

## Catestatin, an endogenous Chromogranin A-derived peptide, inhibits in vitro growth of *Plasmodium falciparum*

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**Abstract** Catestatin, an endogenous peptide derived from bovine chromogranin A, and its active domain cateslytin display powerful antimicrobial activities. We have tested the activities of catestatin and other related peptides on the growth of *Plasmodium falciparum* in vitro. Catestatin inhibits growth of the chloroquine-sensitive strain of

*P. falciparum* 3D7, exhibiting 88% inhibition at 20  $\mu$ M. A similar partial inhibition of parasite growth was observed for the chloroquine-resistant strain, 7G8 (64%) and the multidrug-resistant strain, W2 (62%). In the presence of parasite-specific lactate dehydrogenase, a specific protein–protein interaction between catestatin and plasmepsin II precursor was demonstrated. In addition, catestatin partially inhibited the parasite-specific proteases plasmepsin in vitro. A specific interaction between catestatin and plasmepsins II and IV from *P. falciparum* and plasmepsin IV from the three remaining species of *Plasmodium* known to infect man was observed, suggesting a catestatin-induced reduction in availability of nutrients for protein synthesis in the parasite.

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### Introduction

A series of antimicrobial peptides were derived from the processing of the adrenomedullary chromogranins A and B (CgA and CgB), proenkephalin-A, and ubiquitin, all co-stored and co-secreted with catecholamines upon stimulation of the chromaffin cells [1–7]. Eight pairs of dibasic residues in bovine CgA correspond to major sites for processing in the intragranular matrix [8] to generate several bioactive peptides [9], among them a 21-residue-long cationic peptide (net charge +5) named catestatin (bovine CgA344–364, bCts). This peptide was initially characterized for its inhibitory effect on catecholamine secretion from chromaffin cells of the adrenal medulla [10, 11], and was identified as a product from stimulated chromaffin

cells [12]. The sequence of Cts is highly conserved in mammals (Table 1) [13–19], and cateslytin (bovine CgA344–358, CTL) corresponds to the antimicrobial active domain [2]. The antibacterial potency of the CTL domain has been confirmed in studies of Cts as an antibacterial agent in human skin [20]. A role for these antimicrobial CgA peptides in innate immunity was demonstrated by their presence in secretions of stimulated polymorphonuclear neutrophils (PMNs) [2] and by the fact that Cts activates neutrophils by inducing calcium entry [21].

Malaria is endemic in about 100 developing countries and over 3 billion people live under the threat of malaria infection. Malaria causes over 1 million deaths each year, most of whom are children [22, 23]. The most recent estimates indicate that there are more than 500 million clinical cases of malaria annually on the planet, a number that nearly doubles previous estimates [24, 25], and the disability adjusted life years (DALYs) of malaria are estimated at 45 million [23]. Furthermore, drug resistance of *P. falciparum* has emerged in all classes of antimalarial drugs [26, 27], and increased efforts in antimalarial drug discovery are urgently needed [28, 29]. Because some antiplasmodial agents are host cytotoxic molecules [30], natural non-toxic peptides might represent a new approach for malaria drug research.

Plasmepepsins are aspartic proteases involved in the degradation of the host cell haemoglobin, providing nutrients for parasite growth [31] and other as yet unidentified functions. Novel and potent inhibitors of *Plasmodium* plasmepepsins were identified by using an automated procedure to post-process the results of a large docking screen of commercially available compounds. Efforts to design new inhibitors against malarial plasmepepsins have employed a range of computational tools in the design process, including homology modeling, automated docking, enzyme

reaction simulations, and microscopic calculations of binding free energies [32]. Malaria parasites, both in culture and in animal models, are killed by inhibitors of plasmepepsins, establishing proof of concept that these proteases are important as drug targets [33], especially when combined with the inhibition of falcipains that are also involved in haemoglobin degradation [32]. Previous studies have indicated that several peptidomimetics inhibitors, obtained from combinatorial chemistry based peptides libraries, have a high inhibitory effect on various plasmepepsins [34]. Because some of these inhibitors show sequence similarities with Cts, we investigated the effect of Cts on *Plasmodium* growth.

The aim of the present study was to probe Cts and CTL for their antimalarial activities. Our results show that Cts inhibits growth of several strains of *P. falciparum*, not only the chloroquine-sensitive strain 3D7 but also the chloroquine-resistant (7G8) and the multidrug-resistant (W2) strains. The structural parameters crucial for the antimalarial potency have been identified, as well as a potential plasmodial target protein within the plasmepepsin family [35].

## Materials and methods

### Synthetic peptides

The synthetic peptides were prepared with an Applied Biosystems 433 A synthesizer (Foster City, USA), using the stepwise solid-phase method with 9-fluoromethoxycarbonyl (Fmoc chemistry) [2, 36]. As a negative control, we used the inactive scrambled Cts breaking the putative secondary structure (SLPRRQLPSSAGMRGGKFAYF). For fluorescence studies, rhodaminated peptides were obtained as previously reported [6]. Synthetic peptides were finally purified by HPLC on C18 reverse-phase

**Table 1** Alignment of Cts from different species and sequence similarity (%)

		Sequence Similarity (%)	Ref.
	<b>hCGA<sub>352-372</sub></b>	S S M K L S F R A R A Y G F R G P G P Q L	100 [13]
	<b>bCGA<sub>344-364</sub></b>	R S M R L S F R A R G Y G F R G P G L Q L	90 [14]
	<b>mCGA<sub>364-384</sub></b>	R S M K L S F R T R A Y G F R D P G P Q L	86 [15]
	<b>rCGA<sub>367-387</sub></b>	R S M K L S F R A R A Y G F R D P G P Q L	95 [16]
	<b>eCGA<sub>361-381</sub></b>	R S M K L S F R A R A Y G F R G P G L Q L	90 [17]
	<b>pCGA<sub>343-363</sub></b>	R S M K L S F R A P A Y G F R G P G L Q L	86 [18]
	<b>fCGA<sub>318-335</sub></b>	R S M K I P T K D Q K Y - - - E P A S E E	38 [19]
Homolog residues are in white letter by comparison with human Cts	<b>Consensus sequence</b>	R S M K L S F R A R A Y G F R P G Q L R I K G A	[13]

*h* human, *b* bovine, *m* mouse, *r* rat, *e* equine, *p* pig, *f* frog, – deletion

columns and lyophilized. Purity of the synthetic peptides (>98%) was established by HPLC and MALDI-TOF mass spectrometry, and the concentration was evaluated by automatic Edman sequencing [2] after correction with the Edman degradation yield.

#### Study of peptide activity on *P. falciparum* strains in vitro

We have used three *P. falciparum* strains with different origin and chemo-sensitivities: an African strain sensitive to chloroquine (3D7), a Brazilian strain resistant to chloroquine (7G8), and a South-East Asian strain resistant to chloroquine, quinine, and halofantrine (W2), kindly provided by Prof. S. Picot (EA 3732 Parasitologie, Mycologie Médicale et Pathologie Exotique, Faculté de Médecine, Université Claude Bernard, Lyon, France) and Prof. J. Le Bras (EA 209 Centre National de Référence pour la Chimiosensibilité du Paludisme, APHP, Hôpital Bichat-Claude Bernard, Paris, France). The parasite strains were kept in continuous culture with O<sup>+</sup> erythrocytes (Etablissement Français du Sang, Strasbourg, France) in a malaria culture medium (MCM, pH 7.4) consisting of RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM Hepes (Gibco, Invitrogen, Cergy Pontoise, France), 1 µg/ml hypoxanthine, 0.11 mg/ml Na pyruvate, and 0.02 mg/ml gentamycin in the presence of 10% (v/v) alpha calf serum (Perbio Science, Brebières, France) for the 3D7 strain or 10% (v/v) human group A<sup>+</sup>B<sup>+</sup> serum (Etablissement Français du Sang, Strasbourg, France) for the 7G8 or W2 strain, using an adapted candle jar method as previously described [37–39]. The cultures were evaluated at 1% haematocrit and 1% parasitemia. Ring-form stages and schizonts of *P. falciparum* [40] were also examined. Stock solution of aqueous peptides (1 mM) were prepared and diluted to give final concentrations of 0.02–20 µM in MCM. Antiparasitic activity was assessed at 24, 48, 72, and 96 h of incubation. Scrambled Cts were used as negative controls. In preliminary experiments, scrambled Cts was found to be inactive against the chloroquine-sensitive strain 3D7 (data not shown).

#### Estimation of peptide effects on the parasite infection rate

In order to estimate peptide effects on *Plasmodium* infection rate, the *Plasmodium* cultures were enriched with aged forms (late trophozoites and schizonts) using Plasmion (Laboratoires Fresenius Kabi, France), as previously described [40]. Thereafter, young forms of *Plasmodium* (ring stages) were counted after 24 h of incubation in the presence of 50 µM Cts at 37°C. Control experiments were performed with scrambled Cts at the same conditions. In all

experiments, parasitemia was determined by light microscope (×100), counting the proportion of parasitized red blood cells (RBCs) in about 20 fields of thin blood films prepared from culture. Mean and standard deviation were calculated from three different experiments, each being evaluated by three different readers [28]. In parallel, *Plasmodium*-derived lactate dehydrogenase (pLDH), a parasite-specific intracellular metabolic enzyme, was measured in whole blood in the absence or presence of peptides, using a commercial ELISA kit (DiaMed ELISA Malaria Antigen test; Cressier sur Morat, Switzerland) according to the manufacturer's recommendations [41]. Each peptide was incubated in MCM at 20 µM for 48 h before analysis by pLDH-ELISA. The pLDH test was used to determine the 50% inhibitory concentration (IC<sub>50</sub>). The IC<sub>50</sub> was estimated by linear regression analysis (standard curve) [ICEstimator software (<http://www.antimalarial-icestimator.net/>)]. For each peptide or drug, the data from the ELISA assay correspond to ten different concentrations. For each concentration, the mean of triplicate measurements was obtained and expressed in percent of the enzyme activity obtained with the controls (100%).

#### Analysis of Cts stability in the culture medium by reverse phase HPLC

Cts was incubated in MCM at 20 µM for 30 min to 24 h before analysis by RP-HPLC. The MCM medium and an aqueous solution of 20 µM Cts were used as separate controls. The analyses were performed on a Macherey–Nagel Nucleosil 300-5C18 column (4 × 250 mm) (Hoerd, France) with the Akta Purifier HPLC system (Amersham Biosciences, Les Ulis, France). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.09% (v/v) trifluoroacetic acid in 70% acetonitrile (Carlo Erba, Rodano, Italy) in water (solvent B). Elutions were performed at a flow rate of 700 µL/min using the gradient % B (0–10) for the first 10 min and then % B (10–100) for the remaining 40 min. Peptide absorbance in the eluate was monitored at 214 nm.

#### Haemolytic activity assay

Normal RBCs were washed three times in sterile PBS. After each wash, the tube was centrifuged at 450g for 5 min at 4°C, and then PBS was added to reach a final haematocrit concentration of 2%. Aqueous solutions of peptides were prepared by 10 serial dilutions in sterile PBS from a stock of 1 mM. Diluted peptides (0–300 µM, 150 µL) were added to suspend RBCs in each of 96 wells in a microplate. The haemolytic assay was carried out in triplicate, using 5% SDS as a positive control (100% haemolysis). The plate was incubated with agitation at

37°C for 40 min, centrifuged at 400g during 5 min at 4°C, followed by aspiration of supernatant and finally measuring the absorbance at 438 nm. Haemolysis of each concentration was calculated as a percentage by dividing the optical density (OD) for each concentration by the OD for 5% SDS.

#### Interactions between Cts and *Plasmodium* proteins

*Plasmodium* cultures were enriched with infected RBCs (iRBCs) from 15% in the initial cultures to 80% following Plasmagel treatment (Plasmion; Fresenius Kabi). Lysis of iRBCs with 0.05% saponin was carried out as previously described [42]. The solubilized *Plasmodium* was centrifuged for 5 min at 4,000g and washed three times in PBS. The pellet was sonicated ( $4 \times 10$  s), centrifuged twice at 4,000g, and the second supernatant was kept for further use. The two pellets were incubated with 0.05% Nonidet P-40 (Sigma-Aldrich, St-Quentin, France) and sonicated. The two resulting supernatants were pooled to constitute the *Plasmodium* soluble extract (PSE). The protein content was measured by Bradford assay (Bio-Rad, Marnes-la-Coquette, France). PSE was separated on SDS-10% PAGE under reducing conditions (in Bio-Rad apparatus). After transfer to a PVDF membrane (Bio-Rad), the protein bands were immunodetected using anti-MSP1 Mab (PEM-2; Santa-Cruz Biotechnology, Heidelberg, Germany) and a hyper-immune sera resulting from the pool of several African residents infected by *P. falciparum*, and containing high levels of malaria antibodies measured by a specific ELISA [43]. The secondary antibody was either an anti-human IgG (Roche Diagnostics, Meylan, France) or an anti-mouse IgG (Sigma, St Louis, MO, USA) conjugated to peroxidase, and the complex of antibodies and protein was visualized by ECL-autoradiography (Pierce ECL Western Blotting Substrate; Fisher Bioblock, Illkirch, France). Specific interaction between PSE and Cts was determined by incubating biotin-labelled peptides (100 µg of Cts or scrambled Cts) with 100 µL of streptavidin-conjugated Sepharose beads (GE Healthcare, Orsay, France) under constant shaking for 2 h at 4°C. The beads were centrifuged at 15,000g for 15 min, and washed in PBS containing a cocktail of proteases inhibitors (Roche Diagnostics). The beads were incubated with 100 µg of PSE under constant shaking overnight at 4°C, then washed four times in PBS and centrifuged at 15,000g for 15 min. PSE proteins interacting with the biotinylated Cts were identified by boiling the beads in 30 µL of Laemmli buffer and separating on 10% SDS-PAGE, under reducing conditions (Bio-Rad apparatus). Similar experiments were conducted with biotinylated scrambled Cts as controls. <sup>1</sup>D SDS-PAGE gels were prepared for mass spectrometry analysis.

#### In-gel digestion of separated components

Gels were systematically cut into slices and in-gel digestion was performed with an automated protein digestion system, MassPREP Station (Waters, Manchester, UK). The gel slices were washed three times in a mixture containing 25 mM ammonium bicarbonate: acetonitrile (1:1, v/v). The cysteine residues involved in disulfide bridges were reduced by adding 50 µL of 10 mM dithiothreitol at 57°C and alkylated by adding 50 µL of 55 mM iodoacetamide. After dehydration with acetonitrile, the proteins were cleaved with 40 µL of modified porcine trypsin (12.5 ng/µL; Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate, pH 7.2 at 37°C for 4 h. Finally, the tryptic peptides were extracted with 60% acetonitrile in 5% formic acid, followed by a second extraction with 100% (v/v) acetonitrile.

#### Mass spectrometry analysis of tryptic digests

NanoLC-MS/MS analysis was performed on a nanoACQUITY Ultra-Performance-LC (UPLC; Waters) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (SYNAPT<sup>TM</sup> MS; Waters). Each sample was loaded on a 20 × 0.18 mm, 5 µm Symmetry C18 precolumn (Waters), and the peptides were separated on a ACQUITY UPLC<sup>®</sup> BEH130 C18 column (Waters) (200 × 0.075 mm, 1.7 µm particle size). The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Trapping was performed for 3 min at 5 µL/min with 99% of solvent A and 1% of solvent B. Elution was performed at a flow rate of 400 nL/min, using 1–40% gradient (solvent B) over 35 min at 45°C followed by 65% (solvent B) over 5 min.

The MS and MS/MS analyses were performed on the SYNAPT<sup>TM</sup> (Waters) equipped with a Z-spray ion source in positive mode. The capillary voltage was set at 3.5 kV and the cone voltage at 35 V. The SYNAPT is equipped with a lock-mass system. Mass calibration of the TOF in the 50–2,000 m/z range was achieved using phosphoric acid. In line correction of this calibration was achieved using lock-mass on product ions derived from the [Glu<sup>1</sup>]-fibrinopeptide B (GFP).

The ion (M + 2H)<sup>2+</sup> at m/z 785.8426 is used to calibrate MS data and the fragment ion (M + H)<sup>+</sup> at m/z 684.3469 is used to calibrate MS/MS data.

For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes (MS 0.5 s/scan on m/z range 250–1,500 and MS/MS 0.7 s/scan on m/z range 50–2,000). The three most abundant peptides (intensity threshold 60 counts/s), preferably doubly and triply charged ions, were selected on each MS spectrum for further isolation and CID fragmentation. Fragmentation was performed using argon as the collision gas. To improve

the quality of MS/MS spectra during NanoLC-MS/MS analysis, we empirically derived energy curves depending on the  $m/z$  value of the selected precursor ion. For each  $m/z$  value, two different collision energies were applied.

The complete system was fully controlled by MassLynx 4.1 (SCN 566; Waters). Raw data collected during NanoLC-MS/MS analyses were processed and converted with ProteinLynx Browser 2.3 (23; Waters) into pkl peak list format. Normal background subtraction type was used for both MS and MS/MS with a 5% threshold and polynomial correction of Order 5 and deisotoping was performed.

#### Protein identification

The MS and the MS/MS data were analyzed using a local Mascot server (version MASCOT 2. 2. 0; MatrixScience, UK) and a composite target-decoy database including the protein sequences of the UniProtKB/Swiss-Prot version 55.2 and reversed versions of these sequences (total 719, 884 entries).

Searches were performed with a mass tolerance of 50 ppm for MS mode and 0.2 Da in MS/MS mode. One missed cleavage per peptide was allowed and variable modifications were taken into account such as carbamidomethylation of cysteine and oxidation of methionine.

To minimize false positive identifications, the results were subjected to stringent filtering criteria. For the identification of a protein with two peptides or more, at least two unique peptides had to fit a Mascot ion score above 25. In the case of single peptide hits, the score of the unique peptide must be greater than 50. For estimation of the false positive rate, a target-decoy database search was performed [44, 45]. With this approach, peptides are matched against a database consisting of the native protein sequences found in the database (target) and of the sequence-reversed entries (decoy). The evaluations were performed using the peptide validation software Scaffold (Proteome Software, Portland, USA). This strategy was used to obtain a final catalogue of proteins with an estimated false positive rate below 1%.

#### Plasmepsin kinetic assay

Wild-type recombinant plasmepsins proenzymes (PM II and IV) were expressed and purified as previously described [46, 47]. For the kinetic assays, the enzyme was incubated in 0.1 M sodium acetate, pH 4.5 for 5 min at 37°C to allow autoconversion to the mature enzyme and interaction with the inhibitor. The enzyme (free or inhibitor-complexed form) was mixed with a chromogenic substrate [46] and the initial rates of cleavage were measured using a Cary 50 Bio UV-Visible spectrophotometer for at least six defined substrate concentrations and at least

two different concentrations of inhibitors.  $K_i$  values were determined using SigmaPlot (Systat Software) [48].

#### Statistical analysis

The efficacy of the different peptide concentrations relative to the control was evaluated using the Kruskal–Wallis test. Different substrate concentrations with different rates of inhibition are presented in bar graphs and compared to the control inhibition rate, considering that parasitemia in the control bar will represent 0% inhibition. The test was performed three times to assure reproducible results.  $P$  values < 0.05 for difference from controls were considered significant. Statistical analysis was done using GraphPad InStat software (San Diego, CA, USA).

## Results

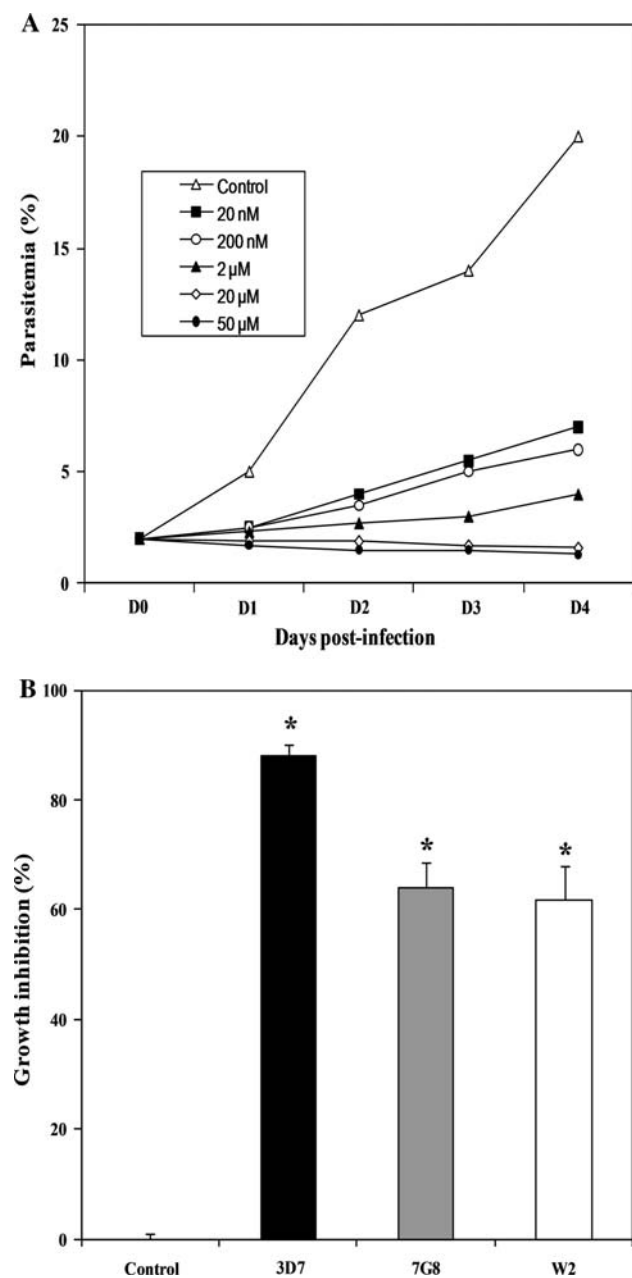
### Cts inhibits *Plasmodium falciparum* growth

Peptide activities against *Plasmodium* strains' growth were measured in vitro. Maximal growth inhibition of the 3D7 strain was reached with 20  $\mu$ M hCts (Fig. 1a). When we assayed hCts at the same concentration on two other strains, 7G8 (CQ resistant) and W2 (multidrug resistant), we reached 64 and 62%, respectively (Fig. 1b). Similar results were observed for ring-form stages and schizonts of the same *P. falciparum* strain (data not shown). The chloroquine-resistant *P. falciparum* strain 7G8 strain was less sensitive to hCts with growth inhibited by 36% at 20 nM and 64% at 20  $\mu$ M (Fig. 2a). Similarly, the growth of the multidrug-resistant *P. falciparum* strain W2 was inhibited by 22 and 62% at 20 nM and 20  $\mu$ M, respectively (Fig. 2b).

The production of pLDH persisted in these non-proliferating stage 48 h later, consistent with a viable state of the growth-inhibited parasite in the presence of hCts (Fig. 2a). We have estimated the half-life of hCts to 12 h by HPLC and Edman sequencing (data not shown). Therefore, we added successive amounts of peptide to cell cultures, but the inhibitory performance was not improved, and we did not observe any kind of haemolytic activity (data not shown). The  $IC_{50}$  was estimated at 14  $\mu$ M by pLDH measurement (Fig. 2b), pointing out that complete growth inhibition never exceeded 90% at any tested concentration higher than 20  $\mu$ M hCts.

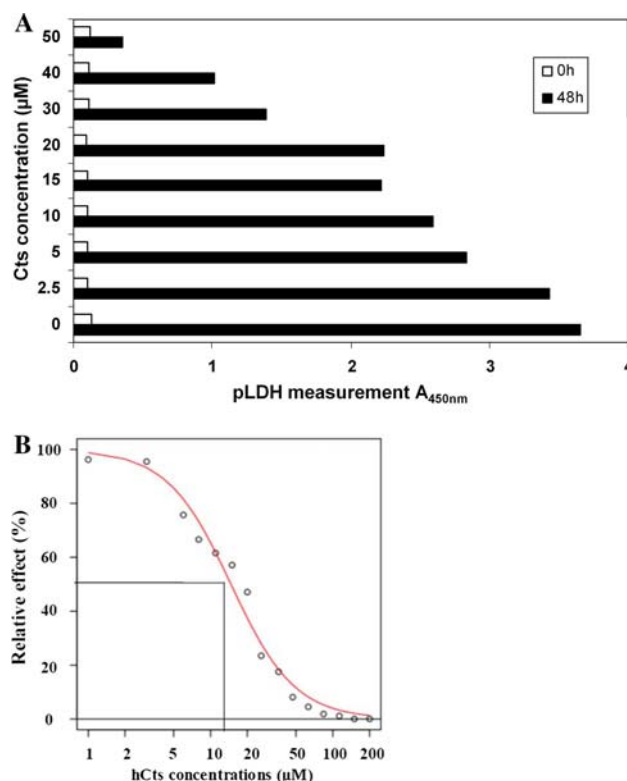
### Structure–activity relationship studies of Cts against the 3D7 strain

Human Cts was more active than the bovine peptide with respect to growth inhibitory effects on the 3D7 strain. The sequence similarity between the two Cts peptides is 90%



**Fig. 1** In vitro action of human Cts (hCGA<sub>352–372</sub>) on the growth of *P. falciparum* strains. **a** Parasitemia (%) was evaluated after adding hCts (20 nM–50 μM) to cultured *P. falciparum* strain 3D7 during 4 days post-infection (D0–D4). Maximal growth inhibition of 3D7 strain was reached with 20 μM; Control corresponds to *P. falciparum* 3D7 strain culture with the inactive scrambled hCts (20 μM). **b** Growth inhibition induced by 20 μM of hCts on *P. falciparum* chloroquine sensitive strain 3D7, chloroquine-resistant strain 7G8, and multidrug-resistant strain W2. Control corresponds to *P. falciparum* 3D7 strain culture with the inactive scrambled peptide (20 μM). Results are expressed as mean and SD from three independent experiments and were compared to control, \**P* < 0.01

[13–19] (Fig. 3). Four substitutions are apparent in positions 1, 4, 11, and 19 of the 21 residues, resulting in a higher cationic charge (+5) in bCts and bCTL than in hCts

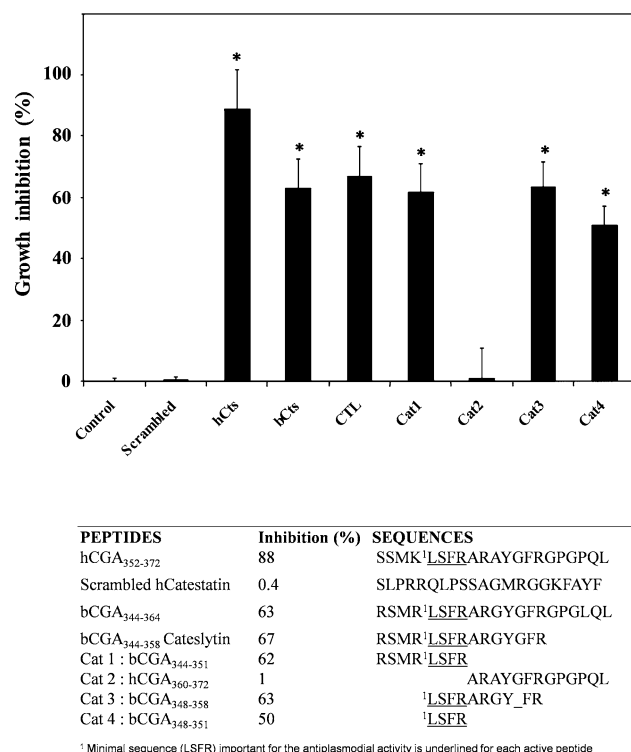


**Fig. 2** **a** Measurement by ELISA (A<sub>450 nm</sub>) of *Plasmodium* lactate dehydrogenase antigen (pLDH), as a reflection of parasite growth. *P. falciparum* 3D7 strain was cultured for 0 and 48 h without Cts and with Cts (2.5–50 μM). **b** hCts IC<sub>50</sub> was measured on *P. falciparum* 3D7 strain with the ICEstimator (<http://www.antimalarial-icestimator.net/>)

(+4) (Fig. 3). Notably, the substitutions of the serine residue in position 1 of hCts to arginine in bCts and bCTL, and of the alanine residue in position 11 of hCts to glycine in bCts and bCTL may be important for the higher inhibitory potency of hCts. In order to identify the region of Cts crucial for the antiplasmodial activity, several truncated peptides were also compared for growth inhibition of the chloroquine-sensitive strain, 3D7 (Fig. 3). The N-terminal region (Cat 1) and the median region (Cat 3) from which a glycine residue in position 13 was deleted were found to be as effective as the intact bCts. In contrast, the C-terminal region (Cat 2) and the scrambled hCts were devoid of inhibitory effects, while the shortest peptide (Cat 4), comprising the highly conserved sequence LSF<sub>13</sub>R, was able to inhibit parasite growth by 50% (Fig. 3). Altogether, these data demonstrate the importance of the LSF<sub>13</sub>R sequence for evaluating antiplasmodial activity.

#### Identification of plasmodial protein targets by mass spectrometry analysis

Soluble extracts of *Plasmodium* cultures were incubated with biotinylated hCts (or scrambled hCts as a negative



**Fig. 3** In vitro inhibition (%) of the growth of *P. falciparum* strain 3D7 by several Cts-derived peptides at 20  $\mu$ M: hCts, bCts, CTL (bCGA<sub>344-358</sub>), Cat 1 (bCGA<sub>344-351</sub>), Cat 2 (hCGA<sub>360-372</sub>), Cat 3 (bCGA<sub>348-358</sub> with deletion of G357), Cat 4 (bCGA<sub>348-351</sub>), and scrambled hCts, Control corresponds to *Plasmodium* growth without peptide. Each peptide was incubated in MCM at 20  $\mu$ M concentration for 48 h before analysis by pLDH-ELISA. Results are expressed as mean and SD correspond to three independent experiments, \* $p < 0.01$

control) and streptavidin-conjugated Sepharose beads overnight at 4°C. The parasite proteins bound to the washed beads were eluted at 100°C and identified by mass spectrometry analysis of components separated by <sup>1</sup>D SDS-PAGE. The gels were systematically cut into 46 bands and treated in-gel with trypsin. The tryptic released peptides were analyzed by nano LC-MS/MS. Three parasite proteins were specifically pulled down by Cts: tubulin beta chain (P14643), proliferating cell nuclear antigen (P31008) and a precursor of plasmepsin II (P46925) (Table 2,

and electronic supplementary data: mass spectrometry-proteomic analysis).

### Inhibition of Plasmepsin activities by Cts

An in vitro assay was established to assess the putative inhibitory potencies of Cts on recombinant PfPM2 and PfPM4 (PfPM: *P. falciparum* plasmepsin) (Table 3). Ki values of 130–140 nM hCts were obtained for both PfPM2 and PfPM4. In contrast, the Ki values for the scrambled hCts for both enzymes were 26 and 40 fold higher, respectively, while the Ki values for the shortest active peptide LSFR were 14- to 15-fold higher than for hCts.

Plasmepsin IV homologues are found in the remaining species of *Plasmodium* known to infect man: *P. ovale*, *P. vivax*, and *P. malariae*. When we compared the Ki values for hCts for these plasmepsins to those obtained for PfPM4 (Table 3), the order of inhibitory potency of hCts was: PfPM4 > PvPM4  $\gg$  PoPM4  $\gg$  PmPM4. For all four *Plasmodium* species, the order of inhibitor potencies was hCts  $\gg$  LSFR > scrambled hCts.

### Discussion

In the present study, we have shown for the first time that hCts, the natural cationic antimicrobial CgA-derived peptide, inhibits *Plasmodium* growth in vitro, not only of the chemo-sensitive strain 3D7, but also of the two drug-resistant strains, 7G8 and W2, of *P. falciparum*. Until 100  $\mu$ M, CTL, the active domain, does not display lytic effect on RBCs and other host cells (human neuroblastoma SKNMC and cardiomyocytes H9C2) (Jenny Briolat, Thesis of the University of Strasbourg, 2006). The parasite remains viable as assessed by persistence of a basic pLDH production in the presence of hCts, also suggesting a role for this CgA peptide in the first line of defence against invasion of this pathogen, and explaining why growth inhibition was never complete. Recently, our group has demonstrated the immunomodulatory properties of Cts by stimulating neutrophils [49]. Although yet to be demonstrated during human or rodent malaria infections, Cts

**Table 2** Putative targets of Cts identified by mass spectrometry

Protein name	Accession numbers	Protein molecular weight (AMU)	Catestatin (%)	Scrambled Catestatin
Tubulin beta chain: <i>Plasmodium falciparum</i> (isolate K1/Thailand)	P14643 TBB_PLAFK,Q7KQL5 TBB_PLAF7	49,732.8	99	0
Plasmepsin-2 precursor: <i>Plasmodium falciparum</i>	P46925 PLM2_PLAFA	51,473.8	99	0
Proliferating cell nuclear antigen: <i>Plasmodium falciparum</i> (isolate K1/Thailand)	P31008 PCNA_PLAFK,P61074 PCNA_PLAF7	30,570.3	98	0

**Table 3**  $K_i$  values in nM of catestatin, scrambled catestatin, and LSFR with plasmepsin II from *P. falciparum*, plasmepsin IV from all four *Plasmodium* species known to infect man

Inhibitor/plasmepsin	PfPM2	PfPM4	PoPM4	PvPM4	PmPM4
$K_i$ (nM)					
Catestatin	130 ± 10	140 ± 10	1,000 ± 120	320 ± 30	5,600 ± 770
Scrambled Catestatin	3,400 ± 310	5,600 ± 780	10,600 ± 1,300	2,800 ± 270	51,000 ± 6,000
LSFR	2,000 ± 200	1,900 ± 190	7,800 ± 850	1,400 ± 150	31,000 ± 2,400

secretion from neuroendocrine cells and PMNs triggered by invading microorganisms [2] may also contribute to the first defence against the *P. falciparum* infection.

Antimicrobial peptides selectively targeting pathogens often present a broad spectrum of action. Several naturally occurring antimicrobial peptides have been tested as potential anti-plasmodium agents, including scorpion, magainin 2, cecropin B, defensin, and dermaseptin S3 and S4 [50–53], as well as the synthetic peptides Vida 1-3, P2WN, ILF, SM1, SB-37, Shiva-1 and 3, and LAH4 [54–59]. Hence, a discussion of the structural requirements for the Cts molecule, a peptide naturally present in humans, on parasite growth, and on the inhibitory potential of the peptide on parasite specific enzymes are important in this context. It is evident from the present experiments that the Cts domain LSFR by itself expresses a growth inhibitory potency (50% inhibition of the 3D7 strain; Fig. 3). Intriguingly, the N-terminal serine residue unique to hCts appeared essential for maximal inhibition (88%) relative to the significantly lower inhibitory effects of bCts and bCTL (63–67%), both containing arginine as a substitute for the N-terminal serine. Moreover, the bovine peptides Cat 1 and Cat 3, both containing the LSFR-sequence, were equipotent with bCts and CTL, while the scrambled hCts and the C-terminal Cat 3 were completely inactive. These findings strongly suggest that the observed growth inhibitory potency on *P. falciparum* is specific for the primary sequence of hCts and dependent on the conservation of the LSFR domain.

Among the synthetic peptides described up to now as inhibitors of *Plasmodium* growth are inhibitors of plasmepsin family members. They were found after homology modeling and molecular docking [32]. Previous computational inhibitor design against malaria plasmepsin II [32] demonstrates that the natural haemoglobin peptide RMFLSF [32–38] is a substrate of the aspartic protease, which rapidly cleaves haemoglobin between F33 and L34. Interestingly, Cts sequence and LSFR both include the short LSF fragment. This observation suggests that Cts might interact with plasmepsin II as a competitive substrate. A non-specific binding of the tetrapeptide cannot be excluded despite the fact that LSFR, but not the scrambled peptide, partially arrested *Plasmodium* growth in vitro. The mechanism underlying this phenomenon will be a topic for

future experiments. Indeed, the identification by mass spectrometry of Cts binding proteins in *Plasmodium* extract show the presence of tubulin beta chain, proliferating cell nuclear antigen and plasmepsin II precursor. It cannot be excluded that Cts might interact with parasite microtubules [60, 61] or proliferating cell nuclear antigen [62] (Table 2), but detection of plasmepsin II precursor attracted interest because, as detailed above, parasite-specific aspartic proteases of the plasmepsin family have a significant role in the processing of haemoglobin. PfPM1, PfPM2, and PfPM4 are localized in the digestive vacuole found in *P. falciparum* [63–65], and the present data made it evident that hCts is able to preferentially inhibit the in vitro activity of recombinant PfPM2 and PfPM4 of *P. falciparum* (Table 3). In previous studies [47], the quadruple-PM KO 3D7 strain showed a significantly slower rate of growth in standard medium similar to the wild-type 3D7 treated with hCts without lysis of parasites. Following these observations, we can suppose that hCts can interact with plasmepsins and inhibit their functions. The corresponding  $K_i$  values of 130–140 nM Cts suggest that these two PMs may be targets for this peptide in this species of *Plasmodium*, in addition to the sequence comparison with the natural substrate and the incomplete *Plasmodium* growth inhibition. In addition, Cts also showed good efficacy as a plasmepsin inhibitor on resistant as well as sensitive *P. falciparum* strains (not shown). Of interest, plasmepsin IV is shared by all four *Plasmodium* species known to infect man. The present experiments have shown that hCts is also able to inhibit, albeit with significantly higher  $K_i$  values, the activity of recombinant plasmepsin IV from *P. ovale*, *P. vivax*, and *P. malariae* in vitro, demonstrating some inter-strain inhibitory capacities (Table 3).  $K_i$  values for a series of synthetic inhibitors of PfPM1 and PfPM2 are available in the literature [66]. The  $K_i$  values for Cts on *P. falciparum* were comparable, if not better, than those reported for most synthetic inhibitors. Cts IC<sub>50</sub> of the 3D7 strain (14  $\mu$ M) is high, when compared to chloroquine (IC<sub>50</sub> = 18–38 nM). However, Cts and its derived peptides may be interesting for the exploration of new antimalarial drugs based upon observed plasmepsin inhibition. Future experiments to demonstrate that Cts is a new actor of innate immunity to fight *P. falciparum* will involve detecting the presence



of Cts in human and in malaria-infected mice in acute infection and determining its role in vivo.

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## Appendix: List of Accession Numbers (Proteins)

hCgA (human Chromogranin A): P10645  
 bCgA (bovine Chromogranin A): P05059  
 mCgA (mouse Chromogranin A): P26339  
 rCgA (rat Chromogranin A): P10354  
 eCgA (equine Chromogranin A): Q9XS63  
 pCgA (pig Chromogranin A): P04404  
 fCgA (frog Chromogranin A): Q9W7A0\_RANRI  
 pLDH (plasmodium Lactate DeHydrogenase): Q27743  
 PfTubulin  $\beta$  chain: P14643  
 Proliferating Cell Nuclear Antigen: P31008  
 PfPlasmepsin II precursor, PfPM2: P46925

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