates an isobole where the two drugs act additively. FIC: fractional inhibitory concentration.


1.2
1
0.8
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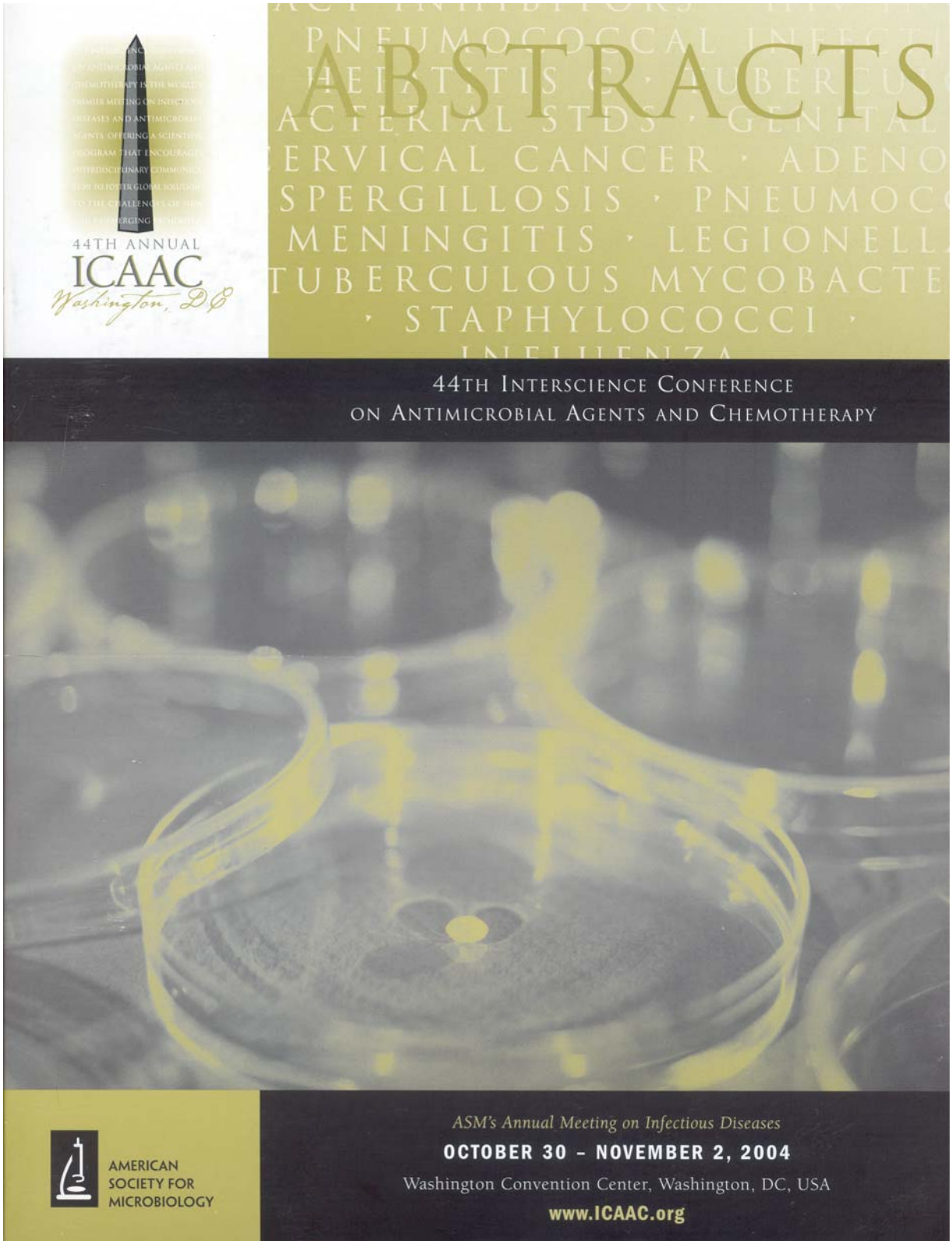
0.4
0.2
0
FIC of Concanamycin A
0
0.2
0.4
0.6
0.8
1
1.2
FIC of Pyronaridine

Figure 1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4 Reversal of Immune Dysregulation with the use of Antihelmintics in Hookworm Infected Patients.

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ound: In recent years the role of the immune response in the pathopysi- of disease states has been increasingly recognized. In a survey of im- gical indices in 57 patients infected with hookworm (*Necator ameri-* in northern Nigeria, low levels of CD3, CD4 and weak MIF indices covered. At the end of the survey, all patients were treated with proven ntics (Mebendazole and Levamisole) and requested to return for fol- Despite a high level of default, after a four-week period, thirty-four had their stool samples analysed for persistence of worms. Of these, nts successfully cleared of worms were assessed to check for any dif- s in their cellular immune status. **Methods:** Stool collected from 20 was analysed by Stoll egg counting technique, on triplicate 3gram mples before and four weeks after antihelmintic treatment. Peripheral mphocytes (PBL) were isolated from whole blood of same patients, by ficoll paque density gradient centrifugation, and stained with monoclonal dies (Becton-Dickinson, USA) conjugated with fluorescein. The cells nalysed in the dark by direct immunoflorescence for CD3, CD4 and mphocyte function was assessed by an-invitro measure of delayed hy- ivity, the leucocyte migration inhibition factor test (MIF). **Results:** The revealed a significant elevation in percentages of CD3, CD4 and MIF, ight depression in CD8 percentages compared to pretreatment values. **sions:** The findings suggest a causal relationship between the presence worms and suppression of cellular immune parameters and function in cted patients. The dysregulation can be restored with elimination of the by antihelmintic therapy.

5 High Prevalence of Co-Infection of HIV and Onchocerciasis in Cameroon: Serum Antibody Levels and the Need for Merging both Programs in Africa.

A. ALEMNJI. *Faculty of Med. and Biomedical Sci., Yaounde,* roon.

ound: Both Onchocerciasis (river blindness) and HIV/AIDS are now nt public health problems in many countries of Africa. The aim of this as to determine the prevalence of co-infection of Onchocerciasis and well as to assess and compare the levels of antibodies among patients in oon. **Methods:** This was a community-based study in Balamba, an On- iasis endemic region in Cameroon. Both skin snip and blood samples llected from all consented inhabitants. Diagnosis of Onchocerciasis e by the skin snip technique while the presence of antibodies to HIV-1 ere confirmed by combination of one rapid test and one ELISA. Simi- erum antibody levels were determined by sandwich ELISA. **Results:** tal of 162 subjects recruited, 54 of them were diagnosed positive for erciasis by skin snip, giving a prevalence of 33.3% (54/162). Also, the nce of HIV among these subjects was 12.9% (21/162). This infection 9% among women (13/73) and 8.9% among men (8/89). Six of the 54 positive for Onchocerciasis were also positive for HIV giving a preva- co-infection of Onchocerciasis and HIV of 11.1% (6/54). Mean serum levels of Onch+ve/HIV+ve, Onch+ve/HIV-ve and Onch-ve/HIV+ve were significantly higher (P<0.001, Student's t-test) when compared of Onch-ve/HIV-ve patients. **Conclusion:** The present study demon- high prevalence of co-infection of Onchocerciasis and HIV. It further significantly high antibody levels, reflecting raised humoral immune es with co-infected patients showing the highest response. Hence the ion of HIV/AIDS care and prevention activities into currently ongoing nt program for Onchocerciasis will save time and scarce resources in with tremendous public health impact.

P-126 Impact of Chloroquine Resistance in Management of Uncomplicated Paediatric Malaria in Nigeria.

I. A. N. OBASI, F. A. F. FAGBENRO-BEYIOKU. *Tropical Disease Res. Unit, Lagos, Nigeria.*

Background: Malaria remains mankinds greatest scourge with its highest morbidity and mortality among children in of sub-Saharan African. The management of malaria becomes traumatic with the emergence and widespread of resistance to chloroquine and other available antimalaria drugs. Chloroquine still remains the only cheap, non-toxic and available option for the treatment of uncomplicated *Plasmodium falciparum* malaria in most African countries. The aim of this study was to determine the efficacy of chloroquine as a therapeutic agent for non-complicated malaria in the face of increasing drug resistance and to recommend new therapeutic options available for paediatric malaria. **Methods:** Four hundred and fifty children aged between 1 and 5 years presenting with clinical signs of acute malaria were selected. Only 222 of these children fulfilled the inclusion criteria and were recruited into the study. They were treated with the standard 3-day chloroquine regime. Parasitaemia was recorded on Day 0 before drug administration and on day 1,2,3,and 7. Resistance was accessed with the "7-day test." **Results:** Two hundred and twenty two children who fulfilled the inclusion criteria were recruited. Resistance to chloroquine was 18.9%. More males (13.5%) showed resistance than female (5.4%). Majority of the resistance to chloroquine was at the R I level (14.8%), while the rest 4.1% of respondents had R II resistance. No R III resistance was observed in this study. **Conclusion:** The high resistance level in this age group is similar to present trends especially in sub-Saharan Africa. The study has revealed an increasing level of resistance to chloroquine as an antimalaria for children in Lagos. Conclusively, there is need for research on new drug substitutes as well as vaccines to combat the scourge of resistance especially in sub-Saharan.

P-127 Mechanism of Action of the Antimalarial Pyronaridine In Vitro.

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Background: Pyronaridine, 2-methoxy-7-chloro-10[3',5'-bis(pyrrolidinyl-1-methyl-)4'-hydroxyphenyl]amino-benzyl-(b)1,5-naphthyridine, a new schizontocidal Mannich base synthesized in China, is undergoing development by WHO as a combination drug with artesunate. We have recently shown that in the test tube pyronaridine interacts with heme similarly to chloroquine (CQ) by inhibiting β-hematin formation and enhancing heme-induced red cell lysis but at 1/100 of CQ concentration (Exp Parasitol 2003; 105: 29). In order to demonstrate that pyronaridine exerts this mechanism of action in vitro, inhibitory studies on *Plasmodium falciparum* growth in culture were conducted with pyronaridine in the presence of antimalarials that inhibit β-hematin formation (CQ, dihydroartemisinin, mefloquine, quinine), with the expectation that these antimalarials would antagonize pyronaridine. Concanamycin A, a macrolide antibiotic inhibitor of vacuolar ATPase derived from *Streptomyces* sp., was also included in the study. **Methods:** *P. falciparum* K1 strain maintained in culture was used for drug testing, employing [3H]- hypoxanthine uptake to monitor parasite growth over 48 hr period under "candle jar"condition. At least three different pyronaridine-drug ratios were tested and the results were expressed as the mean sums of the fractional inhibitory concentrations (FIC), defined as (IC50 of drug A in mixture/IC50 of drug A alone) + (IC50 of drug B in mixture/IC50 of drug B alone). **Results:** The sums of FIC for the antimalarials tested and concanamycin A were > 1 indicating antagonism with pyronaridine, supporting the notion that pyronaridine accumulates within the malaria parasite acidic food vacuole where it inhibits β-hematin formation. **Conclusion:** The mechanism of action of the antimalarial pyronaridine is by inhibiting β-hematin formation within the parasite food vacuole.

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