Distinct effects of HIV protease inhibitors and ERAD inhibitors on zygote to ookinete transition of the malaria parasite.

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Abstract

In an effort to eradicate malaria, new interventions are proposed to include compound/vaccine development against pre-erythrocytic, erythrocytic and mosquito stages of *Plasmodium*. Drug repurposing might be an alternative approach to new antimalarials reducing the cost and the time required for drug development. Previous *in vitro* studies have examined the effects of protease inhibitors on different stages of the *Plasmodium* parasite, although the clinical relevance of this remains unclear. In this study we tested the putative effect of three HIV protease inhibitors, two general aspartyl protease inhibitors and three AAA-p97 ATPase inhibitors on the zygote to ookinete transition of the *Plasmodium* parasite. Apart from the two general aspartyl inhibitors, all other compounds had a profound effect on the development of the parasites. HIVPIs inhibited zygote to ookinete conversion by 75%-90%, while the three AAA-p97 ATPase inhibitors blocked conversion by 50%-90% at similar concentrations, while electron microscopy highlighted nuclear and structural abnormalities. Our results highlight a potential of HIV protease inhibitors and p97 inhibitors as transmission blocking agents for the eradication of malaria.

Keywords

Malaria; ookinete development; HIV protease inhibitors; ERAD inhibitors

Despite all efforts to control and eradicate malaria, the disease still poses a major burden on human health. Drug resistance together with the absence of an effective vaccine has worsened the prospects for a “malaria-free” world. New antimalarial drugs are thus urgently needed, but the ultimate target of blocking malaria is no longer considered a one-goal strategy. Malaria transmission blockage could be theoretically achieved through the effective elimination of gametocytes from the bloodstream rendering human hosts non-infective to mosquitoes, or by interfering with the early parasite development inside the vector (gamete and zygote/ookinete stages).

Thus far though, transmission stages of *Plasmodium* parasites have largely been unexplored in drug screening approaches. Nevertheless, gametes, zygotes and ookinetes in the mosquito midgut lumen, are vulnerable stages, metabolically active, unprotected by the host cell membrane and exposed to the hostile environment of the mosquito midgut. In this study we tested the putative effect of 8 compounds, which belong to 3 distinct classes of inhibitors, on the zygote to ookinete transition that takes place in the mosquito midgut immediately after gamete fertilization and is an attractive target for the development of transmission blocking strategies. For this, we have used the rodent malaria model, *Plasmodium berghei*, where these stages differentiate efficiently *in vitro* under defined experimental conditions [1].

The first class of compounds includes 3 HIV protease inhibitors (HIVPIs); Nelfinavir, Ritonavir and Lopinavir. HIVPIs are specific protease inhibitors of the HIV aspartyl protease, which has an essential role in the generation of mature infectious virus particles [2]. Numerous studies over the last ten years have highlighted the inhibitory effect of HIVPIs on pre-erythrocytic stages and the asexual development of different *Plasmodium* species and also on gametocytogenesis, exflagellation and

viability of gametocytes, while also affecting mosquito infectivity [3-5]. Their effect though on zygote to ookinete transition has not yet been examined. Since HIVPIs inhibit the aspartyl protease of the HIV virus we also included two general aspartyl protease inhibitors; pepstatin A, which is known to inhibit intra-erythrocytic growth of *Plasmodium spp.* but has no effect on *P. gallinaceum* ookinete development *in vitro* [6] and diazo-acetyl-norleucinemethylester (DAN), which has not been previously tested in *Plasmodium spp.* cultures but has been used in *in vitro* proliferation assays in *L. Mexicana*. In the presence of DAN, parasites had alterations in shape, from a long slender form to a spherical one and cell division was blocked [7]. The last family includes 3 compounds, which target the ERAD pathway by inhibiting the action of an AAA ATPase (p97). Previous studies in mammalian cells have shown that Eeyarestatin 1 (Eer) is a bi-modular compound comprised from two functionally independent domains: an aromatic module (CBU) that targets ES to membranes, and a nitrofuran-containing module (5-NA). The compounds tested here were, Eer, CBU-028 and 5-nitrofuryl acrolein (NA), which consists of the nitrofuran domain of Eer [8]. Eer has been shown to inhibit growth of *Plasmodium falciparum* asexual stages with IC_{50} values of 3.5 μM [9].

To assess the transmission blocking potential of these compounds, the *P.berghei* CTRP-GFP strain was used that specifically expresses GFP after zygote formation thus making feasible the identification of toxicity of the tested compounds in early stages of development by FACS analysis [10]. All compounds used were added after formation of the zygote and were present in the *in vitro* cultures throughout ookinete development. Our results showed that only lopinavir at 50 μM and NA at 35 and 50 μM, had a significant toxic effect on parasite survival. The highest concentration of NA has been excluded from further studies. In all other treatments

GFP levels were not statistically different relative to control cultures (Fig. 1A). Giemsa stained smears of purified cultures were used to quantify the effect of each drug on the conversion rate of zygotes to ookinetes and to identify the stage of arrest judged by the morphology of the parasites. 35 μM of nelfinavir and ritonavir decreased development to mature ookinetes by 85% and 75%, respectively. At 50 μM both drugs caused an almost complete arrest at zygote/retort stages. Lopinavir treatment at 35 and 50 μM led to an arrest at stage I-II for the vast majority of parasites (~90%) (Fig. 1B). Eer had also a profound effect on the zygote/ookinete transition at 35 and 50 μM, arresting parasites at stage I-II, a pattern also observed with NA at 10 μM. 35 μM of NA led to an arrest in stage I zygotes (90%). Maximum inhibition exerted by CBU-028 was at 50 μM, while at 35 μM, 50% of parasites progressed to mature ookinetes. From these data it seems plausible that the nitrofuran moiety is responsible for the effect seen on zygote/ookinete transition. Surprisingly Pepstatin A and DAN had no effect on ookinete development at any concentration used in our study.

To determine the infectivity of the drug-treated cells, purified ookinetes from *in vitro* cultures were offered to mosquitoes in standard membrane feeding assays (SMFA). As shown in Fig. 1C, in the presence of HIVPIs very few oocysts are formed in the mosquito midguts suggesting that the ookinetes might have developmental defects that render them unable to establish an infection in the mosquito, as was the case with the p97 inhibitors. Treatment with pepstatin A and DAN, on the other hand, did not result in lower oocyst numbers compared to the DMSO-treated control.

Immunofluorescence assays using specific antibodies were performed to further investigate the defects in ookinetes derived from in vitro cultures to which the the HIVPIs and the p97 inhibitors had been added. The nuclear protein SET displayed no obvious abnormal pattern in treated parasites with the exception of nelfinavir where

the pattern of SET looks fragmented (Fig. 2A). The surface protein p28, in all cases but with varying degrees, was found in the cytoplasm indicating a possible defect in the trafficking of p28 to the surface of the ookinetes (Fig. 2A). The micronemal protein SOAP was present in the treated parasites, exhibiting though a more diffuse pattern throughout the cytoplasm in parasites treated with the different p97 inhibitors (Fig. 2B).

In order to further examine the cellular defects TEM was employed. The apical complex (AC) of HIV-treated parasites was detected in some cases, albeit in early stages of development (Fig. 2C-ritonavir). Micronemes were detectable in the cytoplasm (Fig. 2C, nelfinavir, lopinavir), and IMC was present in treated parasites. In contrast, the nuclear membrane seemed to be distorted in nelfinavir treated parasites, with expanded nuclei. Lopinavir treated parasites are characterized by extensive vacuolation. Parasites treated with the p97 inhibitors show vacuoles throughout the cytoplasm. A key characteristic though in all cases is the extended membrane structures around the nucleus, a possible effect of prolonged ER stress, which has been previously shown to induce changes in the structure of the ER [8, 11].

Our study on the effect of 8 compounds on the early mosquito stages of the malaria parasite have shown a potent negative effect of HIVPIs and p97 inhibitors on ookinete formation. Consequently oocyst formation was significantly decreased when these parasites were offered to mosquitoes. The target(s) of HIVPIs in *Plasmodium* spp have yet to be identified. The initial hypothesis of them targeting the vacuolar aspartyl proteases termed plasmepsins in erythrocytic stages (plasmepsin I, II, II, IV), which are responsible for the haemoglobin degradation, has proven wrong, since their effect persists in mutant knock-out parasites [12]. In the case of *P. falciparum*, two transporters (PfHT and PfCRT) have been suggested as potential targets of lopinavir and saquinavir respectively [13, 14], alongside the plasmepsin family but data are far

from conclusive. The lack of any effect with pepstatin A in our SMFA is not in agreement with the previous study in *P. gallinaceum* but in their experimental set up, infected blood with gametocytes was offered to the mosquitoes instead of purified ookinetes [6]. This might suggest that pepstatin A has an effect on gametocytes or gametogenesis. Overall, our results suggest that a *Plasmodium* aspartyl protease is not the primary target of HIV protease inhibitors on the zygote/ookinete conversion stage.

P97 inhibitors have not been extensively interrogated in *Plasmodium* as only Eer and another reversible inhibitor of p97, DBeQ, have been examined in *in vitro P. falciparum* cultures. This study showed that *Plasmodium* parasites have a very basic ERAD machinery compared to mammalian cells and thus they are much more sensitive to specific compounds that were designed to inhibit their mammalian molecular targets [15].

Although one would expect that HIVPIs and p97 inhibitors are quite distinct in their mode of action and on their molecular targets, they do share some interesting similarities. HIVPIs, mainly nelfinavir, ritonavir and saquinavir, have been repurposed for cancer treatment and clinical trials are currently ongoing with nelfinavir being predominantly the drug of choice [16]. But what is that makes HIVPIs promising candidates for anti-cancer treatment? HIVPIs and particularly nelfinavir have been shown to inhibit the Akt kinase pathway and also induce ER stress leading to activation of the unfolded protein response pathway (UPR). Inhibition of the ERAD pathway with the use of p97 inhibitors also leads to UPR activation [11, 17]. The inhibition of kinases by nelfinavir raises an interesting observation as a similar mis-localisation of p28 (Figure 2A) has been observed in CDPK1 mutant parasites [18]. Could it be that nelfinavir inhibits CDPK1 or other kinases thus affecting trafficking of this surface

antigen? Further studies are necessary to validate this observation. Overall, in *Plasmodium* spp, it seems more plausible that the severe effects of HIVPIs on all stages of the life cycle might be attributed to the off targets of the drugs by inhibition of kinases or by acting as general ER stressors. Further studies are needed in order to identify the potential molecular targets of each class of compounds (HIVPIs or p97 inhibitors).

The early mosquito stages are attractive targets for transmission blocking agents, as they take place soon after uptake of a blood meal by the mosquito and they are still present in the mammalian blood meal. Many groups are pursuing this path with a substantial amount of work been done for the identification of targets and inhibitors. Bumped kinase inhibitors are a good example of potential transmission blocking agents since they can prevent exflagellation of gametocytes in *P. falciparum* in the nM range and preliminary studies show a blockade in oocyst formation in mosquitoes [19]. There are however substantial challenges. Drugs to be given to the human host (HIVPIs for example), should be stable for a long period required for gametocyte maturation and in high concentrations, which will require new formulations and drug delivery strategies. Building on already available drugs, new scaffolds might provide better pharmacokinetic properties, or development of prodrugs could significantly enhance bioavailability. One could envisage HIVPIs *per se*, or as starting points for compound development, to be used for parallel application in malaria/HIV coinfection or as antimalarial drugs and transmission blocking compounds.

The approach of targeting the parasite in the vector is yet a largely unexplored option. Although the vector is not an obvious system for drug delivery, alternative approaches can be pursued to deliver drugs directly to the mosquito [20]. Recent advances in nanoparticle technology in combination with the development of sugar

traps to attract mosquitoes might provide an alternative route for targeting the parasite. Studies in P. falciparum using drug containing liposomes have provided some interesting data and could pave the way for new nanoparticles to be used as carriers for drugs or transmission blocking agents [21] The results presented here suggest that HIVPI's should be interrogated as suitable compounds for such strategies.

Acknowledgments

The work was supported by the grant Transmalaria bloc (contract number GA223736) to T.G.L. Konstantinos Koussis was supported by the Action "Supporting Postdoctoral Researchers" of the Operation Program "Education and Lifelong Learning" (Action's Beneficiary: General Secretariat for Research and Technology), and is co-financed by the European Social Fund (ESF) and the Greek State. The authors would like to thank Mrs. Sandra Siakouli for the excellent technical support with TEM and Dr Yihong Ye (Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, United States of America) for CBU-028 and NA compounds. This work is dedicated to the memory of Thanasis G. Loukeris.

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Figure legends

Figure 1

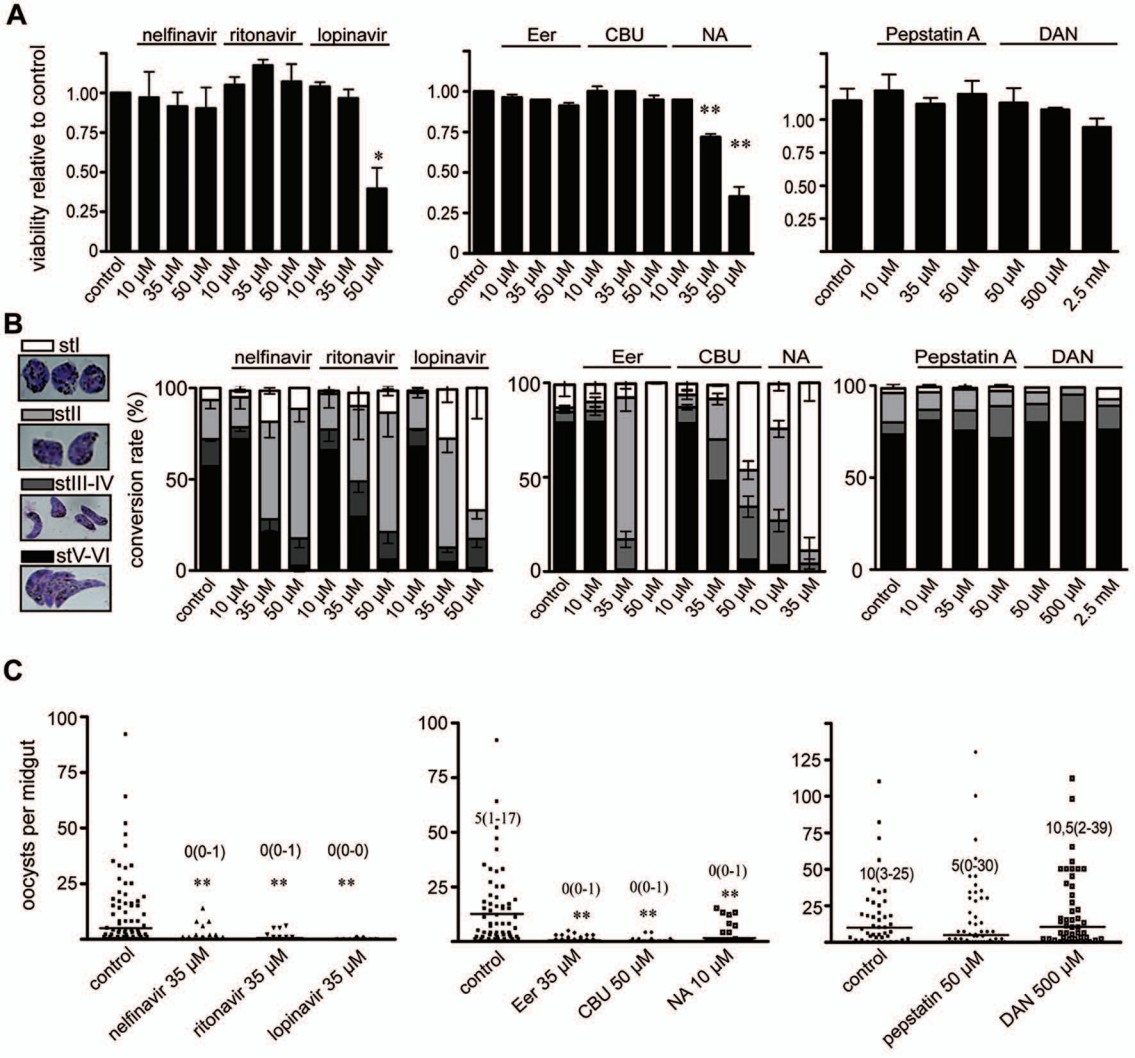
A. Cytotoxic effect of compounds on *P.berghei* ookinete development.
HIVPIs were obtained from Sequoia Research products Ltd. Diazoacetyl-DL-norleucine methyl ester (DAN) and pepstatin A were purchased from Sigma-Aldrich. Stocks (×1000) were prepared in DMSO. Dilutions of the drug were carried out in culture medium. CTRP-GFP expressing *P.berghei* ookinete cultures were analysed using FACS. For the *in vitro* ookinete cultures, blood from 2-3 mice was pooled, leucocytes removed and the blood was diluted 1:10 in ookinete medium (RPMI1640 containing 25 mM HEPES (Sigma), 10% FCS, 100 µM xanthurenic acid, pH 7.5). Samples were dispensed in 6 well-plates, incubated for 1h at 18˚C, after which the drug or DMSO alone, was added. After incubation at 18˚C for 24h, ookinete cultures were enriched and concentrated by Nycodenz gradient centrifugation. Increasing concentrations of the compounds were added after formation of the zygote and were present throughout ookinete development. Cell viability measured in Relative Fluorescence Units is shown relative to control. Bars represent means ± SE from three independent experiments. Asterisk symbols on columns indicate differences between control and compound treated groups. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ (one-way ANOVA followed by Dunett's multiple comparison test).
B. **Phenotypic analysis of compound treated ookinete cultures.** Compounds were added to the ookinete cultures after zygote formation and 18-24hrs after fertilisation purified parasites were stained with Giemsa and observed under light microscope (1000x). Morphologies of Giemsa stained untreated and treated parasites were grouped in four categories (stage I, II, III-IV, V-VI). Representative pictures of each stage are shown. Different morphological shapes were recorded in 12-15 optical fields in three independent experiments. The majority of control parasites have transformed to elongated ookinetes but compound treated parasites failed to reach this stage in the case of HIV-PIs and p97 inhibitors. Aspartyl-protease specific inhibitors do not affect parasite transition to mature ookinetes.
C. **Infectivity of compound treated *P.berghei* ookinetes.** *An. gambiae* mosquitoes were fed using SMFA with DMSO (control) or compound treated *P. berghei* ookinetes. Enriched ookinetes, treated with drugs as mentioned above, were mixed in fresh blood from naive mice at a density of $3\text{-}5x10^3$ ookinetes/µL. Graphs illustrate three independent experiments. Each dot represents the number of oocysts counted per individual *An. gambiae* midgut 6 days after feeding. The horizontal lines indicate the median numbers of oocysts. Error bars represent the interquartile range. Statistical significance between control and compound treated groups is shown when necessary by asterisks and was determined using one-way Anova followed by Dunett's multiple comparison tests (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$). Median number of oocysts with 25%-75% percentile range is indicated above each column.

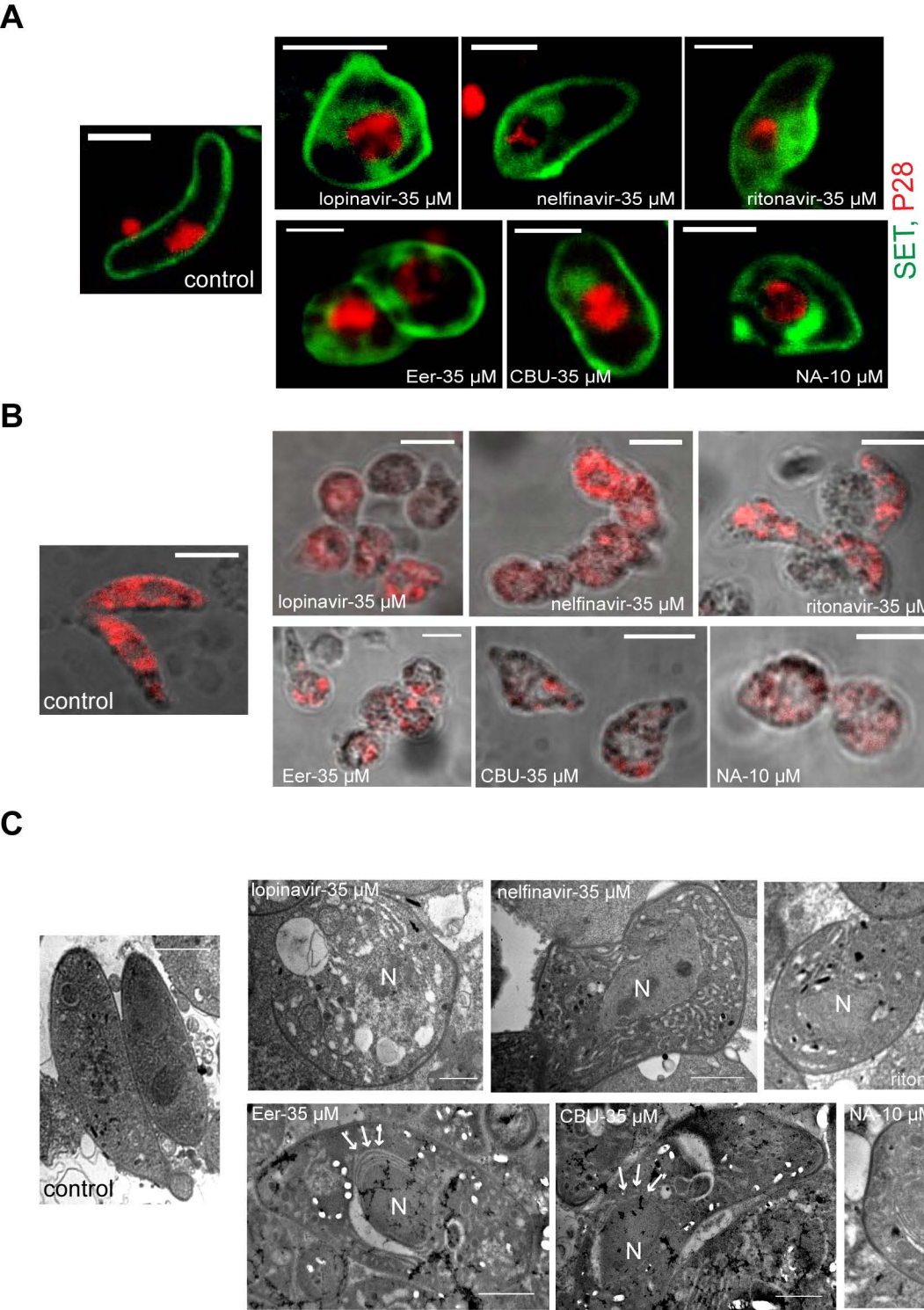
Figure 2

A and B. Expression pattern of nuclear, secreted and micronemal markers in control and treated parasites.
Indirect Immunofluorescence detection of a nuclear (SET), secreted (P28) (**panel A**) and micronemal (SOAP) (**panel B**) protein in control and compound treated parasites. SET distribution did not show any difference in the treated compared to untreated parasites apart from nelfinavir treated ookinetes. P28 localisation was membrane and

cytoplasmic in the treated parasites in contrast with the intense membrane staining of control parasites. Scale bar indicates 5 μM.

C. **Ultrastructural analysis of control and compound treated parasites.** TEM images of HIV-PIs and Eer-related compound treated parasites. All drugs used at 35 μM apart from NA that was used at 10 μM. In the case of nelfinavir treatment parasites exhibit prominent nucleoli (asterisk) and enlarged nuclei. Eer, CBU-028 and NA treated parasites show an extended membrane structure around the nucleus (white arrows). Abbreviations: Nucleus; N. Scale bar indicates 2 μM.


A
nelfinavir
ritonavir
lopinavir
Eer
CBU
NA
Pepstatin A
DAN
viability relative to control
1.00
0.75
0.50
0.25
0
*
**
control
10 µM
35 µM
50 µM
500 µM
2.5 mM
B
stI
stII
stIII-IV
stV-VI
conversion rate (%)
100
50
0
C
oocysts per midgut
100
75
50
25
125
0(0-1)
0(0-0)
5(1-17)
10(3-25)
5(0-30)
10,5(2-39)
nelfinavir 35 µM
ritonavir 35 µM
lopinavir 35 µM
Eer 35 µM
CBU 50 µM
NA 10 µM
pepstatin 50 µM
DAN 500 µM


A
control
lopinavir-35 μM
nelfinavir-35 μM
ritonavir-35 μM
Eer-35 μM
CBU-35 μM
NA-10 μM
SET, P28
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control
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ritonavir-35 μM
Eer-35 μM
CBU-35 μM
NA-10 μM
SOAP
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control
lopinavir-35 μM
nelfinavir-35 μM
ritonavir-35 μM
Eer-35 μM
CBU-35 μM
NA-10 μM
N

Distinct effects of HIV protease inhibitors and ERAD inhibitors on zygote to ookinete transition of the malaria parasite.

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Summary
HIV protease inhibitors and ERAD inhibitors block zygote to ookinete transition in P. berghei highlighting them as potential transmission blocking agents for the eradication of malaria.