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-naphthryidine, a new Mannich base schizontocide originally developed in China and structurally related to the aminoaridine 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 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-naphthryidine, 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), Chavalitshevinkoon 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) are equivocal.

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

Drug combination studies. Fifty percent inhibitory concentration (IC50) 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: (IC50 of drug A in mixture/IC50 of drug A alone) + (IC50 of drug B in mixture/IC50 of drug B alone). The IC50 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 Baemans et al. (3) as described previously (2). Results of the inhibition assay were expressed as IC50 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-2500 PC 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...
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 PC 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 IC₅₀ 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 chloroquine, 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).

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 IC₅₀ 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. 1. Structures of pyronaridine and chloroquine.

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.
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. 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.

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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REFERENCES


