Genetic Background Modulates Falcipain 2a Driven Resistance to Peptidomimetic Inhibitors in *Plasmodium falciparum*

Igor M. R. de Moura[1,2,3], Giovana R. Mendes[1], Vinicius Bonatto[1], Felipe C. P. Martins[4], Sunil K. Narwal[2,3], John Okombo[2,3], James Murithi,[2,3] Tomas Yeo,[2,3] Anna Caroline C. Aguiar[5], Carlos A. Montanari[4], David A. Fidock[2,3], Rafael V. C. Guido[1]

[1] Instituto de Física de São Carlos, Universidade de São Paulo, São Paulo, Brazil
[2] Department of Microbiology and Immunology, Columbia University Irving Medical Center, New York, NY
[3] Center for Malaria Therapeutics and Antimicrobial Resistance, Columbia University Irving Medical Center, New York, NY
[4] Instituto de Química de São Carlos, Universidade de São Paulo, São Paulo, Brazil
[5] Instituto de Saúde e Sociedade, Universidade Federal de São Paulo, São Paulo, Brazil

Malaria caused by *Plasmodium* parasites remains a major global health burden, and the emergence of parasites with reduced susceptibility to artemisinin highlights the need for new antimalarial chemotypes. Peptidomimetic compounds are promising antiplasmodial agents, particularly as protease inhibitors. Here, we investigated a series of 45 dipeptide-like molecules and the mode of action and mechanism of resistance for the most promising compound, Neq1153. Neq1153 inhibited asexual blood stages with a mean IC_{50} of 600 nM and a selectivity index of 350 over HepG2 cells and revealed to be a slow-acting inhibitor. Marked background-dependent differences were observed, with Dd2 parasites exhibiting ~10-fold greater sensitivity than 3D7 to Neq1153, with an IC50 of 70 nM. Drug-drug interaction assays showed antagonism with artesunate and chloroquine, consistent with functional overlap with hemoglobin digestion pathways.

Phenotypic profiling revealed associations between Neq1153 susceptibility and digestive vacuole related proteins. Parasites with increased *pfpm2/3* copy number showed a 2-4 -fold reduction in susceptibility, whereas lower *pfmdr1* copy number correlated with increased sensitivity. Verapamil supplementation did not interfere with Neq1153 efficacy, showing that PfCRT might not be related to Neq1153 phenotype.

In vitro evolution under Neq1153 pressure repeatedly selected alterations in Falcipain 2a (FP2a) in 3 different genetic backgrounds, including copy number amplification and the point mutations S392I in 3D7 and S392R in Dd2. CRISPR/Cas9 editing demonstrated strong genetic background dependence: introduction of FP2a S392R into Dd2 showed resistance, whereas the same mutation in 3D7 background failed to do so. Introduction of S392I in both 3D7 and Dd2 backgrounds failed to promote resistance against Neq1153. This experiment highlights the influence, still not fully understood, of an African and Asian backgrounds in gene edited lines. CRISPR/Cas9 editing is undergoing in different genetic background aiming to understand the genetic background influence on FP2a-mediated response to peptidomimetic inhibitors.

Together, these findings show that parasite genetic background governs both susceptibility and resistance to peptidomimetic inhibitors. Resistance emerges from quantitative, background-dependent phenotypic shifts rather than single mutations alone, underscoring the need to integrate parasite diversity into the development and resistance-risk assessment of FP2a-targeting antimalarials.