Synergistic Interactions of the Antiretroviral Protease Inhibitors Saquinavir and Ritonavir with Chloroquine and Mefloquine against *Plasmodium falciparum* In Vitro[▽]

T. S. Skinner-Adams,^{1,2*} K. T. Andrews,² L. Melville,² J. McCarthy,² and D. L. Gardiner²

University of Queensland, Department of Medicine, Central Clinical Division, Brisbane 4072, Australia,¹ and Queensland Institute of Medical Research and Australian Centre for International and Tropical Health and Nutrition, 300 Herston Road, Herston, Queensland 4029, Australia²

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The antimalarial activity of several antiretroviral protease inhibitor combinations was investigated. Data demonstrate that ritonavir and saquinavir behave synergistically with chloroquine and mefloquine. These data, and interactions with pepstatin-A, E-64, and bestatin, suggest that human immunodeficiency virus protease inhibitors do not target digestive-vacuole plasmepsins.

Although the antimalarial activity of a number of antiretroviral protease inhibitors (PIs; e.g., human immunodeficiency virus [HIV] PIs) in clinical use has been established (1, 15, 20), their mechanism of action against malaria parasites is not known. Understanding the action of these drugs may allow the design of a novel group of potent antimalarials. Our original hypothesis was that, in keeping with their action as aspartyl protease (AP) inhibitors against the HIV virus (21), these agents interfere with a parasite AP (plasmepsin). More specifically, we hypothesized that these drugs interfered with parasite hemoglobin digestion by inhibiting one or more of the digestive-vacuole plasmepsins. Hemoglobin digestion is essential to *Plasmodium* parasites by supplying amino acids for protein synthesis and development, providing space for the growing parasites, reducing the colloid-osmotic pressure of the erythrocyte, and preventing premature cell rupture (9). Known inhibitors of the hemoglobin digestion process with antimalarial activity have been identified (2, 8) and include pepstatin A, an AP inhibitor; E-64 [*N*-(*trans*-epoxysuccinyl)-L-leucine-4-guanidinobutylamide], a cysteine protease inhibitor; and bestatin, an aminopeptidase inhibitor. While the mode of chloroquine action is still debated, it is thought to inhibit parasite growth by preventing heme detoxification (19). In combination studies, vacuole plasmepsin inhibitors have been shown to behave antagonistically with chloroquine and mefloquine (12), while combinations of AP inhibitors with other inhibitors of the hemoglobin digestion process have been shown to behave synergistically (18).

Previous studies have also suggested that HIV PIs, particularly ritonavir, can reduce the efflux of a drug from cells. The synergy of mefloquine when used with HIV PIs against HIV has been proposed to be due, at least in part, to the ability of the HIV PIs to inhibit mefloquine efflux (14). It is unknown whether the HIV PIs may be able to alter drug efflux in *Plas-*

modium falciparum, and if they can, whether this will have any ramifications for the treatment of multidrug-resistant parasites. The aim of the present study was to investigate the antimalarial activity of the HIV PI drugs saquinavir and ritonavir by examining their ability to inhibit the growth of *P. falciparum* when used in combination with chloroquine, mefloquine, pepstatin A, bestatin, or E-64.

P. falciparum clones Dd2, FAC8, and D10 were maintained in modified candle jars as previously described (22). Antimalarial drug combinations were assessed by isobolographic analysis (4). Assays were performed in triplicate in 96-well microtiter plates containing 100 μ l of culture (2% hematocrit and 1% parasitemia) and 100 μ l of drug dilutions or a control. Parasite growth was determined based on tritiated hypoxanthine incorporation. Media and vehicle controls were included on each plate. Experiments were repeated at least twice. Fifty percent effective concentrations (EC₅₀) (Table 1) were determined and isobolograms constructed using data from all experiments. Isoboles were fitted to data using a standard hyperbolic function defined by the parameter *I* (5). Positive values of *I* indicate synergism, and negative values indicate antagonism; addition occurs when *I* equals 0. The significance of the difference between *I* and 0 was assessed with Student's *t* test.

Chloroquine behaved synergistically with saquinavir and ritonavir against the chloroquine-resistant line Dd2. *I* values of

TABLE 1. EC₅₀ of individual drugs against *P. falciparum* clones during combination studies

Drug	EC ₅₀ against indicated clone ^a		
	Dd2	D10	FAC8
Chloroquine	55–85 nM	NT	NT
Mefloquine	60–91 nM	64–65 nM	23–33 nM
Ritonavir	0.85–6.1 μ M	1.1–2.8 μ M	7–12 μ M
Saquinavir	0.96–7.2 μ M	5.5–6.2 μ M	NT
Pepstatin	NA	14–18 μ M	NT
E-64	2.9–6.6 μ M	NT	NT
Bestatin	5.1–14 μ M	NT	NT

^a EC₅₀ values are reported as ranges determined from all isobologram experiments. NT, not tested; NA, never achieved.

* Corresponding author. Mailing address: Malaria Biology Laboratory, Infectious Diseases and Immunology Division, Queensland Institute of Medical Research, Herston, QLD 4029, Australia. Phone: 61 07 3362 0419. Fax: 61 07 3362 0104. E-mail: tinaS@qimr.edu.au.

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FIG. 1. Isobolograms describing the interaction of saquinavir or ritonavir with chloroquine or mefloquine when tested against *P. falciparum* isolate Dd2. The test of each combination was repeated on two occasions in triplicate. All data points are shown. FIC, fractional inhibitory concentration.

1.3 and 2.2 for saquinavir and ritonavir, respectively (Fig. 1), were demonstrated. As was found with chloroquine, combinations of mefloquine with saquinavir and ritonavir were synergistic, with I values of 1.3 and 2.2, respectively (Fig. 1). The synergistic interaction of mefloquine with ritonavir was inde-

pendent of the parasite line used. Although demonstrating the smaller I value of 0.9, mefloquine and ritonavir also behaved synergistically against D10 and FAC8 (Fig. 2). Combinations of saquinavir and pepstatin were suggestive of synergy ($I = 0.6$), whereas combinations of saquinavir and E-64 ($I = -4.5$)

FIG. 2. Isobolograms describing the interaction of mefloquine and ritonavir when assessed against the *P. falciparum* isolates D10 and FAC8. The test of each combination was repeated on two occasions in triplicate. All data points are shown.

FIG. 3. Isobolograms describing the interactions of saquinavir with the known hemoglobin digestion inhibitors pepstatin, E-64, and bestatin and of chloroquine in combination with pepstatin when assessed against *P. falciparum*. The test of each combination was repeated on two occasions in triplicate. All data points are shown.

or saquinavir and bestatin ($I = -2.9$) were antagonistic (Fig. 3). As Dd2 was resistant to pepstatin with an EC_{50} of $>20 \mu\text{M}$ (Table 1), all combinations with this agent were tested with D10. EC_{50} for saquinavir and ritonavir were within the range normally achieved in humans (20). EC_{50} values for all drugs are shown in Table 1.

Although a full understanding of the action of chloroquine is lacking, evidence suggests that chloroquine kills sensitive malaria parasites by preventing heme polymerization (19). Other mechanisms of action have been suggested but are considered less important (10). The synergistic interaction of chloroquine with ritonavir and saquinavir suggests that these HIV PIs do not inhibit a digestive-vacuole plasmepsin. Digestive-vacuole plasmepsins have been shown to carry out the initial cleavage of heme from hemoglobin (3). If this process is inhibited, chloroquine would be prevented from accessing heme, resulting in antagonism, as described with other plasmepsin inhibitors (12). The antagonistic action of saquinavir with E-64 and bestatin also supports this theory. Combinations of PIs, especially those of the aspartyl and cysteine class, have been shown to behave synergistically (18). Similar to the way that other drug combinations inhibit sequential steps in a metabolic pathway, it is believed that the inhibition of sequential steps in the hemoglobin digestion pathway maximizes antimalarial activity. Combinations of E-64 with pepstatin, a known AP inhibitor, are reported to be synergistic (2, 18). The antagonistic nature of the E-64 and saquinavir combination has also been supported by a recent study demonstrating that E-64 and the HIV PI lopinavir behave antagonistically against *P. falciparum* (16). The mildly synergistic nature of the pepstatin and saquinavir combination provides additional evidence to suggest that the HIV PIs are not targeting vacuole plasmepsins, or if they are, that other factors are contributing to the action of these antimalarials. These data also support the observations of others who have shown that when food vacuole plasmepsins are “knocked out,” a lethal phenotype is not observed (11, 13). Further studies will be necessary to ascertain the primary target of these drugs.

Although extrapolation of our in vitro data to the in vivo setting should be undertaken with caution, data describing the synergistic interactions of saquinavir and ritonavir with meflo-

quine and chloroquine are encouraging and suggest that if chosen correctly, antimalarial and antiretroviral combinations may be useful in the field. The results with combinations of mefloquine with the HIV PIs suggest that these drugs may be more effective against mefloquine-resistant parasites (Table 1; Fig. 1 and 2), and although the use of mefloquine in areas such as sub-Saharan Africa is likely to be restricted by cost, this finding may hold true for other antimalarial drugs. As is true of mefloquine, the development of malaria parasite resistance to quinine, the artemisinin drugs, and chloroquine has been associated with drug efflux (6, 7). Although additional studies are required to determine whether HIV PIs alter drug transport, our data suggest that *Pfmdr1* copy number is not associated with the synergistic action of the HIV PIs with mefloquine. The HIV PI-mefloquine combinations were more effective against Dd2 (2 copies of *Pfmdr1*) than against D10 (1 copy) or FAC8 (3 copies) (17). Unless they can be shown to be effective against chloroquine-resistant parasites, HIV PI and chloroquine combinations may be of limited clinical value.

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