Short Report: HIV Protease Inhibitors, Indinavir or Nelfinavir, Augment Antimalarial Action of Artemisinin *in vitro*

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Abstract. Most malaria endemic regions are co-infested with HIV infection. Treatment of one may affect outcome of the other in co-infected individuals. HIV protease inhibitors, indinavir or nelfinavir, are important antiretroviral drugs and artemisinin is central to malaria treatment. We show these protease inhibitors augment the antimalarial activity of artemisinin against *P. falciparum in vitro*.

Menace of malaria looms over one-fifth of the world population in more than 80 malaria endemic countries. Over one million people die annually due to *Plasmodium falciparum* malaria.¹ Most of these deaths occur in resource poor settings. The increased morbidity and mortality has been mainly due to widespread emergence of drug resistant *P. falciparum* strains. After the emergence of chloroquine resistance in *P. falciparum*, the only sure cure of malaria rests largely on artemisinin based combination therapies.¹ Artemisinin is now the only vital drug for effective treatment of malaria. Any resistance to it could be a fatal setback to malaria control programs. Recent appearance of artemisinin tolerant parasites at the Thai-Cambodia border is a cause of concern.^{2,3} Most of the malaria-endemic countries are also burdened and co-infested by pandemic human immunodeficiency virus.^{4,5} An estimated 40 million HIV-infected cases exist in Africa alone with an annual mortality of over 3 million people.⁶ Malaria is more prevalent, severe, and recurrent in HIV infected people than those without the virus.^{4,7} This co-infection would also augment the spread of geographic boundaries of malaria infection in HIV prevalent areas.⁸ HIV infection is long lasting, requiring almost life long antiretroviral treatment, whereas malaria is self limiting. Treatment of one infection may affect outcome of the other in co-infected individuals. Novel antiretroviral treatments are being steadily introduced.⁹ The effect of HIV protease inhibitors on antimalarial potency of artemisinin has not been reported. This prompted us to evaluate the interaction of HIV protease inhibitors, indinavir or nelfinavir, on the antimalarial efficacy of artemisinin on chloroquine sensitive (3D7) and chloroquine resistant (RKL 303) strains of *P. falciparum in vitro*.

Erythrocytic stages of chloroquine-sensitive *P. falciparum*, strain 3D7, and chloroquine-resistant RKL 303 strain were cultured and maintained as stocks on human B⁺ erythrocytes by candle-jar method¹⁰ at 37°C. Antimalarial potential of eight antiretroviral drugs (Matrix Laboratories Ltd., Secunderabad, India) and artemisinin (Sigma-Aldrich, St. Louis, MO) (Table 1) was evaluated by exposing parasites to graded concentration of drugs by the modified 48 hour test¹¹ with the aid of candle jar method.¹⁰ Synchronized ring stage parasites¹² were used in all the experiments. Percentage parasitemia and inhibition of parasite multiplication rate in relation to control was determined at the end of experiment by examining Giemsa-stained blood films. Counting of parasites was done in random adja-

cent microscopic fields, equivalent to about 3,000 erythrocytes at 1,000× magnification. The complete culture medium was prepared by adding sterile 5% sodium bicarbonate to Roswell Park Memorial Institute-1640 (Sigma-Aldrich) and supplemented with 10% B⁺ serum. Drug dilutions were prepared in antibiotic-free complete culture medium from the drug stock solution of 1 mg/mL in dimethyl sulfoxide (DMSO) 50% inhibitory concentration (IC₅₀) values were computed from log dose-response curve (Microsoft Excel, Microsoft Corp., Redmond, WA) and presented in Table 1. HIV protease inhibitor drugs, indinavir and nelfinavir, show promising antimalarial efficacy at adequate (clinically relevant) concentrations. These *in vitro* results on inhibition of parasite multiplication rate by antiretroviral drugs reported here are comparable with that of other.¹³ Each of these drugs was further investigated for its antimalarial interaction in combination with artemisinin *in vitro* using fixed ratio method. For combination assay, artemisinin was combined with either of the drugs in six fixed-ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5.^{14,15} The first and the last ratio of these six preparations had the artemisinin and antiretroviral drug alone at a concentration approximately 8× their respective IC₅₀ value against a particular parasite strain. Further dilutions of these combinations have been described in plate preparation elsewhere.^{14,15} After 48 hours, thin blood film slides from each triplicate experimental well and from control were prepared separately for counting the parasitemia. The fractional inhibitory concentration (FIC) for each drug in triplicate for six fixed-dose ratios was calculated by the following formula:

$$\text{FIC} = \frac{\text{Concentration of drug in combination to produce IC}_{50}}{\text{Concentration of drug alone required to produce IC}_{50}}$$

The sum FICs (Σ FIC) value for drugs of the six fixed-dose preparations were determined to classify the drug-drug interaction and isobologram construction. The Σ FIC < 1 represents synergism, Σ FIC ≥ 1 and < 2 represents additive interaction, Σ FIC ≥ 2 and < 4 represents slight antagonism, whereas Σ FIC ≥ 4 represents marked antagonism.^{15–17} The isobolograms obtained from fixed ratio combinations against two strains of *P. falciparum* are presented in Figure 1. The points appearing below the additive line of concentration 1 and 6 represent synergism (Σ FIC < 1). The drug-drug interaction studies have previously shown antagonism between known antimalarial drugs, artemisinin and pyrimethamine,¹⁸ using this method we have also found synergism between pyrimethamine and sulfadoxine (data not presented). Indinavir synergizes the antimalarial action of artemisinin irrespective of the parasite strains (Figure 1A, Table 2), in most combination ratios. Similarly artemisinin and nelfinavir interaction shows synergism in

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TABLE 1

Mean IC_{50} values ($\pm$ standard error) of artemisinin and antiretroviral drugs against *Plasmodium falciparum* (3D7 and RKL 303 strains) *in vitro*

Drug	Mean $IC_{50} \pm SE^*$	
	3D7 strain	RKL 303 strain
Artemisinin	3.97 nM $\pm$ 0.08	3.43 nM $\pm$ 0.32
Indinavir	2.33 μ M $\pm$ 0.1	2.65 μ M $\pm$ 0.16
Nelfinavir	9.00 μ M $\pm$ 0.08	8.28 μ M $\pm$ 0.79
Abacavir	61.04 μ M $\pm$ 3.31	58.26 μ M $\pm$ 2.25
Lamivudine	54.96 μ M $\pm$ 1.3	51.61 μ M $\pm$ 3.14
Stavudine	81.01 μ M $\pm$ 3.23	102.57 μ M $\pm$ 2.82
Zidovudine	67.97 μ M $\pm$ 3.78	75.83 μ M $\pm$ 9.94
Efavirenz	67.67 μ M $\pm$ 4.48	34.41 μ M $\pm$ 2.38
Nevirapine	56.56 μ M $\pm$ 4.5	37.42 μ M $\pm$ 1.93

* Standard error ($N = 3$).

nearly all combinations against both chloroquine resistant and chloroquine sensitive *P. falciparum* parasites (Figure 1B, Table 2), except at combination 4 (i.e., 2:3 drug ratios) where the interaction was additive.

Malaria and HIV infection are among the most important infectious diseases of man. HIV protease inhibitors reduce viral load in patients. Guidelines for the optimal use of antiretroviral therapy include the use of protease inhibitors, indinavir and nelfinavir, as antiretroviral agents for treatment of advanced HIV infection on the basis of their strong clinical

TABLE 2

Interaction between artemisinin and indinavir or nelfinavir tabulated as sum FICs (Σ FICs), against *P. falciparum* (3D7 and RKL 303 strains) at six fixed-dose preparations

Combination preparation	Strain	Artemisinin-Indinavir	Artemisinin-Nelfinavir
	Used	Σ FICs, Interaction*	Σ FICs, Interaction*
1	3D7	1.02, ADD	1.13, ADD
	RKL 303	1.01, ADD	1.00, ADD
2	3D7	0.49, SYN	0.74, SYN
	RKL 303	1.42, ADD	0.36, SYN
3	3D7	0.95, SYN	0.79, SYN
	RKL 303	0.42, SYN	0.72, SYN
4	3D7	0.42, SYN	1.45, ADD
	RKL 303	0.85, SYN	0.45, SYN
5	3D7	0.66, SYN	0.47, SYN
	RKL 303	0.49, SYN	0.36, SYN
6	3D7	1.08, ADD	1.03, ADD
	RKL 303	1.09, ADD	1.00, ADD

* Standard error ($N = 3$).

SYN = synergy; ADD = additive.

benefit and/or restrained sustainability of plasma viral load.¹⁹ Combination antiretroviral regimens containing nelfinavir are commonly recommended to HIV-infected pregnant women.²⁰ Risk of antimalarial treatment failure persists in immune suppressed patients. Effect of these HIV inhibitors on the antimalarial efficacy of drugs is best studied using *in vitro* *P. falciparum* culture system, devoid of immune interference. Synergistic

FIGURE 1. Isobolograms describing the interaction between artemisinin with indinavir (A) or nelfinavir (B) against *P. falciparum* isolate 3D7 and RKL 303. Each combination was done in triplicate and data point shows mean FIC of each drug in six different combination ratios with standard error bars. The line of additivity joins both axes at mean FIC_{50} values of combination 1 and 6.

interactions of other HIV protease inhibitors in combination with chloroquine or mefloquine have been reported.⁵ Intraerythrocytic parasite digests hemoglobin and converts the toxic heme generated into non-toxic crystalline hemozoin. The *P. falciparum* genome sequence has revealed the existence of 10 aspartic proteases (plasmepsins), some of which are involved in hemoglobin digestion.^{5,13} Plasmepsins inhibition by HIV protease inhibitors has already been suggested.¹³ However, which specific enzymes are inhibited remains to be determined. In silico docking experiments with some HIV protease inhibitors have been shown to fit in the active site of *P. falciparum* aspartyl proteases.²¹ An endoperoxide bridge in artemisinin structure interacts with iron in the infected erythrocyte to form a free toxic radical that destroys proteins in nanodomain neighborhood, that are crucial to the parasite's survival, leading to demise of the parasite.²² Artemisinin inhibits an essential calcium adenosine triphosphatase enzyme on endoplasmic reticulum of falciparum, disrupting calcium homeostasis.²³ This multi-targeted attack on the parasite by artemisinin may even be responsible for inhibiting proteolytic activity of some of the hemoglobin digesting enzymes. The exact mode of action of artemisinin on parasites remains elusive as does that of indinavir or nelfinavir. Synergistic interaction observed between these two different groups of drugs may be due to protease inhibition of different antimalarial metabolic steps in hemoglobin digestion. In conclusion, indinavir or nelfinavir aid artemisinin in antimalarial activity *in vitro*.

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