AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Killing of *Trypanozoon* Parasites by the Equine Cathelicidin eCATH1

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***Trypanozoon* parasites infect both humans, causing sleeping sickness, and animals, causing nagana, surra, and dourine. Control of nagana and surra depends to a great extent on chemotherapy. However, drug resistance to several of the front-line drugs is rising. Furthermore, there is no official treatment for dourine. Therefore, there is an urgent need to develop antiparasitic agents with novel modes of action. Host defense peptides have recently gained attention as promising candidates. We have previously reported that one such peptide, the equine antimicrobial peptide eCATH1, is highly active against equine Gram-positive and Gram-negative bacteria, without cytotoxicity against mammalian cells at bacteriolytic concentrations. In the present study, we show that eCATH1 exhibits an *in vitro* 50% inhibitory concentration (IC_{50}) of 9.5 μM against *Trypanosoma brucei brucei*, *Trypanosoma evansi*, and *Trypanosoma equiperdum*. Its trypanocidal mechanism involves plasma membrane permeabilization and mitochondrial alteration based on the following data: (i) eCATH1 induces the rapid influx of the vital dye SYTOX Green; (ii) it rapidly disrupts mitochondrial membrane potential, as revealed by immunofluorescence microscopy using the fluorescent dye rhodamine 123; (iii) it severely damages the membrane and intracellular structures of the parasites as early as 15 min after exposure at 9.5 μM and 5 min after exposure at higher concentrations (19 μM), as evidenced by scanning and transmission electron microscopy. We also demonstrate that administration of eCATH1 at a dose of 10 mg/kg to *T. equiperdum*-infected mice delays mortality. Taken together, our findings suggest that eCATH1 is an interesting template for the development of novel therapeutic agents in the treatment of trypanosome infections.**

Eukaryotic pathogens of the subgenus *Trypanozoon* cause deadly diseases in humans and animals. Dourine is a contagious disease of breeding *Equidae* caused by *Trypanosoma equiperdum* that is directly transmitted from animal to animal during mating. Dourine is notifiable to the World Organisation for Animal Health (OIE) (1). An infected mare may also transmit the infection to its foal via its milk or through udder lesions (2). Dourine is widespread throughout Asia (3), Africa (e.g., Ethiopia) (4), the Middle East, South America, and Eastern Europe (e.g., Russia) (5; World Animal Health Information Database, accessed 28 April 2015 [http://www.oie.int/wahis2/public/wahid.php/Wahidhome/Home]). The only Western European country in which it has been observed in recent decades is Italy, where outbreaks were reported in the 1970s and 1980s and again in 2011, with sporadic reports of isolated cases in the late 1990s (6). For effective disease control, the OIE recommends slaughtering infected animals. *Trypanosoma brucei* causes nagana in domestic and wild animals and sleeping sickness, or human African trypanosomosis (HAT), in humans. Nagana and HAT are fatal diseases occurring in sub-Saharan Africa that are transmitted by tsetse flies. Whereas one of the infectious agents of nagana is *T. brucei brucei*, HAT is caused by the two subspecies *T. brucei gambiense* (the chronic form) and *T. brucei rhodesiense* (the acute form) that have acquired the ability to infect humans (7). *Trypanosoma evansi* is mainly spread by blood-sucking flies through mechanical transmission and is responsible for a widely distributed disease called surra in domestic and wild animals found in Asia, Africa, South America, and even Europe (2, 8). These diseases are medically and economically devastating and have consequences on animal movement restrictions worldwide.

There are no vaccines against human and animal trypanosomoses, and chemotherapy relies on a small set of drugs. These drugs become increasingly ineffective due to the development of drug resistance, and their use has drawbacks arising from high levels of toxicity and long treatment periods (9, 10). Accordingly, the development of new chemotherapeutic agents is urgently needed to overcome these problems. An ideal antiparasitic agent should have a broad spectrum of activity, should be largely unaffected by mutations in the target pathogen, and should be nontoxic *in vivo* (11). Effectors with such potential are the host defense peptides, also called antimicrobial peptides (AMPs).

Antimicrobial peptides are structurally diverse, most often cationic proteins of a length between 10 and 100 amino acid residues that are components of the innate immune systems of organisms within all kingdoms (12). AMPs have a myriad of functions and are known to interact with and disrupt microbial surface membranes or to interfere, after membrane permeation, internally with cellular functions, in each case leading to cell death within minutes. Their peculiar mode of action is thought to greatly hamper

Received 13 May 2015 **Returned for modification** 29 June 2015
Accepted 6 January 2016
Accepted manuscript posted online 11 January 2016
Citation Cauchard S, Van Reet N, Büscher P, Goux D, Grötzinger J, Leippe M, Cattoir V, Laugier C, Cauchard J. 2016. Killing of *Trypanozoon* parasites by the equine cathelicidin eCATH1. Antimicrob Agents Chemother 60:2610–2619. doi:10.1128/AAC.01127-15.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.01127-15.

the emergence of mutants resistant to these compounds (13). The cathelicidins are a family of AMPs, some of which are produced and stored in the neutrophils of many mammalian species (14). All members of the cathelicidin family contain a C-terminal cationic domain and an N-terminal cathelin-like portion that must be cleaved to release the active C-terminal peptide. In the horse, three cathelicidins have been investigated to date, namely, eCATH1, eCATH2, and eCATH3 (15, 16; reviewed in reference 17). The synthetic eCATH1, which is an α-helical equine antimicrobial peptide composed of 26 amino acid residues, has been shown to have the most potent antimicrobial activity against bacteria (16). Previous work in our laboratory revealed that eCATH1 was not cytotoxic against mammalian cells at bacteriolytic concentrations (18), that it maintained its potent activity even at physiological salt concentrations (18), and that it effectively killed intracellular bacterial cells in an *in vivo* model of *Rhodococcus equi*-infected mice (19), supporting the idea that eCATH1 is a strong candidate for possible use as a therapeutic in animals.

Continuing the investigation of host defense peptides from the horse, the present study was undertaken in order to assess the *in vitro* trypanocidal activity of eCATH1 against the *Trypanozoon* parasites *T. brucei brucei*, *T. evansi*, and *T. equiperdum*. Moreover, we investigated the effect on membrane permeability of eCATH1 using fluorescent assays and monitored its lytic effect at the ultrastructural level using high-resolution microscopic techniques. Finally, an acute infection model of *T. equiperdum*-infected mice was developed to assess the *in vivo* efficacy of eCATH1.

MATERIALS AND METHODS

Ethics statement. Experiments on animals were approved by the ANSES/ENVA/UPEC's Animal Ethics Committee under license 10/01/12-8 and by the Animal Experimentation Ethics Committee of the Institute of Tropical Medicine Antwerp under licenses BM2012-1 and BM2013-7.

Peptide. eCATH1 (KRFGRLAKSFLRMRILLPRRKILLAS) was synthesized (Biosyntan, Germany) and highly purified (>95%) by reverse-phase high-performance liquid chromatography (HPLC), resulting in a single sharp chromatographic peak. Peptide analysis by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry gave a single peak at 3,142.4 *m/z*. The peptide was dissolved and diluted in 10 mM acetic acid at 2 mg/ml. Stock solutions were kept at −20°C until use.

Parasite culture. One of the major problems with studying *T. equiperdum* is the difficulty in isolating it from its original host and the difficult adaptation of the parasite to *in vitro* mass propagation. To obtain a population of bloodstream form trypomastigotes of *T. equiperdum* BoTat 1.1, a male Wistar rat (CURB, France) was inoculated intraperitoneally (i.p.) with a thawed cryopreserved stock of *T. Equiperdum*-infected rodent blood. A matching method was used to monitor parasitemia in tail blood (20). At first-peak parasitemia, when the blood contained 10^8/ml long, slender bloodstream form trypomastigotes, the rat was anesthetized, and the blood was collected by cardiac puncture with a heparinized syringe. Trypanosomes were separated from the blood by the mini-anion exchange centrifugation technique (mAECT) (21) and centrifuged at 1,000 × *g* for 15 min. The parasites were then resuspended at a concentration of 10^5 cells/ml in HMI-9 medium supplemented with 15% fetal calf serum and used to initiate *in vitro* culture according to Van Reet et al. (22). Briefly, for adaptation of *T. equiperdum* to *in vitro* culture, several primary cultures were initiated at 1×10^4 cells/ml by inoculating 50 μl of this suspension in 450 μl of medium in a 48-well plate. Cultures were incubated at 37°C with 5% CO_2 and monitored daily by phase-contrast inverted microscopy or counting in disposable counting chambers (Fast-Read 102; Biosigma). To establish continuous cultures, primary cultures showing a density increase in the range of 2×10^5 to 10×10^5 cells/ml were subpassaged to densities of 2×10^4 to 10×10^4 cells/ml. A culture was considered continuous when 10 consecutive subpassages could be made. Continuous cultures of *T. evansi* RoTat 1.2 and *T. b. brucei* AnTat 1.1^E were maintained similarly in HMI-9 medium (22).

Viability assays. Cultures were scaled up in 25-cm^2 culture flasks by inoculating 4.5 ml of fresh medium with 0.5 ml of an exponentially growing culture. When the parasite density reached 10^6 cells/ml, the parasite suspension was diluted into 30 ml of fresh medium and incubated for an additional 24 h at 37°C with 5% CO_2. AMP susceptibility assays were performed as previously described (23) with minor changes. In brief, 100 μl of parasite culture (10^5 cells/ml) was incubated in 96-well culture plates with eCATH1 at concentrations from 90 μg/ml (28.5 μM) to 0.123 μg/ml (0.04 μM) in 3-fold dilutions. After 24 h of incubation, 10 μl of Presto-Blue (Invitrogen) was added to each well, and the culture plate was further incubated for an additional 24 h. Absorbance was read spectrophotometrically at 570 nm and 600 nm (reference wavelength for normalization) according to the manufacturer's recommendations. Sigmoidal inhibition curves were determined, and the concentration required to inhibit 50% of cell growth (IC_{50}) was calculated. Assays were performed three times in duplicates over a 1-year period. Melarsoprol was used as a reference drug.

SYTOX Green assay for cell membrane permeabilization. Compromised membranes of parasites were assessed according to Kulkarni et al. (24) with minor modifications. Briefly, 10^7 parasites/ml were incubated in the dark with 1 μM SYTOX Green (Invitrogen) in HMI-9 medium for 15 min. After addition of eCATH1, fluorescence was measured every 5 min for up to 1 h. Fluorescence was measured at excitation and emission wavelengths of 485 and 535 nm, respectively (Infinite 200 Pro; Tecan). Parasites treated with the peptide diluent alone were used as negative controls. Control for maximum fluorescence (100%) was obtained by the addition of Triton X-100 to a final concentration of 0.1%.

Measurement of mitochondrial activity using fluorescent rhodamine 123. Trypanosomal mitochondrial activity was measured using the cell-permeant cationic fluorescent dye rhodamine 123 (Invitrogen) that is sequestered by active mitochondria. Parasites (10^7/ml) were incubated at 37°C for 10 min with rhodamine 123 at a final concentration of 500 nM, and subsequently eCATH1 was added. At intervals, trypanosomes were analyzed by immunofluorescence microscopy (1,000× oil immersion objective) using a Zeiss microscope fitted with an epifluorescence attachment and digitally photographed and filmed (Canon EOS 600D).

Electron microscopy. Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 0.03 M $CaCl_2$ at 4°C for 4 h and washed in the same buffer. For scanning electron microscopy (SEM), the cells were placed onto poly-L-lysine-coated coverslips (Thermanox), washed in cacodylate buffer, gradually dehydrated in ethanol, and critical-point dried (CPD 030; LEICA Microsystems). The samples were sputtered with platinum and examined by use of a scanning electron microscope (JEOL 6400F). For transmission electron microscopy (TEM), the cells were postfixed in 1% osmium tetroxide for 1 h and washed in cacodylate buffer. The cells were then gradually dehydrated in ethanol and propylene oxide (100%), embedded in Embed 812 resin, and polymerized at 60°C for 24 h. Ultrathin sections were collected onto grids and were stained with uranyl acetate and lead citrate for contrast. All grids were observed by use of a JEOL 1011 transmission electron microscope, and images were taken using an Orius 200 camera and DigitalMicrograph (Gatan) software at the Electron Microscopy Center of the University of Caen Basse-Normandie (CMABio; France).

Assessment of *in vivo* trypanocidal activity of eCATH1 in mice. Six female 6-week-old OF-1 mice were used per experimental group (Charles River, Belgium). Mice were infected by a 0.2-ml i.p. injection of 2.5×10^3 trypomastigotes of *T. equiperdum* BoTat 1.1, obtained from the blood of an infected mouse, in phosphate-buffered saline with glucose (PSG; 7.5 g/liter $Na_2HPO_4 \cdot 2H_2O$, 0.34 g/liter $NaH_2PO_4 \cdot H_2O$, 2.12 g/liter NaCl, 10 g/liter D-glucose, pH 8). Treatment with eCATH1 started at day 2 postin-

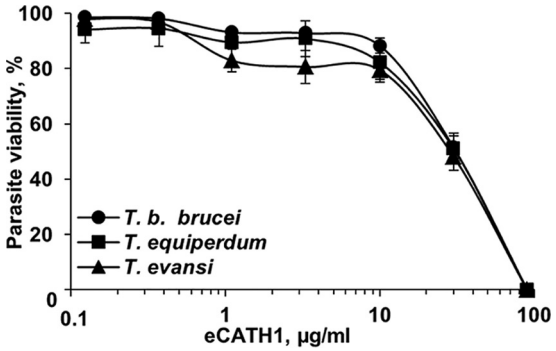

FIG 1 Antiparasitic activity of eCATH1. Parasites were incubated for a total of 48 h with serial peptide dilutions, and cell viability was determined by PrestoBlue assay. Mean values ± standard deviations of one representative experiment of at least three are represented.

fection, immediately after confirmation of parasitemia, and eCATH1 was administered i.p. at a dose of 0, 1, 5, or 10 mg/kg of body weight for four consecutive days. Melarsoprol was administered similarly at a dose of 10 mg/kg in 50% polyethylene glycol. Parasitemia was monitored every other day during treatment.

Statistical analysis. The statistical analyses to assess the effect of peptide activity in mice were performed using one-tailed Fisher's exact test. A P value of <0.05 was considered to be significant. Analyses were carried out using GraphPad Prism, version 6.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

eCATH1 exhibits *in vitro* trypanocidal activity against all species of *Trypanozoon*. To assess the *in vitro* trypanocidal activity of eCATH1, three species of *Trypanozoon*, *T. b. brucei* AnTat 1.1^{E}, *T. evansi* RoTat 1.2, and *T. equiperdum* BoTat 1.1, were exposed to various concentrations of the peptide. After cultures were incubated for 48 h, the metabolic activity of the organisms was determined using PrestoBlue substrate. The antiparasitic activity of eCATH1 was similar against all *Trypanozoon* parasites tested *in vitro*, with an estimated 50% inhibitory concentration (IC_{50}) value of 30 μg/ml (9.5 μM) (Fig. 1).

eCATH1 induces rapid influx of the vital dye SYTOX Green following membrane permeabilization. Because most AMPs, such as eCATH1, are highly cationic and interact with and compromise cell membranes, we measured the permeability of the surface membranes of trypanosomes using SYTOX Green, a vital dye (molecular weight [MW], ~600) that enters cells following membrane permeabilization and fluoresces upon interaction with DNA. The influx of the dye is prevented in intact membranes but not in those parasites having lesions with a size large enough to allow the entrance of the dye (25). *T. equiperdum* trypomastigotes were exposed to different concentrations of eCATH1 for 60 min in the presence of SYTOX Green. When parasites were treated with eCATH1 at a concentration above 1 μM, rapid and sustained increases in fluorescence could be observed (Fig. 2), which occurred in a dose-dependent manner, suggesting a high affinity of the peptide to the plasma membrane. The kinetics of the SYTOX Green influx demonstrated also that the membranes became compromised immediately after the contact with the peptide. At its IC_{50}, the fluorescence value for eCATH1 reached within 20 min ~60% of that representing the maximal permeabilization, achieved by employing the detergent Triton X-100 at a final concentration of 0.1%. At 19 μM (2× IC_{50}) and 28.7 μM (3× IC_{50}) eCATH1, the fluorescence levels were similar, reaching ~70% and 80% of the maximal permeabilization, respectively, within 30 min, and no viable parasite was detected by phase-contrast microscopy analysis at the end of the peptide exposure time; control parasites, however, remained viable (data not shown).

Rhodamine 123 release revealed a rapid disruption of the mitochondrial membrane potential. Rhodamine 123 is a cell-permeant fluorescent dye that accumulates in mitochondria. It is thought that an attraction of cationic rhodamine 123 molecules by the relatively high negative electric potential across the mitochondrial membrane is the basis for the selective staining of mitochondria in living cells. Loss of fluorescence staining may indicate mitochondrial membrane disruption (11). We assessed the dye retention of the cells by fluorescence microscopy after incubating *T. b. brucei*, *T. Equiperdum*, and *T. evansi* with rhodamine 123 and subsequently after treating the same populations with increasing con-

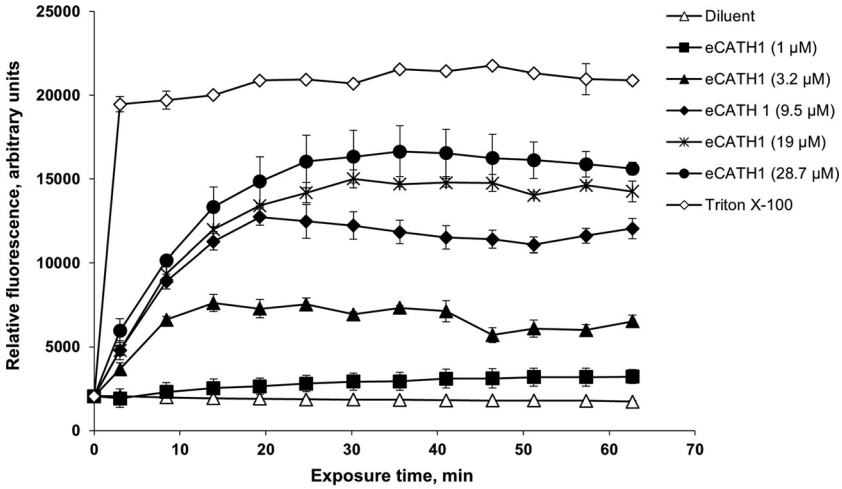

FIG 2 Kinetics of membrane permeabilization induced by eCATH1. *T. equiperdum* trypomastigotes were treated with increasing amounts of eCATH1 and analyzed for membrane permeability using SYTOX Green. The control with maximum permeabilization was obtained with 0.1% Triton X-100. The values represent the means ± standard deviations from triplicate samples of a single experiment, representative of three independent experiments.

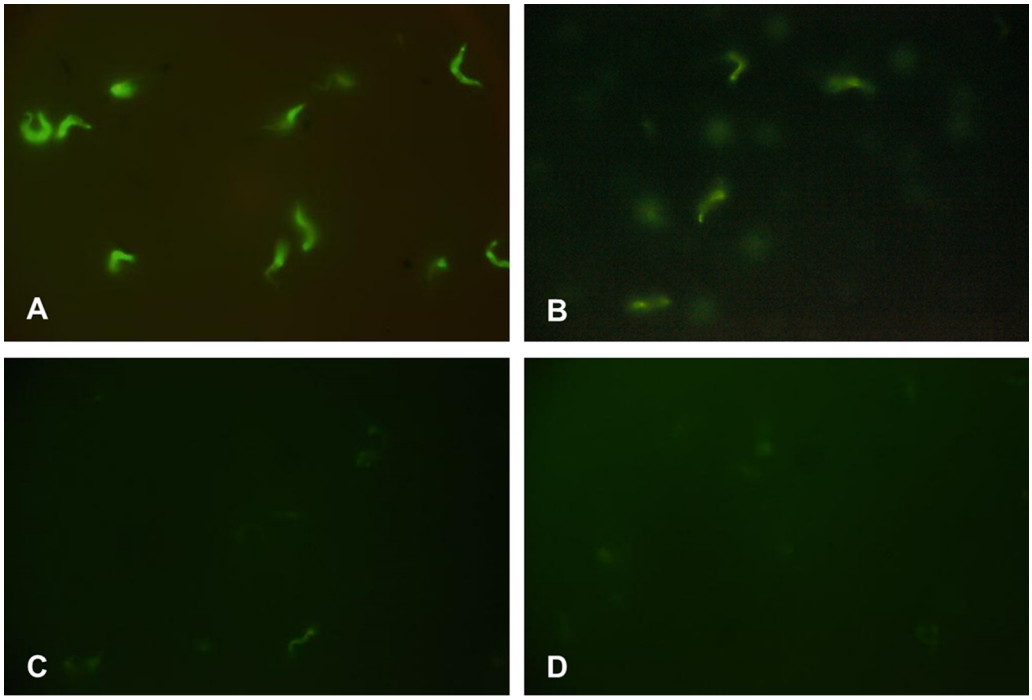

FIG 3 Analysis of trypanosomal mitochondrial activity by fluorescence microscopy before and after treatment with eCATH1. *T. equiperdum* parasites were incubated first with rhodamine 123 and examined (A). Untreated parasites showed bright fluorescence distributed along their mitochondria. Trypanosomes were then treated with different concentrations of eCATH1, 9.5 μM (B), 19 μM (C), and 28.7 μM (D), and examined after 15 min. A substantial reduction of fluorescence levels was observed, indicating that the mitochondrial potential was disrupted, releasing the dye. Trypanosomes were visualized at a magnification of ×1,000.

centrations (0, 9.5, 19, and 28.7 μM) of eCATH1. Figure 3 displays the results obtained with *T. equiperdum* as a representative.

Trypanosomes that were treated with rhodamine 123 alone displayed bright fluorescence distributed along the mitochondrion (Fig. 3A). Rhodamine 123-labeled trypanosomes were then treated with eCATH1 or peptide diluent for 15 min and photographed again. Trypanosomes treated with the peptide diluent alone remained brightly fluorescent, at a level similar to that observed in the control parasites (data not shown), which is consistent with a normal mitochondrial membrane potential. In contrast, parasites treated with eCATH1 at the IC_{50} for 15 min displayed a faint and diffuse fluorescent signal (Fig. 3B). At higher peptide concentrations (2× IC_{50} and 3× IC_{50}), fluorescence was no longer observed (Fig. 3C and D), indicative of a strong mitochondrial depolarization resulting in release of the dye. Treatment of *T. b. brucei* and *T. evansi* with eCATH1 gave similar results (data not shown).

Changes in parasite motility as a function of concentration and time exposure to eCATH1 were also observed. eCATH1 caused constricted motility compared to that of untreated parasites displaying normal motility, and death. Examples of untreated trypanosomes (*T. evansi*) are shown in Movie S1 in the supplemental material, while those exhibiting constricted motility are exemplified by the trypanosomes treated with the IC_{50} of eCATH1 and are presented in Movie S2. At higher concentrations (2× IC_{50} and 3× IC_{50}), dead trypanosomes without fluorescence were observed, as shown in Movie S3.

eCATH1 severely damages the parasite membrane and intracellular structures. The facts that eCATH1 is trypanocidal against all three species of parasites under study and that this process starts at the trypanosome cell membrane prompted us to test whether eCATH1 induced alterations in the parasite surface membrane and intracellular structures that are substantial enough to be detected by high-resolution microscopic techniques. Accordingly, we analyzed eCATH1-treated cells by scanning electron microscopy (SEM) and transmission (TEM) electron microscopy. eCATH1 was first assayed on *T. b. brucei* and *T. equiperdum* trypomastigotes at concentrations causing 80% inhibition of parasite proliferation (2× IC_{50}) for 15 min and 60 min. Trypanosomes were then fixed and examined by SEM at magnifications from ×5,000 to ×30,000 (Fig. 4). After only 15 min of treatment with eCATH1, the structural integrity of both parasite species was dramatically changed compared to that of untreated parasites. Peptide-treated cells lost their typical elongated shape, developing a rounded and swollen morphology, particularly in the case of *T. equiperdum*, which is consistent with the decrease in their viability. The magnitude of the effect was time dependent, with a nearly complete detachment of the flagellum from the trypanosomal body after treatment for 60 min.

Next, the ultrastructural alterations of the trypomastigotes induced by eCATH1 were visualized by TEM. In order to detect an initial effect of eCATH1-mediated trypanocidal activity rather than to demonstrate the total destruction of the cells, parasites were treated here with eCATH1 at the IC_{50} (9.5 μM) for 15 min or at 19 μM for only 5 min. As a control, parasites were incubated with peptide diluent alone for the same time periods as indicated above. As depicted in Fig. 5, TEM revealed that incubation of trypomastigotes with 9.5 μM eCATH1 for 15 min caused ultra-

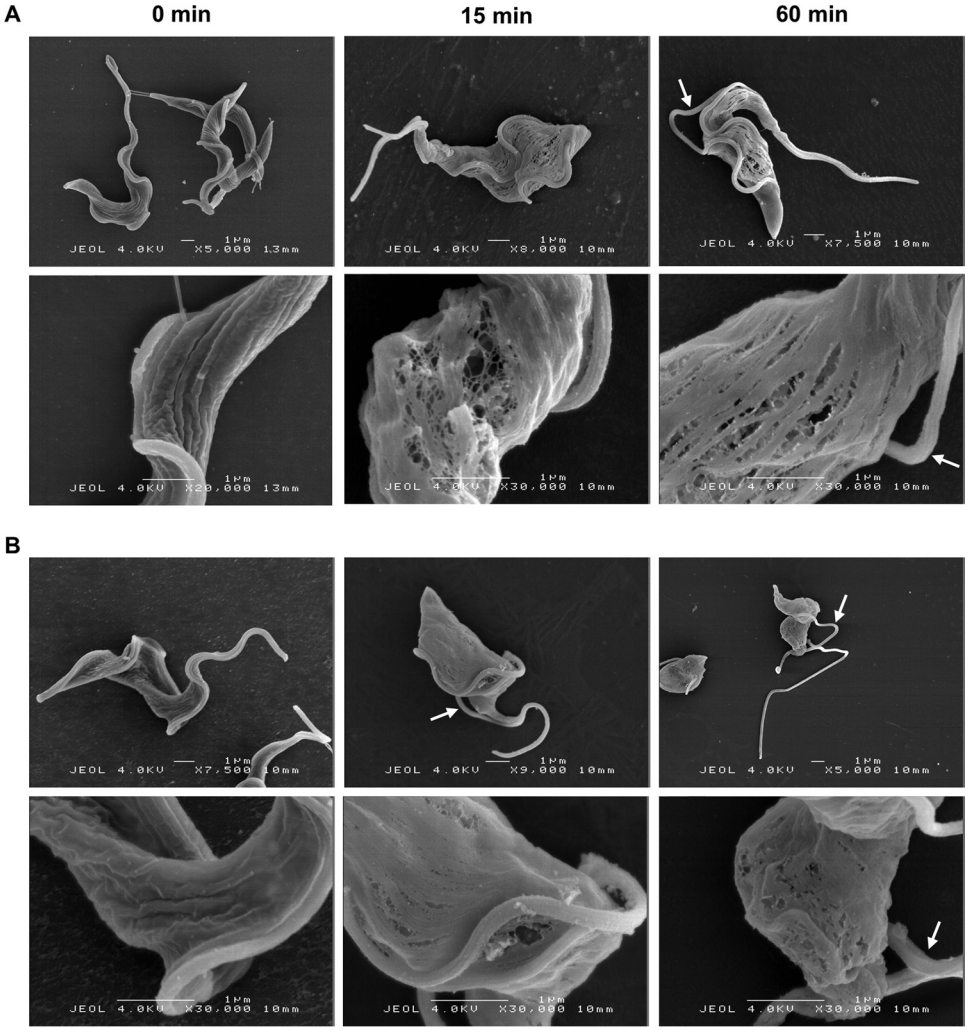

FIG 4 eCATH1 induces structural alterations of the surface membrane of *T. b. brucei* (A) and *T. equiperdum* (B) trypomastigotes as evidenced by SEM. eCATH1 was added to the parasites (2×10^7/ml) at a concentration that caused ~80% inhibition of proliferation (19 μM) for 15 min or 60 min before parasites were fixed and processed for microscopy. Scanning electron micrographs are displayed at low (upper panels) and high (lower panels) magnifications. Parasites assumed a more rounded morphology with increasing times of treatment. Membrane disruption with detachment of the flagellum (indicated by arrows) can be observed. The micrographs are representatives of three independent experiments with similar results.

structural changes that appeared already after 5 min and that were even more dramatic when the concentration was raised to 19 μM. We observed that a 15-min incubation with 9.5 μM eCATH1 resulted in trypanosome body swelling (Fig. 5H) and cytoplasmic vacuolization, membrane disruption, membrane blebbing of organelles, and disorganization and loss of membrane microtubules (Fig. 5B) in contrast to trypanosomes exposed to peptide diluent alone (Fig. 5A and G). Plasma membrane blebbing (Fig. 5L) and disruption (Fig. 5E) were evidenced after exposure of the trypanosome at 19 μM eCATH1 for 5 min. Detachment of large areas of the plasma membrane was also observed (Fig. 5F). At this high concentration, parasites appeared ghost-like, with damage visualized as organelle blebbing (Fig. 5K), as observed at the IC_{50}, and depletion of intracellular structures (Fig. 5E), whereas organelles and plasma membranes of untreated parasites remained intact in both trypanosome species (Fig. 5D and J).

***In vivo* administration of eCATH1 to *T. equiperdum*-infected mice delays mortality.** To further investigate the trypanocidal activity of eCATH1, we administered the peptide to mice during an acute *T. equiperdum* infection. At day 2 postinfection, mice developed parasitemias in the range of 1×10^6 to 4×10^6 trypomastigotes/ml. After two injections of eCATH1, only the mice treated with the highest dose of eCATH1 showed a minor reduction (30%) in parasitemia (mean, ~1.8×10^8 trypomastigotes/ml) compared to the level in control mice injected with peptide diluent (mean, ~2.7×10^8 trypomastigotes/ml). No parasites were detected in mice treated with melarsoprol. However, a significant delay in mortality was observed in the mice treated with eCATH1 compared to mortality in control mice (Fig. 6). This was more striking for the mice treated with the highest dose of eCATH1 ($P = 0.03$). Whereas all control mice died at 5 days postinfection, 33% of mice treated with 1 or 5 mg/kg eCATH1 and 67% of those treated with 10 mg/kg eCATH1 survived longer. However, because of the already advanced parasitemia, all mice treated with the peptide died at day 6 postinfection. All mice treated with melarsoprol survived until the end of a 60-day follow-up period.

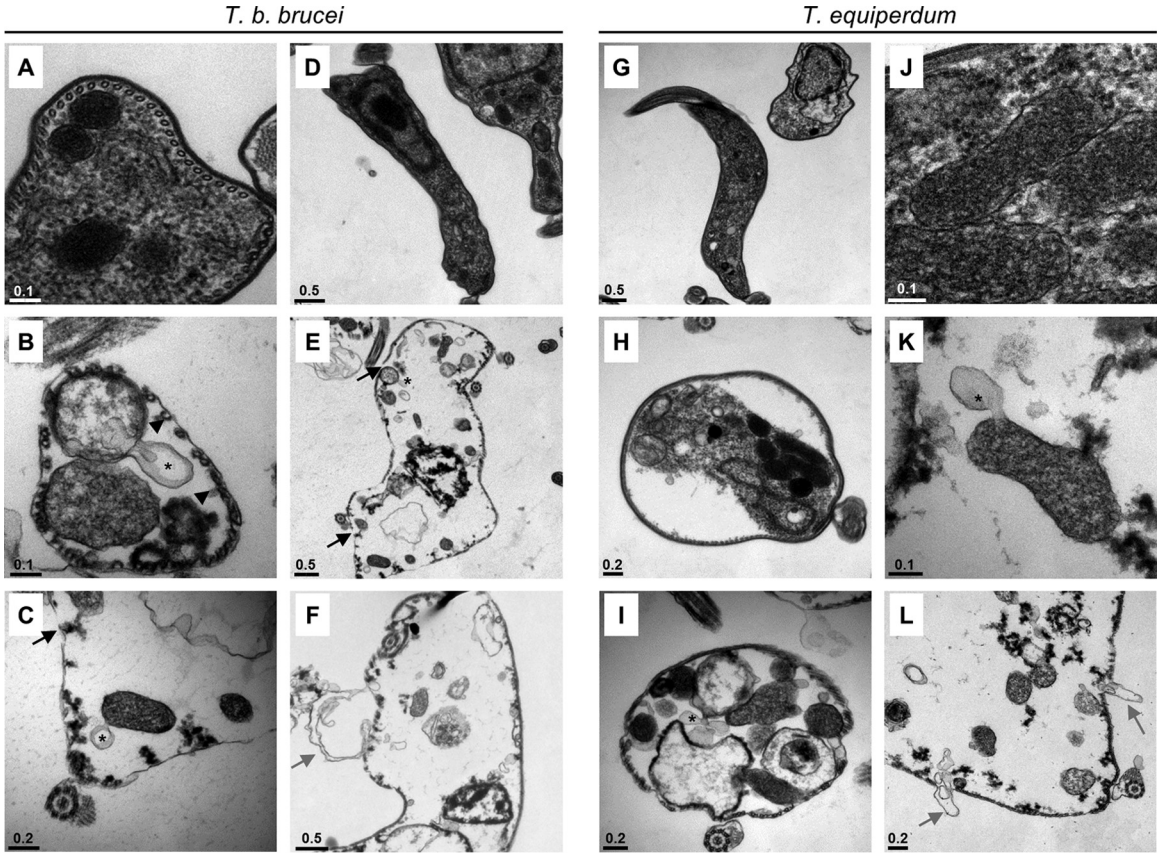

FIG 5 Exposure of *T. b. brucei* and *T. equiperdum* trypomastigotes to eCATH1 results in ultrastructural alterations as evidenced by TEM. Parasites were treated with eCATH1 at IC_{50} (9.5 μM) for 15 min (B, C, H, and I) or at a concentration that caused ~80% inhibition of proliferation (19 μM) for 5 min (E, F, K, and L). Control parasites at the indicated times were incubated with the peptide diluent alone (A, D, G, and J). Membrane disruption (black arrows), membrane blebbing (gray arrows), and depletion of electron-dense cytoplasmic material can be observed. Arrowheads indicate alteration of membrane microtubules, and asterisks mark membrane blebbing of organelles. The results are representative of two independent experiments performed with similar results. Bars indicate measurements in micrometers.

DISCUSSION

Identification of new trypanolytic agents with nontraditional modes of action, low cytotoxicity, and a broad spectrum of activity is critical for the design of more efficient therapeutic strategies in trypanosomoses. Host defense peptides have many of these features and hence were chosen for the work reported here. In this study, we report for the first time that equine cathelicidin eCATH1 displays trypanocidal activity against pathogens causing dourine, surra, and nagana; thereby, we substantially expand the antimicrobial spectrum of eCATH1 reported to date by including protozoan parasites.

Compared to the vast number of reports about antibacterial activity of host defense peptides, there are few studies dealing with their effects on parasites. Nonetheless, structurally diverse mammalian antimicrobial peptides (AMPs) have been found to be toxic against trypanosomes *in vitro*, and these include the cathelicidins BMAP-27 and BMAP-18 (11), protegrin-1 (26), the human defensins (26, 27), the porcine NK-lysin and its cationic core region NK-2 (28), and the vasoactive intestinal polypeptide (29). On the basis of antiparasitic activity defined as the concentration of AMP that either kills parasites and/or decreases their ability to replicate, McGwire and Kulkarni (30) have proposed a scoring scale ranging from 0 (>50 μM) to 4+ (<1 μM). Accordingly, the 50% inhibitory concentration (IC_{50}) of 9.5 μM found for eCATH1 against *T. b. brucei*, *T. evansi*, and *T. equiperdum* allows us to consider eCATH1 as an effective AMP with a score of 2+. However, comparisons to other IC_{50}s of AMPs should be carefully analyzed, given the different protocols described in the literature for their evaluation (26, 31–33). The concentrations of eCATH1 used in this study that revealed trypanocidal activity *in vitro* were 2-fold to 15-fold higher than those sufficient to kill various Gram-positive and Gram-negative bacteria (34). Such a difference between the activities against bacteria and parasites had also been observed previously for cathelicidins (26). This may reflect differences in the assay conditions used in this study, such as the presence of 15% fetal calf serum in the culture medium. In addition, as trypanosomes are typically 20 μm and bacterial cells are approximately 1 to 2 μm in size, a greater number of peptides may be required to produce toxic effects in parasites than in bacterial cells when the same density of microorganisms is used. In general, the properties of cellular membranes, such as their surface charge and the composition of the phospholipid bilayer, including cholesterol content, vary immensely, which also may account for variations in affinity between the cationic α-helical peptide eCATH1 and the target cell membrane and in the subsequent membrane perturbation, conferring a level of selectivity to the effect of the

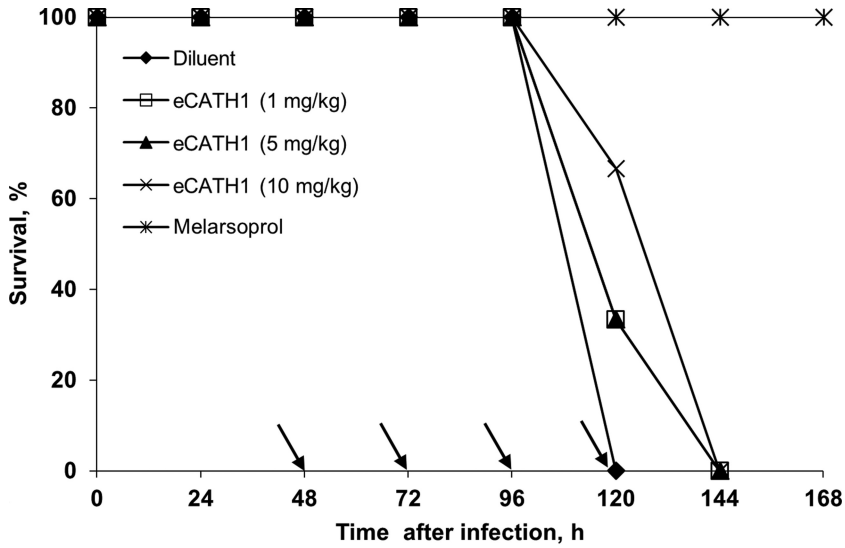

FIG 6 *In vivo* administration of eCATH1 increases survival of mice infected with *Trypanosoma equiperdum*. eCATH1 (1, 5, or 10 mg of peptide/kg/day) was administered intraperitoneally to OF-1 mice infected with *T. equiperdum* BoTat 1.1 bloodstream form parasites. Controls included mice treated with peptide diluent alone (negative control) or with 10 mg of melarsoprol/kg/day (positive control). Six mice were used for each group. Arrows indicate time of treatment administration. Note that 1-mg/kg and 5-mg/kg lines overlap.

peptide. Particularly, trypanosomes possess a surface coat of variable surface glycoproteins (VSGs) with a rapid turnover that may confer additional protection against proteins binding to the parasite's surface.

The SYTOX Green assay and the electron microscopy images argue in favor of plasma membrane disruption as a major initial mechanism involved in eCATH1-mediated trypanocidal activity, as occurs with other cationic antimicrobial peptides (26, 35, 36). This phenomenon is thought to occur via the binding of the peptide to the parasite cell membrane, causing membrane destabilization that can initiate microbial death by inducing autophagic, necrotic, or apoptotic cell death (24, 36). Our data demonstrated that eCATH1 rapidly altered the permeability of the plasma membrane, resulting in a dose-dependent influx of the vital dye SYTOX Green into the cells. At 19 μM (2× IC_{50}) and 28.7 μM (3× IC_{50}), eCATH1 induced the highest fluorescence intensity within 30 min, which was then sustained, suggesting long-term effects on the membrane of parasites. The morphological patterns observed in SEM and TEM are consistent with the results obtained in membrane permeabilization experiments. Dramatic changes in the structural integrity of *T. equiperdum* and *T. b. brucei*, including a rounded and swollen morphology and membrane blebbing and disruption, were evidenced after exposure for only 5 min at 2× IC_{50} of eCATH1. In agreement with the current study, similar observations have also been reported in previous studies assessing the effect of other antimicrobial peptides, namely, protegrin-1 on *T. brucei* (26) and BMAP-18 on *T. b. brucei* and *T. b. rhodesiense* (11). The disruption of membrane integrity probably leads to osmotic instability, causing cell swelling and eventual lysis. The loss of intracellular compartmentalization and contents also may reflect the ability of the peptides to gain entrance to the cell and directly disrupt internal membranes, as suggested in previous work for protegrin-1 (26).

Indeed, while it is well established that AMPs associate with and destabilize cell surface membranes, it appears that they can also enter cells and associate with intracellular organelles, giving rise to pleiotropic effects on metabolic and bioenergetic pathways (11, 37, 38). Trypanosomatids have a single mitochondrion exhibiting unique structural and functional characteristics that are remarkably distinct from those of host mammalian cells. These features make this organelle an important target for new pharmaceutical agents (32, 39). In the present study, after incubation with eCATH1, the parasites showed a rapid dose-dependent decrease of rhodamine fluorescence, revealing mitochondrial membrane potential disruption. Changes in parasite motility as a function of concentration and time exposure to eCATH1 were also observed, compared to motility in untreated parasites, consistent with bioenergetic system disruption. However, it has also been proposed that small hydrophobic peptides (SHP) could target the membrane fluidity of bloodstream form *T. brucei* parasites, resulting in membrane rigidification and subsequent changes in trypanosome motility and thus contributing to a general poisoning of the cell by SHP (40). Altogether, the results obtained with these *in vitro* assays support the idea that eCATH1 could potentially be used to treat trypanosome-infected animals. In this regard, we further investigated the parasiticidal activity of this peptide in mice infected with *T. equiperdum*. Although a few studies have demonstrated the use of AMPs in animal models of infection by trypanosomatids (26, 35, 41–43), to our knowledge, this is the first study assessing the therapeutic potential of eCATH1 in a *T. equiperdum* infection model. We found that eCATH1 at the highest dose of 10 mg/kg prolongs the life of infected mice. However, treatment with the peptide was insufficient to completely cure an acute infection, and treated mice succumbed to late-stage parasitemia 24 h after the end of the treatment, suggesting that the concentration of eCATH1 was not sufficient to eradicate the parasites. It has previously been shown that the antimicrobial activity of eCATH1 is slightly diminished under physiological salt conditions (18) but is still potent against a model of *Rhodococcus equi*-infected mice (19). Thus, it would be

interesting to test whether a higher dose of eCATH1 and/or a prolonged treatment would clear the infection. One limitation of our study is that we did not assess parasitemia immediately after treatment, e.g., at 2 h and 5 h posttreatment as performed by McGwire et al. (26), in order to get insight into the kinetics of the *in vivo* trypanocidal activity of the peptide and an eventual initial decrease in parasitemia.

This study has revealed that eCATH1 displays many advantages for a potential clinical application. Indeed, trypanosomatids will presumably be less prone to induce resistance to eCATH1 due to its mode of action, which targets not only the plasma membrane but also intracellular organelles. Also, it would be important to test the peptide in strains resistant to current veterinary drugs to support its hypothetical future implementation. Moreover, an important criterion in the search of new compounds with trypanocidal activity is their low cytotoxicity to host cells. In this regard, the cytotoxic potential of eCATH1 on epithelial cells was assessed previously (18) and showed that eCATH1 had no cytotoxic effect on RK13 and Vero cell lines (IC_{50} of >100 μg/ml). Also, positively charged side chains of cationic antimicrobial peptides are generally thought to provide the initial long-range electrostatic attractive forces that guide them toward the negatively charged membranes (44). Trypanosomatids exhibit a negatively charged surface whose value is species specific and varies according to the development stage (45). According to Lanham (46), the bloodstream forms of trypanosomes belonging to different species could be arranged in the following order of negative surface charge: *T. cruzi* > *T. lewisi* > *T. vivax* > *T. congolense* > *T. b. rhodesiense*, *T. b. gambiense*, *T. b. brucei*, and *T. evansi*. Furthermore, it has previously been reported that eCATH1 is capable of killing bacteria inside macrophages (19) and, thus, may have the potential to gain access to intracellular parasites. There is, hence, considerable potential to broaden this approach to diseases caused by related trypanosomatids, such as *T. cruzi* or *Leishmania* infections, which represent a challenge as they are primarily intracellular in mammals and as AMP-based drugs need to gain access to intracellular compartments to kill parasites at concentrations that are parasiticidal.

Regarding the next steps of this work, we will try to improve druggability by developing methodological strategies to increase the peptide half-life or gain further insight into the pharmacokinetic and pharmacodynamic properties to improve peptide dosing and treatment. For that purpose, we plan to use chemical modifications such as PEGylation. These approaches seek to achieve the following benefits: protecting protein/peptide from degradation, extending *in vivo* half-life, providing prolonged drug release, augmenting drug efficacy while reducing side effects, reducing administration frequency, and lowering drug dosage. Other advantages include alleviation of pain associated with frequent injections and a significant reduction in the cost of treatment (47). Also, the high cost of manufacturing peptides is arguably the principal problem that remains to be solved for widespread clinical use of this class of antimicrobial therapeutics. One promising approach is to decrease the size of the peptide by designing several eCATH1-derived peptides with improved activity while maintaining reduced cytotoxicity. We have already tested these shortened peptides on several Gram-positive and Gram-negative bacteria (unpublished data), and they will soon be tested on trypanosomes to confirm the feasibility of this approach. This could reduce the cost of peptide synthesis by 30%. Regarding the feasibility of scale-up and the cost of goods, it is probably too early to give an estimation, but scaling up will certainly greatly lower the price at an industrial level compared to what we used at the research level (13 €/mg for a purity of >95%). It will also depend on the peptide drug formulation that will be chosen *in fine*. Another approach to reduce the cost of clinical application would be to lower the concentration of eCATH1 needed for therapeutic use by linking it with a high-affinity nanobody/antibody to directly target the parasite.

Regarding the issue of exploitation of this peptide and its derivatives in the pharmaceutical business, it is likely that the target market will be similar to that of a drug such as Cymelarsan, i.e., Asia, the Middle East, South America, and Eastern Europe. The costs of commercialized drugs are still too high to allow them to be easily used in developing countries, e.g., Ethiopia. In the current stagnating pharmaceutical industry, peptides are considered to have added value by representing a potential solution to more efficacious disease treatment. In addition, peptides promise to combine the lower production costs of conventional (small-molecule chemical) drugs with the high specificity of (the larger) biological entities. A recent review reported that 86% of the approved peptide drugs work through the parenteral route (47). In fact, for large animals (especially horses) parenteral administration is very often preferred to the oral route for practical reasons. However, the expansion of alternative routes, such as the oral route, is likely. Finally, the obstacles described above will have to be overcome in order to consider the equine antimicrobial peptide eCATH1 for therapeutic use as a realistic possibility.

ACKNOWLEDGMENTS

We thank Camille Bois, Nicolas Bebronne, and Fabien Duquesne for technical assistance. We are grateful to Michel Laurentie for advice in statistical analyses. We also thank Sandrine Petry for her support in this study.

FUNDING INFORMATION

This work was supported by public grants from the European Regional Development Fund, the Regional Council of Low Normandy, the Institut Français du Cheval et de l'Equitation, and the French Agency for Food, Environmental and Occupational Health Safety.

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