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Identification of Synthetic and Natural Host Defense Peptides with Leishmanicidal Activity

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***Leishmania* parasites are a major public health problem worldwide. Effective treatment of leishmaniasis is hampered by the high incidence of adverse effects to traditional drug therapy and the emergence of resistance to current therapeutics. A vaccine is currently not available. Host defense peptides have been investigated as novel therapeutic agents against a wide range of pathogens. Here we demonstrate that the antimicrobial peptide LL-37 and the three synthetic peptides E6, L-1018, and RI-1018 exhibit leishmanicidal activity against promastigotes and intramacrophage amastigotes of *Leishmania donovani* and *Leishmania major*. We also report that the *Leishmania* protease/virulence factor GP63 confers protection to *Leishmania* from the cytolytic properties of all L-form peptides (E6, L-1018, and LL-37) but not the D-form peptide RI-1018. The results suggest that RI-1018, E6, and LL-37 are promising peptides to develop further into components for antileishmanial therapy.**

Protozoan parasites belonging to the genus *Leishmania* are a global health problem, especially in resource-poor areas of Africa, Asia, the Americas, and Europe. *Leishmania* parasites cause leishmaniasis, which is classified as a neglected tropical disease. An estimated 20 million people are affected worldwide, with 1.3 million new cases each year and 20,000 to 30,000 deaths occurring annually (1). Transmission of *Leishmania* to the mammalian host occurs during a blood meal of infected sand flies of the genera *Phlebotomus* or *Lutzomyia* (2). Clinical manifestations of leishmaniasis vary depending on the infecting *Leishmania* strain. *Leishmania major* primarily causes a cutaneous form, with infected individuals developing characteristic but self-healing open sores. In contrast, *Leishmania donovani* infection can lead to a more invasive visceral leishmaniasis, also called kala-azar, which is potentially fatal if untreated.

Leishmania parasites have a complex, digenetic life cycle, alternating between an extracellular, flagellated promastigote form that develops in the gut of a sand fly, and an intracellular nonmotile amastigote form that replicates in the macrophages of mammalian hosts.

To date, no *Leishmania* vaccine is available, and current therapy is based on traditional pentavalent antimonials with considerable adverse side effects (3). Recently, oral miltefosine was approved by the U.S. Food and Drug Administration, while liposomal amphotericin B is in clinical trials for treatment against cutaneous and visceral leishmaniasis (4, 5). In addition, the increasing incidence of drug resistance in *Leishmania* renders currently available treatment options ineffective and further drives the need for new therapeutic agents.

Cationic host defense (antimicrobial) peptides have been identified as an important part of the host innate immune response in all living species and have broad-spectrum antimicrobial, including antileishmanial, activity (6, 7). The actions of host defense peptides range from direct killing of invading pathogens to immune response modulation (8). A variety of host defense peptides are currently being evaluated in preclinical and clinical trials as novel therapeutics to treat a range of pathogens (7, 9, 10). Certain cationic peptides, such as cyclic hexapeptide gramicidin S and the cationic lipopeptides polymyxin B and colistin, are already in clinical use, for topical infections and multidrug-resistant Gram-negative bacteria, respectively (11–13). Investigations are ongoing to discover new host defense peptides with anti-parasitic activity and low host cell cytotoxicity (9, 14, 15). For example, we demonstrated that the antimicrobial peptide D-BMAP-28 has leishmanicidal activity against *L. major* promastigotes and amastigotes (16). The modes of action of leishmanicidal antimicrobial peptides are starting to be understood (8). The selectivity of host defense peptides for pathogens over host cells makes them attractive candidates for the development of novel peptide-based drugs, either for use alone, or in combination with already licensed therapeutics (16, 17). Here we demonstrate that four cationic peptides, including the host defense peptide LL-37 and synthetic peptides E6, L-1018, and the retroinversion (RI) form of L-1018, RI-1018, a peptide containing all D-amino acids with reversed amino acid sequence with respect to its L form, exhibit leishmanicidal activity against promastigotes and intramacrophage amastigotes of *L. donovani* and *L. major*. We also show that the leishmanial virulence factor GP63 confers protection to *Leishmania* promastigotes from the cytolytic properties of all L-form peptides (E6, L-1018, and LL-37), but not RI-1018. Our results suggest that these peptides, particularly if in their RI form, show potential for development of new drug candidates for antileishmanial therapies.

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MATERIALS AND METHODS

Strains and culture conditions. *Leishmania donovani* (strain 1S from Sudan, WHO designation MHOM/SD/00/1S-2D) and *L. major* (MHOM/IL/80/Friedlin) strains were cultured in M199 medium (HyClone, Scientific) supplemented with 10 mM hemin, 40 mM HEPES, and 10% heat-inactivated fetal calf serum (FCS). The generation of *L. major* KO strain (wild-type strain with seven copies of *gp63* genes knocked out) and *L. major* KO+GP63 strain (the *gp63* mutant complemented with one functional copy of the *gp63* gene) are described in Kulkarni et al. and Joshi et al. (18, 19). THP-1 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 medium supplemented with 2.05 mM L-glutamine (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA), at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Viability assays by flow cytometry analysis. A total of 1×10^7 mid-log-phase *Leishmania* parasites were pelleted and resuspended in 100 μl complete M199 medium containing host defense peptides at a final concentration of 20 μM. M199 medium without FCS was used for serum-free assays. *Leishmania* parasites were incubated at 26°C for different times as indicated in the result section for each experiment. Following incubation with or without host defense peptide, the following staining protocols were used. To stain dead cells, propidium iodide (PI) was added to the *Leishmania* suspension at a final concentration of 50 μg/ml and incubated in the dark for 5 min. To stain live cells, fluorescein diacetate (FDA) was added to the *Leishmania* suspension at a final concentration of 10 μg/ml, and cells were incubated for 15 min in the dark and then washed twice with 1× phosphate-buffered saline (PBS). FDA-stained cells were fixed using a 0.4% paraformaldehyde (PFA) solution prior to flow cytometry analysis. The percentage of PI- or FDA-stained cells was determined using BD FACSCalibur and FlowJo analysis software. Heat-killed *Leishmania* parasites were used as a control to differentiate live versus dead promastigotes. Data were normalized so that the viability of untreated cells was set at 100%. Experiments were done in three independent replicates, unless otherwise stated.

Resazurin-based assay for measurement of mitochondrial redox activity. To test the mitochondrial redox activity of *Leishmania* promastigotes in the presence and absence of host defense peptides, the reduction rate of resazurin to resarufin was determined using alamarBlue solution (Invitrogen) as a substrate. Briefly, mid-log promastigotes were resuspended in 1× PBS, and twofold serial dilutions were prepared in a 96-well plate, ranging from 5×10^7 cells/ml to 0.3×10^7 cells/ml. Host defense peptides were added to a final concentration of 20 μM. After incubation for 1.5 h at 26°C, 10 μl alamarBlue (Invitrogen) was added to each well containing promastigote suspension and incubated for further 2.5 h at 26°C. Fluorescence was determined with a Tecan Infinite PRO plate reader, setting excitation at 550 nm and emission at 600 nm. Data were analyzed using i-control software (Tecan). Data from the linear portion of each *Leishmania* dilution series were used to compute the percentage of mitochondrial redox activity. Data were normalized so that the viability of untreated cells was set at 100%. Experiments were done in three independent replicates.

Macrophage infection assay to measure amastigote replication. A total of 3×10^5 THP-1 cells/well were seeded on a 4-well Nunc Lab-Tek chamber slide (Thermo Scientific). Phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) was added to induce differentiation of THP-1 cells into macrophages. Cells were allowed to differentiate and adhere to the slides for 48 h at 37°C in 5% CO_2. Macrophages were subsequently washed with 1× PBS. Infections (multiplicity of infection [MOI] of 10 for *L. donovani* and MOI of 20 for *L. major* strains) were performed with metacyclic *Leishmania* cells. For *L. major* strains, metacyclic cells were separated from procyclic cells as described elsewhere (20) using 50 μg/ml concanavalin A beads instead of peanut agglutinin. After 24 h of incubation, nonadherent *Leishmania* cells were washed away, and infected macrophages were cultured in complete RPMI 1640 medium for another 24 h for parasites to fully adapt to the intracellular environment. After 24 h of incubation, host defense peptides were added to the infected macrophages at a final concentration of 20 μM (in the presence of serum), and infected macrophages were cultured for an additional 48 h. Subsequently, macrophages were fixed in a 4% PFA solution for 5 min and mounted for epifluorescence microscopy. All samples were treated with ProLong Gold antifade reagent with 4′,6′-diamidino-2-phenylindole (DAPI) (Invitrogen). Images were taken using a BX61 microscope (Olympus) and analyzed using ImagePro 6.0 software. The numbers of DAPI-stained intracellular amastigotes per macrophage were counted, and the mean values of 100 infected macrophages were calculated. Data were normalized so that the infectivity of untreated THP-1 macrophages was set at 100%. The viability of THP-1 macrophages was determined using alamarBlue substrate (Invitrogen) as described above. Experiments were done in three independent replicates.

Statistical analysis. Statistical analysis was done using GraphPad Prism software. Groups of untreated and treated *Leishmania* were compared either by one-way analysis of variance (ANOVA) with Bonferroni posttests or by two-way ANOVA with repeated measures, as indicated in the figure legends. A P value of <0.05 was considered statistically significant.

RESULTS

A library screen identified host defense peptides with leishmanicidal activity. To identify new peptides with potential to develop further into drug candidates against promastigotes and amastigotes of the *Leishmania* parasite, we screened a library of 23 natural and synthetic cationic peptides for leishmanicidal activity. The library consisted of peptides that were shown to possess immune-modulatory and/or antimicrobial activity (see the selected references for each peptide in Table 1). The leishmanicidal activity of peptides was tested by a flow cytometry viability assay using propidium iodide (PI) staining (21). For this initial screen, *L. donovani* promastigotes were cultured in the presence or absence of host defense peptides listed in Table 1 at a final concentration of 20 μM in complete M199 medium and subsequently stained with PI to determine the viability. The peptides RI-BMAP28 and D-BMAP-28 that we reported previously to demonstrate antileishmanial activity were used as positive controls (16). Heat-killed *L. donovani* promastigotes were used as an additional negative control. In the initial screen, incubation with LL-37, L-1018, and BMAP-28 and RI-BMAP-28 reduced the viability of *L. donovani* promastigotes by >50% compared to the untreated control. RI-1018 and E6 resulted in a reduction of viability of approximately 40%. The remaining host defense peptides assayed had either no effect on the viability or reduced viability by less than 25% and, therefore, were not investigated further (Table 1).

LL-37, L-1018, RI-1018, and E6 had leishmanicidal activity against *L. donovani* promastigotes. To validate the leishmanicidal activity of the host defense peptide LL-37 and the three synthetic peptides L-1018, RI-1018, and E6 against *L. donovani* promastigotes, two different viability assays were used: (i) a flow cytometry assay in combination with fluorescein diacetate (FDA) (i.e., live-cell) staining and (ii) a redox activity assay that determines the redox activity of mitochondria using resazurin (22). Both assays were done in M199 medium in the absence of heat-inactivated FCS (Fig. 1). FCS was omitted because recent studies have shown that direct cytotoxic activities of host defense peptides are diminished in the presence of serum (23–25). Consistent with the results of the library screen, *L. donovani* promastigotes grown in the presence of E6, LL-37, L-1018, and RI-1018 at a final concentration of 20 μM had a significant reduction in viability as determined by FDA staining, as well as a significant reduction in

TABLE 1 Peptides used in this study and their leishmanicidal activity[a]

Peptide[b]	Amino acid sequence	Chirality	% live cells	Reference(s)
RI-BMAP-28	GIRIIPVIIPGYKKWARLIKRGLSRLGG-NH_2	D	0.4	16
D-BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRIG-NH_2	D	0.5	16
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH_2	L	27.4	22
E6	RRWRIVVIRVRR-NH_2	L	58.3	22, 43
W3	VRWIVAVRIWRR-NH_2	L	77.8	32
HHC10	KRWWKWIRW-NH_2	L	81.8	44
HHC36	KRWWKWWRR-NH_2	L	92.7	45
E2	RIWVIWRR-NH_2	L	98.4	22
HH2	VQLRIRVAVIRA-NH_2	L	98.7	46
L-1037	KRFRIRVRV-NH_2	L	95.9	47
L-1018	VRLIVAVRIWRR-NH_2	L	32.2	46
RI-1018	RRWIRVAVILRV-NH_2	D	60.0	46
L-1012	IFWRRIVIVKKF-NH_2	L	98.4	48
RI-1012	FKKVIVIRRWFI-NH_2	D	98.6	
L-JK6	VQWRRIRVWVIR-NH_2	L	98.3	48
D-JK6	VQWRRIRVWVIR-NH_2	D	98.2	48
RI-JK6	RIVWVRIRRWQV-NH_2	D	98.6	48
L-JK4	VQLRRIRVWVIR-NH_2	L	99.5	48
D-JK4	VQLRRIRVWVIR-NH_2	D	98.9	48
RI-JK4	RIVWVRIRRLQV-NH_2	D	99.4	48
L-1002	KRIRWVILWRQV-NH_2	L	99.0	46
RI-1002	VQRWLIVWRIRK-NH_2	D	96.6	46
RI-1035	KRWRWIVRNIRR-NH_2	D	92.6	46
RI-JK3	RIVRVRIARLQV-NH_2	D	99.1	48

[a] The amino acid sequence of each peptide and their leishmanicidal activity against *L. donovani* promastigotes after 4 h of treatment at a final concentration of 20 μM are shown. Data are normalized to heat-killed *L. donovani* promastigotes, which was set at 100%. RI-BMAP-28 and D-BMAP28 served as positive controls (16). The data shown are from a single experiment.

[b] Only human LL-37 is a natural peptide; all other peptides are synthetic. Peptides with the L- prefix or with no designation (no L-, R-, or RI- prefix) are L-amino acid peptides. Peptides with the D- prefix contain only D-amino acids, while RI peptides are retroinversion peptides containing D amino acids with the sequence reversed compared to the L amino acid equivalent. Peptides were grouped according to their ability to kill *L. donovani* promastigotes.

mitochondrial redox activity, compared to the untreated control (Fig. 1). Among the four peptides tested, L-1018 appeared to have the weakest leishmanicidal activity in both assays. Note that differences in the leishmanicidal activity between the L-form peptide L-1018 and the corresponding RI-form peptide were observed, which were statistically significant ($P \leq 0.01$) when evaluating the viability of *L. donovani* promastigotes by FDA staining, but not when viability was based on mitochondrial redox activity.

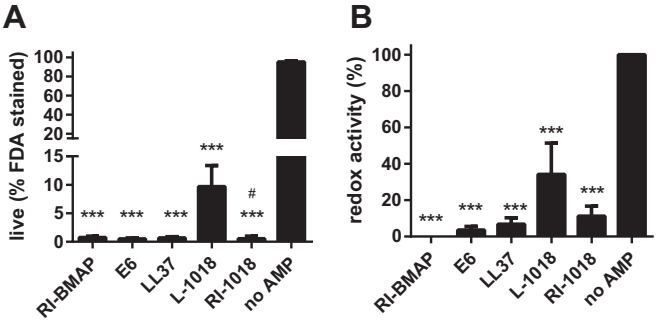


FIG 1 Effects of E6, LL-37, and RI-1018 on cell viability measured by fluorescein diacetate (FDA) staining and mitochondrial redox activity in *L. donovani* promastigotes. (A and B) Percentage of FDA-stained (live) promastigotes (A) and redox activity (B) of mid-log *L. donovani* promastigotes in the presence and absence of peptide (20 μM) after 4 h of incubation. The promastigotes were incubated with no antimicrobial peptide (AMP) as a control. The mean values from three independent experiments are shown. Error bars represent standard deviations. Statistical analysis was done by one-way ANOVA with Bonferroni posttests. Values that were significantly different are indicated as follows: ***, $P < 0.001$ versus untreated control; #, $P < 0.01$ versus L-1018.

GP63 conferred resistance to *Leishmania* promastigotes against the leishmanicidal activity of L-form peptides LL-37, L-1018, and E6. It has been shown that the *Leishmania* virulence factor GP63, a zinc-dependent metalloprotease expressed on the cell surface, confers protection to the parasite from the cytolytic properties of host defense peptides (18). A previous study from our laboratory demonstrated resistance to GP63 activity of the RI and D forms of the antimicrobial peptide BMAP-28, while the natural form of the peptide, L-BMAP-28, was susceptible (16). To test whether GP63 also influenced the antileishmanial properties of LL-37, L-1018, RI-1018, and E6, kill curves were established using three different *L. major* strains, including a wild-type strain, an isogenic mutant lacking all seven copies of *gp63* genes (*L. major* KO) (19), as well as the *gp63* mutant complemented with one functional copy of the *gp63* gene (*L. major* KO+GP63) (19). Antileishmanial activity of peptides E6, LL-37, RI-1018, and L-1018 against the three *L. major* strains was assayed at 5, 10, 15, and 20 μM using the flow cytometry assay and PI staining. Based on the dose-response curves, 50% lethal dose (LD_{50}) values were calculated. As shown in Fig. 2, the GP63 mutant strain *L. major* KO was highly susceptible to all four peptides tested. Interestingly, in comparison to its isogenic mutant, *L. major* KO, the *L. major* wild-type strain was significantly less susceptible to killing by the L-form peptides E6, LL-37, and L-1018 (Fig. 2A, B, and D). In contrast, there was no difference in susceptibility of the wild-type and mutant strain toward RI-1018 (Fig. 2C), consistent with the concept

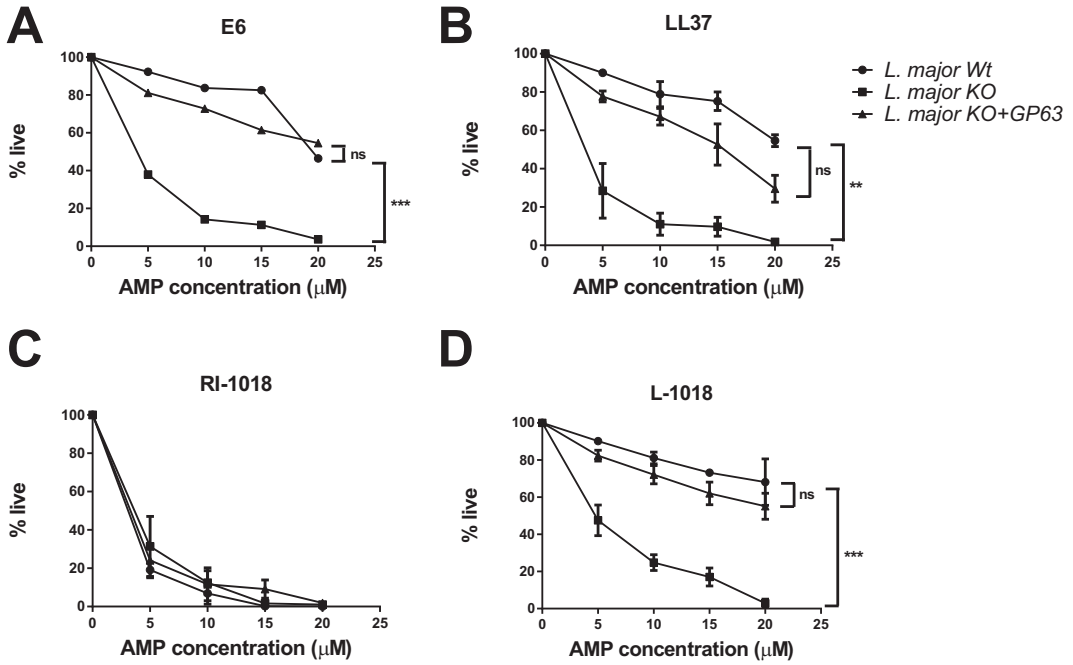


FIG 2 Susceptibility of *L. major* promastigotes toward L-form peptides depends on GP63 expression. (A to D) Percentages of live cells of wild-type (Wt) *L. major*, *L. major* KO, and *L. major* KO+GP63 in the presence or absence of increasing concentrations (5 μM, 10 μM, 15 μM, and 20 μM) of host defense peptides E6 (A), LL-37 (B), RI-1018 (C), and L-1018 (D). The mean values from three independent experiments are shown. Error bars represent standard deviations. Statistical analysis was done by two-way ANOVA with repeated measures. ns, no statistically significant difference; **, $P \leq 0.01$ versus wild type; ***, $P \leq 0.001$ versus wild type.

that D-form peptides should be resistant to degradation by the GP63 protease. The susceptibilities of the complemented strain *L. major* KO+GP63 to the four tested peptides were similar to those of the wild-type strain, confirming that the difference in the susceptibility of the different *L. major* strains toward the L-form peptides was indeed due to the expression of the zinc-dependent metalloprotease GP63 (Fig. 2). Consistent with these findings, LD_{50} values of the L-form peptides E6, LL-37, and L-1018 were >10 times higher in the wild-type and complemented *L. major* strains than in the *L. major* KO strain lacking a functional *gp63* gene, whereas no considerable difference in the LD_{50} of the RI-form peptide RI-1018 was found between the different *L. major* strains with or without a functional *gp63* gene (Table 2).

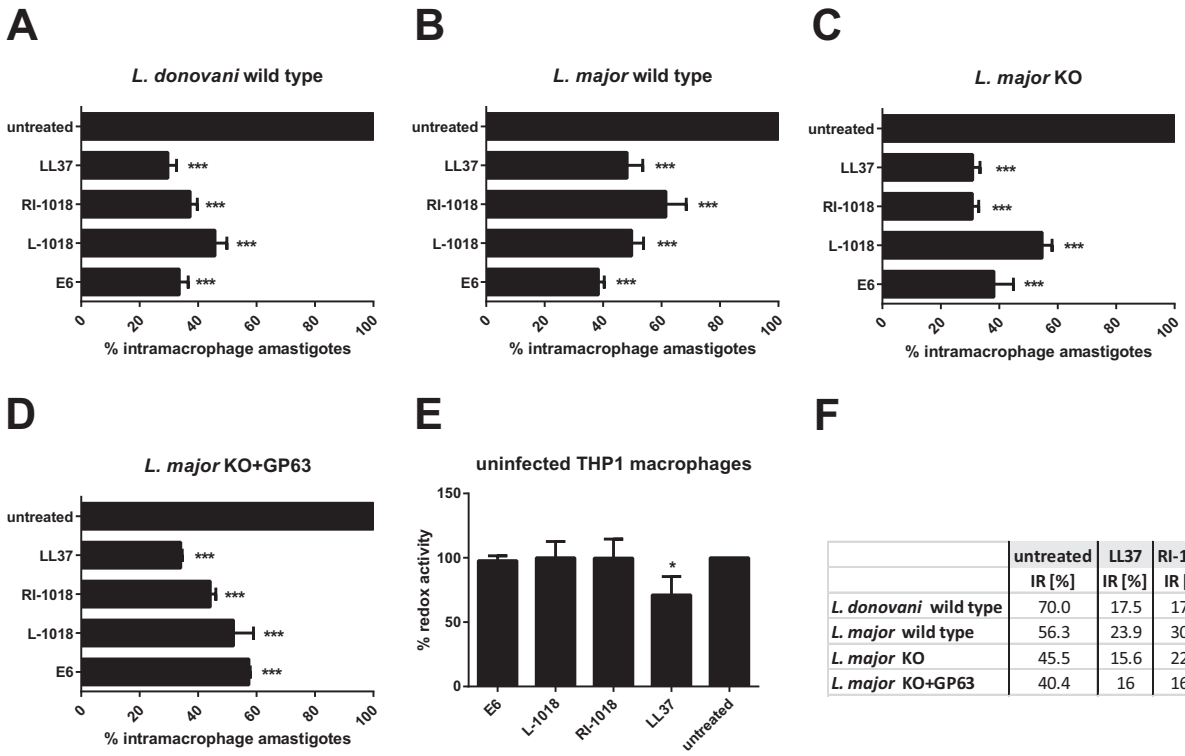
E6, LL-37, RI-1018, and L-1018 have leishmanicidal activity against intramacrophage *L. donovani* and *L. major* strains. The susceptibility of *Leishmania* promastigotes does not necessarily translate into leishmanicidal activity against the disease-causing, intramacrophage amastigotes due to the morphological and biochemical differences between the two life stages of the *Leishmania* parasite and the location of amastigotes within the phagolysosome of the macrophage. Therefore, we assayed for leishmanicidal activity of E6, LL-37, RI-1018, and L-1018 against intramacrophage amastigotes. For this purpose, THP-1 cells were differentiated to macrophages in the presence of phorbol 12-myristate 13-acetate and subsequently infected with metacyclic *L. donovani*, wild-type *L. major* strain, the gp63 mutant strain *L. major* KO, or the complemented strain *L. major* KO+GP63. At 24 h postinfection (i.e., after differentiation into amastigotes), host defense peptides (20 μM) were added, and the infected cells were incubated for an additional 48 h. *Leishmania* replication at the endpoint was determined by DAPI staining of intracellular parasites. Compared to the untreated control, the number of intramacrophage *L. donovani* amastigotes was significantly reduced in the presence of E6, L-1018, RI-1018, and LL-37 (Fig. 3A). Similarly, a significant reduction in the number of intramacrophage *L. major* amastigotes was observed when THP-1 cells were cultured in the presence of 20 μM E6, L-1018, RI-1018, and LL-37 (Fig. 3B). The susceptibility of intramacrophage *L. major* amastigotes toward each of the four tested peptides appeared to be similar when comparing the wild-type *L. major*, *L. major* KO strain, and the complemented strain *L. major* KO+GP63, suggesting that GP63 expression plays a negligible role in the susceptibility of intramacrophage amastigotes to the four tested peptides. This is consistent with reduced expression of GP63 in the amastigote form (26).

To confirm that our four peptides had no toxic effect toward host cells, differentiated THP-1 cells were either treated with 20 μM E6, L-1018, RI-1018, or LL-37 or left untreated in the absence of a *Leishmania* infection using the same experimental conditions described above. Macrophage viability was measured by determining the redox activity using the resazurin assay. No loss in

TABLE 2 LD_{50} values of E6, L-1018, RI-1018, and LL-37 against *L. major* promastigotes after 4 h of incubation

	LD_{50} (μM)[a]			
L. major strain	E6	RI-1018	L-1018	LL-37
Wild type	20.9	0.6	31.5	21.2
KO	2.8	2.1	1.5	1.4
KO+GP63	22.1	0.9	22.3	15.6

[a] The LD_{50} values are based on experimental data presented in Fig. 2.



	untreated IR [%]	LL37 IR [%]	RI-1018 IR [%]	L-1018 IR [%]	E6 IR [%]
L. donovani wild type	70.0	17.5	17.7	31.1	29.1
L. major wild type	56.3	23.9	30.3	20.7	26.4
L. major KO	45.5	15.6	22.5	21.3	25
L. major KO+GP63	40.4	16	16.4	29.5	24.5

FIG 3 Leishmanicidal activity of E6, RI-1018, LL-37, and L-1018 against intramacrophage amastigotes. (A to D) Rates of intramacrophage replication of wild-type *L. donovani* (A), wild-type *L. major* (B), its isogenic mutant *L. major* KO (C), and *L. major* KO+GP63, which was complemented with a functional copy of *gp63* (D). Data shown in panels A to D are normalized to THP-1 macrophages that were infected with the respective *Leishmania* strain in the absence of peptides. (E) Mitochondrial redox activity of differentiated, uninfected THP-1 macrophages upon 48 h of culture in the presence of E6, L-1018, RI-1018, and LL-37 at a final concentration of 20 μM. Data depicted in panel E were normalized to the values of an untreated control, which was set at 100%. The mean values from three independent experiments are shown. Error bars represent standard deviations. Statistical comparison was done by one-way ANOVA with Bonferroni posttests. ***, $P \leq 0.001$ versus untreated control; *, $P \leq 0.05$. (F) Infection rates (IR) are shown as percentages.

viability of macrophages was observed when cultured in the presence of E6, RI-1018, and L-1018 at a final concentration of 20 μM, whereas culturing of macrophages in the presence of 20 μM LL-37 led to a slight but significant reduction in the mitochondrial redox activity compared to the untreated control (Fig. 3E).

DISCUSSION

The host defense peptide (LL-37) and three synthetic peptides (RI-1018, L-1018, and E6) demonstrated antileishmanial activity against intramacrophage amastigotes. These peptides, particularly if in their RI form, show potential for development of new drug candidates for antileishmanial therapies. The development of novel antileishmanial therapeutics has been hampered by a lack of suitable drug targets owing to the complex life cycle of the *Leishmania* parasite, including an extracellular promastigote and an intracellular amastigote life stage, with differences in metabolism, protein expression, and membrane composition (27). An ideal antileishmanial drug candidate likely needs to combine direct killing of the amastigote life form and triggering of immune-modulatory activities in host cells, while the potential for development of drug resistance needs to be low. Antimicrobial peptides combine these features and are therefore a promising new class of therapeutics against parasitic diseases (8, 16, 28).

For the development of an active compound against cutaneous leishmaniasis, a topical administration would be favorable that overcomes the challenge to deliver the active compound to the dermis (29). In contrast, for the treatment of visceral leishmaniasis, a lipid-based, oral application of an antimicrobial peptide formulation might overcome the barriers preventing absorption of the active compound as demonstrated in the study of Wasan et al., with oral amphotericin B against murine visceral leishmaniasis (30).

LL-37 is a naturally derived, human host defense peptide with broad immune-modulatory effects (31), whereas E6 and L-1018 are synthetic peptides developed from the bovine bactenecin peptide (namely, its linearized form Bac2A) through amino acid exchange (32). Both LL-37 and Bac2A are cationic peptides that have been implicated to play an important role in the host defense against infection (33, 34). The detailed mechanism as to how *Leishmania* parasites escape host defense mechanism *in vivo* and are able to replicate and survive within macrophages is not fully understood. It was proposed that the leishmanial surface metalloprotease GP63 mediates degradation of host defense peptides and thus protects against antimicrobial peptide-induced killing (16, 18). A similar immune defense strategy has been demonstrated for a wide range of bacterial pathogens, including *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus*, and *Streptococcus pyogenes*, that are protected from host defense peptide attacks by protease degradation (35). Besides this role in the early stage of infection, GP63 was shown to have an impact on macrophage signaling and innate

immune response (36). Interestingly, the expression levels (0.1% in amastigotes compared to 1% in promastigotes) as well as post-translational modifications and localization of GP63 differ dramatically in amastigotes and promastigotes (36). The role of GP63 in amastigotes is still under discussion; despite lower expression on the surface of amastigotes, it might be more exposed on the surface of the intracellular form of the parasite through the absence of lipophosphoglycan (LPG), which forms a dense glycocalyx in promastigotes (37). In addition, expression of other surface molecules such as proteophosphoglycan (PPG) is absent or low in amastigotes compared to promastigotes (37). PPG was reported to play a role in the susceptibility of *Leishmania* promastigotes toward cationic antimicrobial peptides (37). The data presented here support the hypothesis that GP63 plays a role in resistance of *Leishmania* spp. against antimicrobial host defense peptides. We have shown that promastigotes of a mutant strain of *L. major* lacking a functional *gp63* gene exhibit increased susceptibility to L-form peptides, such as L-1018, E8, and LL-37, compared to the parental wild-type strain or a complemented strain in which GP63 function has been restored. In contrast, no difference in sensitivity to L-form peptides was observed between amastigotes of the *L. major* wild-type strain and mutant strain lacking a functional *gp63* gene, suggesting that GP63 is primarily functional in the promastigote life form. This is in agreement with the findings of Kelly et al., who showed that *gp63* gene expression is downregulated in *Leishmania* during the amastigote life stage (38).

With recent peptidomimetic technology, a new class of host defense peptides has been developed, optimizing the peptide's antipathogenic activity and overcoming the low *in vivo* stability of most host defense peptides due to degradation by proteases (39). Strategies to overcome protease sensitivity include the incorporation of the D form, rather than the naturally occurring L form, of amino acids and in addition reversing the sequence and thus the chirality of amino acids in peptidomimetics (designated "RI" for retroinversion from peptides) (8). Indeed, here we demonstrate enhanced activity of RI-1018 over its L-form peptide, most likely due to resistance to proteolytic degradation, since folding of these peptides into their active α-helical form would result in different backbone twists but the positioning of most amino acids in the same place in three-dimensional space (40). Similar findings were made by Lynn et al. who demonstrated enhanced leishmanicidal activity of the D isomer as well as RI-form peptides of BMAP-28 against wild-type *L. major* and *L. major* KO promastigotes (16).

Once promastigotes are transmitted to humans by the sand fly, the parasite invades macrophages and differentiates into the amastigote form to proliferate and establish a permanent infection. Previous studies have shown that host defense peptides are able to enter macrophages and mediate killing of *Brucella abortus* (41). Here we tested E6, LL-37, L-1018, and RI-1018 for activity against *L. donovani* and *L. major* amastigotes utilizing a macrophage infection assay (16). All four peptides, E6, L-1018, RI-1018, and LL-37, were effective against intracellular amastigotes, while showing little or no toxicity toward host cells. Interestingly, in the intracellular amastigote assay for *L. donovani*, LL-37 was the most potent peptide tested, followed by E6, RI-1018, and L-1018. When comparing these results with those of the library screen, a data set where leishmanicidal activity was also tested in the presence of serum, E6 and RI-1018 were even more potent against amastigotes in infected cells than against axenic promastigotes, while LL-37 had very similar leishmanicidal activity and L-1018 had slightly less leishmanicidal activity. The higher leishmanicidal activity in intracellular amastigotes could be explained by the fact that the peptides tested may have, in addition to a direct leishmanial killing effect, an indirect activity by activating host defense mechanisms to kill the intracellular parasite. In conclusion, the host defense peptide LL-37 and the three synthetic peptides E6, L-1018, and RI-1018 were effective against *L. donovani* and *L. major* promastigotes and intracellular amastigotes. The action of leishmanial proteases could be overcome by incorporation of the D form, rather than the naturally occurring L form, of amino acids and by reversing the sequence and thus the chirality of amino acids in peptidomimetics, thereby enhancing their antileishmanial activity (8). Indeed, among the peptides tested in this study, RI-1018 appears to be the most promising candidate to develop further for antileishmanial treatment, since it is highly active against amastigotes and promastigotes, resists proteolytic degradation by the leishmanial zinc-dependent protease GP63, and shows no toxicity toward host cells even at the highest concentration tested in this study. On the basis of our observations and that of others (42), it is tempting to speculate that E6 can be optimized further by generating a D or RI isoform to enhance its antileishmanial activity and protease resistance.

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A.K.M. wrote the manuscript, designed the experiments, and performed experiments. S.C. performed experiments. R.E.W.H. designed experiments, wrote the manuscript, and contributed antimicrobial peptides for this study. W.R.M. designed experiments, wrote the manuscript, and contributed lab consumables.

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