Gametocytocidal activity of pyronaridine and DNA topoisomerase II inhibitors against multidrug-resistant

Plasmodium falciparum in vitro

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Received 12 November 1998; accepted 6 October 1999

Abstract

Gametocytocidal activities of pyronaridine and DNA topoisomerase II inhibitors against two isolates of multidrug-resistant Plasmodium falciparum, KT1 and KT3 were determined. After sorbitol treatment, pure gametocyte cultures of Plasmodium falciparum containing mostly young gametocytes (stage II and III) obtained on day 11 were exposed to the drugs for 48 h. The effect of the drugs on gametocyte development was assessed by counting gametocytes on day 15 of culture. Pyronaridine was the most effective gametocytocidal drug against P. falciparum isolates KT1 and KT3 with 50% inhibitory concentration of 6 and 20 nM, respectively. Moreover, the 50% inhibitory concentration of pyronaridine was lower than that of primaquine which is the only drug used to treat malaria patients harboring gametocytes. Prokaryotic (norfloxacine) and eukaryotic (amsacrine and etoposide) DNA topoisomerase II inhibitors were only effective against asexual but not sexual stages of the malaria parasites. Pyronaridine has both schizontocidal and gametocytocidal activities against the human malaria parasite, P. falciparum. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Gametocytocidal activity; Pyronaridine; DNA topoisomerase II inhibitors; Plasmodium falciparum

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PII: S1383-5769(99)00028-8
1. Introduction

Malaria still remains one of the major health problems in tropical countries, and among the four species of malaria parasites infecting humans, *Plasmodium falciparum* is the most virulent. Chemotherapy has played a major role in alleviating suffering and in reducing mortality caused by *P. falciparum* infection. Unfortunately, resistance to most currently used antimalarials has appeared in *P. falciparum* and continues to increase in many parts of the world [1]. Most of the currently used antimalarial drugs affect only the asexual stage of the parasite, except primaquine which can also act as a gametocytocidal drug.

Pyronaridine, a 9-anilino-aza-acridine synthesized in the 1970s, has been developed primarily from Chinese research efforts and has been used in China for more than 15 years [2,3]. It is highly effective against chloroquine-sensitive and -resistant strains of *P. falciparum* [4,5] and good antimalarial activity has also been reported in Thailand [6]. Pyronaridine is a highly active blood schizontocide and has already undergone extensive trials in human against both *P. falciparum* and *P. vivax* [7,8]. Although an effect of pyronaridine on the ultrastructure of malaria parasite has been reported [9,10] and our previous study showed that decatenation activity of *P. falciparum* DNA topoisomerase II was inhibited by pyronaridine [11], the mechanism of action of pyronaridine is still not known.

Since inhibition of *P. falciparum* sexual stage should not be overlooked, and only one drug, primaquine, is currently used to combat *P. falciparum* gametocytes, a search for new gametocytocidal drugs is urgently needed. Therefore, in this study, in vitro gametocytocidal activities of pyronaridine and a number of DNA topoisomerase II inhibitors were determined against two isolates of gametocyte-producing *P. falciparum* from Thailand.

2. Materials and methods

2.1. Parasites

Gametocyte-producing isolates, KT1 and KT3, of *Plasmodium falciparum* were collected from two infected patients at Thong Pha Phum District, Kanchanaburi Province, Thailand. Multi-drug resistant K1 strain originally taken from this province [12] was used as a control parasite for determination of drug resistance. KT1 and KT3 isolates were successfully cultured in our laboratory for at least 3 years and continuously produced gametocytes under our culture conditions [13,14]. Morphological and functional maturation of KT3 isolate have already been reported [13] and KT1 isolate behaved similarly.

2.2. Cultivation of *Plasmodium falciparum*

*P. falciparum* KT1 and KT3 isolates were cultured continuously in RPMI medium supplement with 15% human plasma using human erythrocytes (O,Rh-) previously treated with PIGPA (Pyruvate, Inosine, Glucose, Phosphate, Adenine) solution [13]. In gametocyte cultivation, 50 mg/l (final concentration) of hypoxanthine were also added. Culture dishes were placed in candle jars and incubated at 37°C. The culture medium was changed every 3 days.

2.3. Test of in vitro drug sensitivity against asexual stage of *P. falciparum* KT1 and KT3 isolates

Mefloquine, amsacrine, etoposide and primaquine were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1% (v/v). Chloroquine, cycloguanil and pyronaridine were dissolved in sterile distilled water. Pyrimethamine was dissolved in 0.5% lactic acid and the final concentration of lactic acid in culture was not allowed to exceed 0.0005%. Norfloxacin was diluted with 0.1 M HCl and the final concentration of HCl was less than 1 μM. The stock drug solutions were diluted to the desired concentrations with culture medium.

The activities of drugs against *P. falciparum* KT1 and KT3 were measured as 50% inhibitory concentration (IC50) by incubating 1.5% erythrocyte suspension containing 0.5% initial parasitemia with drugs for 24 h at 37°C. [3H]Hypoxanthine (0.25 μCi, 6.2 Ci.mmol, Amersham, UK) was then added to each sample and parasite cultures were incubated for an additional 24 h. IC50
values were recorded as the concentration of drug required to inhibit by 50% the incorporation of [3H]hypoxanthine into parasite DNA, compared with untreated control.

2.4. Test of in vitro gametocytocidal effect

After synchronization of *P. falciparum* growth with sorbitol treatment [15], gametocyte culture was started with 1% initial parasitemia containing mostly ring forms in 2% erythrocyte suspension. The medium was changed on day 4, 6 and 8 of the culture. Pure gametocytes of *P. falciparum* KT1 and KT3 isolates were obtained by adding 2.5 volumes of 5% (w/v) sorbitol to packed erythrocytes for 5 min once a day, starting from day 9 until day 11 of culture. Approximately 450 μl aliquots of this suspension were transferred to a 24-well plate which contained 50 μl of drug in each well. After 24 h incubation, drug was again replaced and cultures were incubated for an additional 24 h. All wells then received complete medium without drug and cultivations were continued for 2 more days. Thin blood films were prepared on day 15 of cultivation and gametocytes were counted per 10,000 erythrocytes. The effect of each drug concentration was investigated in triplicates. Gametocytocidal activity of drug was recorded as the concentration of drug that inhibited gametocytes by 50% as compared with untreated control.

3. Results

3.1. In vitro antimalarial drug sensitivity of the asexual stage of *P. falciparum* KT1 and KT3 isolates

Asexual parasites were cultured in the presence of five known antimalarials for 48 h and IC₅₀ values of these drugs against *P. falciparum* KT1 and KT3 isolates were determined by measuring uptake of [3H]hypoxanthine compared with K1 strain. Both *P. falciparum* isolates were resistant to chloroquine, pyrimethamine and cycloguanil, but were still sensitive to mefloquine compared with K1 strain (Table 1). The IC₅₀ of the drugs against KT1 isolates were not significantly different from those of KT3 except for that of cycloguanil in KT3 isolate which was approximately six times less than that in KT1 isolate. Primaquine was also able to inhibit asexual parasite growth only at a higher concentration than chloroquine and mefloquine.

3.2. In vitro activity of pyronaridine and DNA topoisomerase II inhibitors against the asexual stage of *P. falciparum* KT1 and KT3 isolates

IC₅₀ of pyronaridine and DNA topoisomerase II inhibitors, namely, amsacrine, etoposide and norfloxacin, were determined. All compounds could inhibit parasite growth in vitro and IC₅₀ were found to be between 0.002 and 43 μM (Table 2). Pyronaridine showed the highest activity against the asexual stage of both parasite isolates (IC₅₀ = 2 nM). Among the DNA topoisomerase II inhibitors, eukaryotic DNA topoisomerase II inhibitors, amsacrine and etoposide, were more active against both *P. falciparum* isolates than norfloxacin, a prokaryotic DNA topoisomerase II (gyrase) inhibitor.

3.3. Pure gametocyte cultures of *P. falciparum* KT1 and KT3 isolates

Gametocytes of KT1 and KT3 isolates produced in cultivation could undergo a maturation process and stages I–V identified on the basis of morphology [13]. After sorbitol treatments on day 9, 10 and 11, there was 99% reduction in the
number of asexual parasites. Pure gametocyte cultures of *P. falciparum* KT1 and KT3 isolates were obtained on day 11 with an average number of gametocytes of 305 and 392 per 10,000 erythrocytes, respectively. These gametocytes consisted of 19% stage II, 60% stage III and 21% stage IV and were used in the drug treatment studies.

### 3.4. In vitro gametocytocidal effects of pyronaridine and DNA topoisomerase II inhibitors against *P. falciparum* KT1 and KT3 isolates

Gametocytes of *P. falciparum* KT1 and KT3 isolates could be inhibited by pyronaridine and eukaryotic DNA topoisomerase II inhibitors (amsacrine and etoposide) whereas norfloxacin did not have any effect on gametocytes even when the concentration was raised to 100 μM (Table 3). Pyronaridine was the most potent inhibitor against gametocytes in cultures (IC$_{50}$ of 6 and 20 nM against KT1 and KT3, respectively), being approximately $3 \times 10^3$–$3 \times 10^5$ times more effective than amsacrine and etoposide. Moreover, IC$_{50}$ of pyronaridine was lower than IC$_{50}$ of primaquine.

### 4. Discussion

Gametocyte-producing *P. falciparum* KT1 and KT3 isolates used in this study can be looked upon as multidrug-resistant parasites because of their resistance to chloroquine, pyrimethamine

and cycloguanil. Both isolates were still sensitive to mefloquine.

The study of topoisomerases has expanded into the realm of pharmacology and clinical medicine through identification of bacterial topoisomerase II (DNA gyrase) as a target of antibiotics and toxins and of eukaryotic DNA topoisomerase II as a target of a large number of anticancer agents [16]. In vitro activities of pyronaridine and DNA topoisomerase II inhibitors against the asexual stage of recently acquired *P. falciparum* KT1 and KT3 isolates were investigated and the results showed that both prokaryotic and eukaryotic DNA topoisomerase II inhibitors could inhibit asexual parasite growth, similar to previous studies conducted on the established multidrug-resistant *P. falciparum* K1 strain [11]. Pyronaridine was the most potent inhibitor with IC$_{50}$ values for both KT1 and KT3 isolates of 2 nM, not significantly different from that for *P. falciparum* K1 strain (IC$_{50} = 2.7$ nM) [11]. The present results indicate that chloroquine-, pyrimethamine- and cycloguanil-resistant *P. falciparum* showed no cross resistance to pyronaridine whereas pyronaridine-resistant *P. falciparum* exhibits cross resistance to chloroquine and piperaquine [17].

Divo et al. [18] have reported on the antimalarial activity of fluoroquinolones against asexual stage of chloroquine-sensitive and -resistant *P. falciparum*. Previous study showed that the decatenation activity of *P. falciparum* DNA topoisomerase II is inhibited by fluoroquinolones [19]. Growth inhibition of asexual stages of *P.
falciparum KT1 and KT3 isolates by norfloxacin is consistent with these observations. Mitochondrial DNA topoisomerase II of P. falciparum may be a possible target for DNA gyrase inhibitors.

Gametocytocidal activity of pyronaridine was very much higher than that of the other DNA topoisomerase II inhibitors tested (amsacrine, etoposide and norfloxacin). Since the gametocytes used in this study were predominantly stage III, in which only RNA and protein synthesis occur, it is not surprising that known DNA topoisomerase II inhibitors showed very low gametocytocidal efficacy because their target plays a role in DNA synthesis which occurs only in stage I and II gametocytes.

Although we have shown that pyronaridine can inhibit P. falciparum DNA topoisomerase II in vitro, it now appears that DNA topoisomerase II is not the specific target of pyronaridine based on two lines of evidence. Firstly, pyronaridine strongly inhibited gametocyte growth in spite of the lack of DNA synthesis in this stage. Secondly, using an assay for detection of DNA cleavage, we have shown that pyronaridine was not able to inhibit asexual P. falciparum topoisomerase II in situ [20]. Pyronaridine has recently been demonstrated to inhibit malaria parasite heme polymerization as well [21].

This is the first report of the gametocytocidal effect of pyronaridine on P. falciparum. It is 100 times more effective than primaquine which is the only gametocytocidal antimalarial in clinical use. The dual role of pyronaridine as a schizontocidal and gametocytocidal drug should make it highly attractive for clinical application.

Acknowledgements

We are grateful to Ms Somsri Kajorndechakait and Mr Saiyud Incheng for technical assistance. This work was supported by a grant (RSA/3/2538) from The Thailand Research Fund. P.W. is a Senior Research Scholar of The Thailand Research Fund.

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