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Short Communication

Inhibition of *Plasmodium falciparum* cysteine proteases by the sugarcane cystatin CaneCPI-4

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ABSTRACT

Malaria is a disease caused by *Plasmodium* parasites that affects hundreds of millions of people. *Plasmodium* proteases are involved in invasion, erythrocyte egress and degradation of host proteins. Falcipains are well-studied cysteine peptidases located in *P. falciparum* food vacuoles that participate in hemoglobin degradation. Cystatins are natural cysteine protease inhibitors that are implicated in a wide range of regulatory processes. Here, we report that a cystatin from sugarcane, CaneCPI-4, is selectively internalized into *P. falciparum* infected erythrocytes and is not processed by the parasite proteolytic machinery. Furthermore, we demonstrated the inhibition of *P. falciparum* cysteine proteases by CaneCPI-4, suggesting that it can exert inhibitory functions inside the parasites. The inhibition of the proteolytic activity of parasite cells is specific to this cystatin, as the addition of an anti-CaneCPI-4 antibody completely abolished the inhibition. We extended the studies to recombinant falcipain-2 and falcipain-3 and demonstrated that CaneCPI-4 strongly inhibits these enzymes, with IC_{50} values of 12 nM and 42 nM, respectively. We also demonstrated that CaneCPI-4 decreased the hemozoin formation in the parasites, affecting the parasitemia. Taken together, this study identified a natural molecule as a potential antimalarial that specifically targets falcipains and also contributes to a better understanding of macromolecule acquisition by *Plasmodium falciparum* infected RBCs.

Malaria is an important human infection, resulting in 429 thousand deaths in 2015 [1]. Resistance to drugs appears early in the parasite population and inhibits the control of this disease. Approximately 100 proteases were identified in the *Plasmodium* genome [2], as proteolysis is tightly associated with intraerythrocytic cycle events, such as invasion and egress of erythrocytes, degradation of host proteins and inactivation of host immune defense mediators [3]. Three cysteine proteases from *P. falciparum*, falcipain-1, falcipain-2 and falcipain-3, have been well-characterized [4,5]. Localized in the food vacuole, falcipains-2 and falcipain-3 are essential for hemoglobin digestion, which is crucial for amino acid acquisition [4,5] and osmotic stability of the parasite [6].

Cystatins are competitive inhibitors of cysteine proteases and represent a major class of proteases inhibitors, the cystatin superfamily [7]. Phytocystatins or plant cystatins are considered a distinct family [8] and can be found in a variety of superior plants. Several biological functions have been attributed to phytocystatins, including regulation of endogenous proteinases and defensive roles against insects and fungi [9,10].

Sugarcane cystatins were first described by Reis et al. [11], who identified 25 cystatin-like sequences in the Sugarcane Expressed Tags Project (SUCEST) database [12]. Four sugarcane cystatins (CaneCPI-1, CaneCPI-2, CaneCPI-3 and CaneCPI-4) were recombinantly expressed and c [13–15]. Among them, CaneCPI-4 showed the highest inhibitory activity against human cathepsins B and L, attenuating the invasive activity of breast cancer cells [15]. In addition, CaneCPI-4 showed high inhibitory potential against a recombinant cathepsin L from *Sphenophorus levis* (*Sl-CathL*), a sugarcane pest [16].

Here, we evaluated the ability of CaneCPI-4 to inhibit *P. falciparum* development in *in vitro* cultures by reducing the cysteine protease proteolytic activity on hemoglobin. Isolated parasites or infected erythrocytes were incubated with recombinant CaneCPI-4 (MW = 13 kDa) labeled with the fluorescent probe FITC (isocyanate of fluorescein). The protein uptake in the cytoplasm of isolated parasites (Fig. 1A) and

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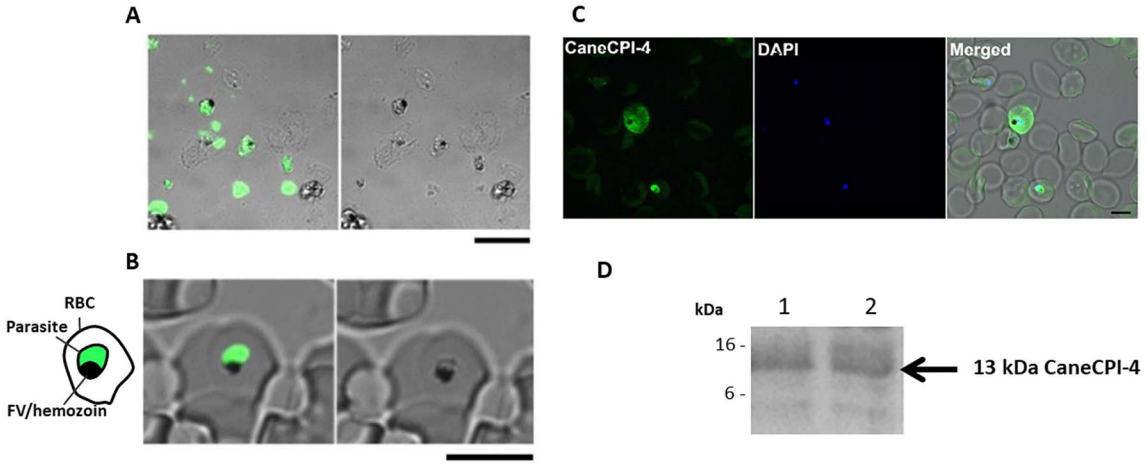

Fig. 1. Uptake and degradation resistance of CaneCPI-4 by *Plasmodium falciparum*. Fluorescent isothiocyanate isomer 1 (FITC 10 μg/μL) was conjugated to CaneCPI-4 according to the manufacturer's specifications (Sigma). (A) Isolated *P. falciparum* and (B) infected erythrocytes were resuspended in PBS buffer (10^3 cells/mL), and 1 mL of this suspension was incubated with 0.6 μM CaneCPI-4-FITC for 30 min, at 37 °C. The cells were placed on microscopy glass bottom dishes (MatTek Corp, Ahland, MA, USA) pre-treated with poly-L-lysine. The images were acquired on Confocal Zeiss LSM 780 (Carl Zeiss, Germany), with λ_{EX} = 488 nm and λ_{EM} = 505–550 nm and 100 × oil immersion objective. As a control, uncoupled FITC was incubated in the same conditions. (C) For immunofluorescence assays, the infected erythrocytes were incubated with CaneCPI-4 for 2 h, washed, fixed and incubated overnight at 4–8 °C with CaneCPI-4 antibody (1:250) and revealed with FITC-goat anti rabbit (1:250, Invitrogen) mouse maintained for 2 h at room temperature. The fluorescence was analyzed by confocal microscopy with λ_{EX} = 488 nm and λ_{EM} = 505–550 nm and 100 × oil immersion objective. Scale bar 10μm. DAPI staining (blue) (D) Lane 1: CaneCPI-4 control. Lane 2: CaneCPI-4 (2 μg/200 μL) incubated at 37 °C with isolated *P. falciparum* (10^7 cell/mL) in 50 mM sodium phosphate, pH 6.5, for 1 h in the presence of inhibitors of metalloproteases (0.5 mM *ortho*-phenanthroline), serine proteases (0.5 μM PMSF) and aspartyl proteases (1 μM pepstatin). The samples were centrifuged for 2 min (400 g), and the supernatants were submitted to SDS-PAGE (15%) and electroblotted onto a nitrocellulose membrane (Millipore). Following the blocking and washing steps, the membrane was incubated overnight with the primary antibody anti-CaneCPI-4 (1:1000) in PBS with 5% non-fat milk. The secondary anti-mouse IgG coupled to HRP (Millipore) was incubated for 1 h in PBS with 5% non-fat milk. The bands were visualized by chemiluminescence after reaction with TMB™ membrane substrate (KPL). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inside *P. falciparum*-infected erythrocytes (Fig. 1B) was assessed by fluorescence microscopy. Fluorescent-labeled CaneCPI-4 (CaneCPI-4-FITC) was selectively internalized by the parasites, while non-infected erythrocytes did not show any labeling (Fig. 1B). The cystatin internalization was also confirmed by CaneCPI-4 antibody recognition by confocal imunofluorescence (Fig. 1C). These data corroborate previous findings that *P. falciparum* can uptake proteins and peptides from extracellular medium [17,18]. Furthermore, *P. falciparum* did not cleave the cystatin as shown by Western blotting analysis (Fig. 1D), suggesting that the whole protein is resistant to proteolysis in iRBC until reaches the parasite cytoplasm.

To test this hypothesis, isolated parasites in suspension (10^7 cells/mL) were incubated with CaneCPI-4, and the intracellular proteolytic activity was determined using the fluorogenic substrate *Z*-Phe-Arg-MCA (Z = carbobenzoxy; MCA = 7-amino-4-methylcoumarin) (Fig. 2). This approach is advantageous because the inhibitor was assayed against the proteases in their natural environment, the parasite cytoplasm. We observed that at least 1 μM of CaneCPI-4 inhibited approximately 70% of the intracellular protease activity, while 10, 25 or 50 μM of the inhibitor nearly abolished the proteolytic activity (Fig. 2A). The inhibition is specific to the cystatin, as the addition of an anti-CaneCPI-4 antibody completely blocked the inhibition (Fig. 2B).

CaneCPI-4 showed strong inhibitory activity against recombinant falcipain-2 and falcipain-3, with IC_{50} values of 11.9 ± 0.4 nM and 42.3 ± 0.8 nM, respectively (Table 1, Supplementary material). Falcipains are involved in hemoglobin cleavage, with generation of two fractions: the heme and the globin. The heme group is toxic to the parasite, and the detoxification is mediated through hemozoin formation (malaria pigment) [4,5]. As CaneCPI-4 inhibits falcipains, we analyzed the intracellular hemozoin formation in *P. falciparum* incubated with CaneCPI-4 (2 μg/200 μL). A significant reduction in hemozoin density was observed in *P. falciparum in vitro* (Fig. 2C) and no changes in morphology of food vacuole were observed in CaneCPI-4 treatment (data not shown). Furthermore, we investigated the effect of CaneCPI-4 in *P. falciparum* development and/or replication during the erythrocytic cycle (Fig. 2D) and values are summarized in the Table 2 presented in supplementary material. To this end, transgenic parasites expressing the reporter Nano-Luciferase, which is exported to red blood cells [19], were cultivated in presence of 6 μM CaneCPI-4 for up to 96 h, which is approximately two reinvasion cycles. As a control, corresponding to 100% growth, parasites were cultivated in the same medium without the cystatin. Growth was reduced by approximately 39% (p = 0.0003) after 96 h but not after 48 h or 72 h (Fig. 2D). Lower CaneCPI-4 concentrations did not affect growth under the conditions assayed (data not shown).

Taken together, our results indicated for the first time that cystatins may be used to inhibit *P. falciparum* cysteine proteases, as CaneCPI-4 inhibit falcipain activity at nM range and consequently reduce the parasite proteolysis compromising the cell development. The cystatin action identified in the present work involves the inhibition of a key biochemical process, such as the amino acid availability from hemoglobin, which directly compromises parasite growth. Furthermore, CaneCPI-4 is also a promising molecule for *P. falciparum* studies *in vivo*, as it does not present cytotoxicity in HUVECs [20], which is an important advantage compared to commercial cysteine protease inhibitors.