Amino Acids (2012) 43:2265–2277
DOI 10.1007/s00726-012-1304-0

ORIGINAL ARTICLE

Enhanced leishmanicidal activity of cryptopeptide chimeras from the active N1 domain of bovine lactoferrin

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Received: 8 March 2012 / Accepted: 17 April 2012 / Published online: 29 April 2012

Abstract Two antimicrobial cryptopeptides from the N1 domain of bovine lactoferrin, lactoferricin (LFcin17–30) and lactoferrampin (LFampin265–284), together with a hybrid version (LFchimera), were tested against the protozoan parasite *Leishmania*. All peptides were leishmanicidal against *Leishmania donovani* promastigotes, and LFchimera showed a significantly higher activity over its two composing moieties. Besides, it was the only peptide active on *Leishmania pifanoi* axenic amastigotes, already showing activity below 10 μM. To investigate their leishmanicidal mechanism, promastigote membrane permeabilization was assessed by decrease of free ATP levels in living parasites, entrance of the vital dye SYTOX Green (MW = 600 Da) and confocal and transmission electron microscopy. The peptides induced plasma membrane permeabilization and bioenergetic collapse of the parasites. To further clarify the structural traits underlying the increased leishmanicidal activity of LFchimera, the activity of several analogues was assessed. Results revealed that the high activity of these hybrid peptides seems to be related to the order and sequence orientation of the two cryptopeptide moieties, rather than to their particular linkage through an additional lysine, as in the initial LFchimera. The incorporation of both antimicrobial cryptopeptide motifs into a single linear sequence facilitates chemical synthesis and should help in the potential clinical application of these optimized analogues.

Keywords *Leishmania* · Leishmanicidal activity · Antimicrobial peptides · Membrane permeabilization · Lactoferricin · Lactoferrampin · LFchimera

Abbreviations

AMP	Antimicrobial peptide
Di-LFampin	LFampin265–284-K-LFampin265–284
Di-LFcin	LFcin17–30-K-LFcin17–30
DMNPE-D-luciferin	D-Luciferin, 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester
FITC	Fluorescein isothiocyanate
Fmoc	9-fluorenylmethyloxycarbonyl
IC_{50}	Peptide concentration causing 50 % inhibition of MTT reduction at 4 h incubation
LC_{50}	Peptide concentration causing 50 % inhibition of *Leishmania* proliferation
LF	Lactoferrin
LFampin265–284	Lactoferrampin 265–284

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LFampin–LFcin	LFampin265–284–LFcin17–30
LFchimera	LFcin17–30-K-LFampin265–284
LFchimera-R	LFampin265–284-K-LFcin17–30
LFcin17–30	Lactoferricin 17–30
LFcin–LFampin	LFcin17–30–LFampin265–284
MALDI-TOF	Matrix-assisted laser desorption ionization, time-of-flight analysis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PI	Propidium iodide
RP-HPLC	Reverse phase-high performance liquid chromatography

Introduction

Eukaryotic antimicrobial peptides (AMPs) are part of the primitive immune defense mechanism acting as a first chemical barrier against invading pathogens. These peptides are often multifunctional, acting in concert with other immune mechanisms, and have evolved in nature throughout centuries to protect their hosts against diverse pathogens, such as bacteria, fungi, virus, protozoa and even cancer cells (Nguyen et al. 2011; Reddy et al. 2004; Teixeira et al. 2012; Yeung et al. 2011). Despite their huge structural diversity, most of them share a cationic character and the adoption of amphipathic structures when in contact with membranes. These characteristics are directly linked to their microbicidal mechanism, as most of them act as membrane-active peptides, disrupting the plasma membrane of pathogens (Castanho 2010). Due to their high cationic character, they preferentially act by electrostatic interaction with the exoplasmic leaflet of prokaryote or lower eukaryote membranes richer in anionic phospholipids than the more zwitterionic membranes of higher eukaryotes (Bastos et al. 2008; Brogden 2005; Lohner 2009; Lohner and Blondelle 2005; Wassef et al. 1985). This membrane-associated lethal mechanism makes AMPs less prone to induce resistance than many antibiotics under current use (Brogden 2005; Hancock and Sahl 2006; Lohner 2009; Teixeira et al. 2012), since it would require extensive reorganization of membrane structure and composition, hence affecting a multitude of transport systems and enzymes embedded in the phospholipid matrix. In addition to the membrane-associated mechanism, an increasing body of evidence shows some AMPs to be also active on intracellular targets (Brogden 2005; Henriques et al. 2006).

Given their small size and few post-translational modifications, AMPs are particularly amenable to chemical synthesis, which may involve the inclusion of non-native structural elements for improved activity. AMPs can in fact be designed de novo by genomic mining and algorithmic prospection (Fjell et al. 2011), or result from proteolysis of proteins, giving rise to so-called antimicrobial cryptopeptides (Cho et al. 2009; Ibrahim et al. 2002; Mak et al. 2007; Pellegrini 2003).

Within cryptopeptides, the most paradigmatic example is lactoferricin (LFcin), released by pepsin digestion of lactoferrin (LF), either artificially or as consequence of LF digestion in the stomach (Kuwata et al. 1998). Antimicrobial properties of LFcin have been extensively studied on Gram-negative and Gram-positive bacteria, fungi, protozoa and viruses (Gifford et al. 2005; Jenssen and Hancock 2009; León-Sicairos et al. 2006; Oo et al. 2010; Sánchez-Gómez et al. 2011; Yamauchi et al. 1993), and their antitumoral and antiendotoxic activities have also been referred (Chan et al. 2006; Gifford et al. 2005; Mader et al. 2007; Yamauchi et al. 1993). Further trimming of the bovine LFcin17–41 led to LFcin17–30 as its best analogue, with higher cationicity and bactericidal activity than its human counterpart (Groenink et al. 1999). The N1 domain contains also lactoferrampin (268–284), active on a broad range of pathogens (van der Kraan et al. 2004, 2005b). Systematic studies using truncation and/or extension of the initial lactoferrampin resulted in LFampin265–284 as the shortest and most active peptide, especially against *Candida albicans* (Adao et al. 2011; Haney et al. 2009; van der Kraan et al. 2005a, b, 2006). LFcin and LFampin fragments are spatially close in LF (Fig. 1), making it plausible that they cooperate in many of the beneficial properties of this protein. To test whether these peptides would form a functional unit, a chimeric peptide (LFchimera) containing LFcin17–30 and LFampin265–284 was synthesized by Bolscher et al. (2009). In order to mimic the internal spatial topology of LF, both chimera moieties were linked through their C-carboxy terminals to the α- and ε-amino groups, respectively, of an additional C-amidated lysine, leaving two N-terminals as free ends (Fig. 1). LFchimera displays a strong activity against a wide variety of multi-drug resistant pathogens, even when assayed at high ionic strength or in rich growth medium (Bolscher et al. 2009, 2012; Flores-Villaseñor et al. 2010; León-Sicairos et al. 2009; López-Soto et al. 2010).

Protozoans have received far less attention than other pathogens as potential AMP targets, even though a variety of in vivo and in vitro assays suggest that AMPs may constitute a powerful tool for developing new antiparasitic therapies or complementing current ones [for reviews see (Cobb and Denny 2010; Rivas et al. 2009)]. Some of the AMPs studied here, namely LFcin17–30, LFampin265–284 and LFchimera, have been successfully tested against *Entamoeba histolytica* (López-Soto et al. 2010).

The protozoan parasite *Leishmania* is responsible for a set of infections known as leishmaniasis, with a broad range of clinical manifestations that rank second only to malaria in mortality and morbidity (WHO 2010).

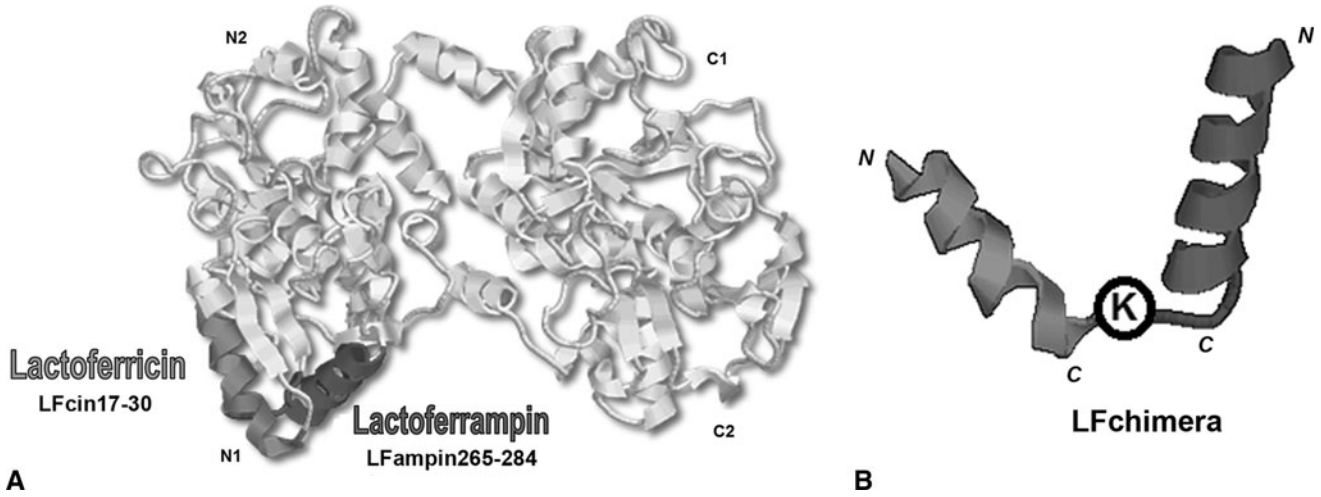

Fig. 1 Lactoferrin peptides. **a** Ribbon representation of bovine lactoferrin (PDB ID: 1BLF) with LFcin17–30 and LFampin265–284 highlighted in *light grey* and *dark grey*, respectively. **b** Design of LFchimera (Bolscher et al. 2009), composed by LFcin17–30 (*light grey*) and LFampin265–284 (*dark grey*) linked via an additional lysine (K)

Leishmania has a digenic cycle with two major forms, the promastigote flagellated form typical of the invertebrate vector and the intracellular amastigote, dwelling inside the mononuclear phagocytic cells of a wide variety of mammals. Among the protozoans, *Leishmania* promastigotes are endowed with powerful defenses against AMPs (Ilgoutz and McConville 2001). Although amastigotes appear more vulnerable to AMPs, due to the much less developed glycocalyx (Cobb and Denny 2010; Kima 2007; McConville and Ralton 1997), their location inside phagocytic cells and the substantial changes in the membrane composition adopted by this form, are barriers that partially prevent AMPs action (Luque-Ortega and Rivas 2010; Wang et al. 2011). Despite these drawbacks, *Leishmania* is more susceptible to AMPs than mammalian cells, as shown for chimeric cecropin A-melittin peptides (Chicharro et al. 2001; Fernández-Reyes et al. 2010).

In this work, we have thoroughly studied the effect of three LF-peptides, LFcin17–30, LFampin265–284 and LFchimera, against *Leishmania donovani* promastigotes and *Leishmania pifanoi* axenic amastigotes, and investigated the lethal mechanisms by which these peptides exert their antimicrobial activity. Further, we provide activity data for a number of analogues of LFchimera, the most active peptide of the initial set.

Materials and methods

Peptide synthesis and characterization

All peptides in Table 1 were prepared by solid phase peptide synthesis using Fmoc-protected amino acids (Orpegen Pharma GmbH, Heidelberg, Germany) in a MilliGen 9050 synthesizer (MilliGen/Biosearch, Bedford, MA, USA). For the LFchimera peptide, where the LFcin17–30 and LFampin265–284 sequences are, respectively, built on the α- and ε-amino groups of a C-terminally amidated lysine, the synthesis started with Fmoc-Lys (iv-Dde) linked to NovaSyn®TGR resin (0.23 mmol/g) (Merck KgA, Darmstadt, Germany) and proceeded as described previously (Bolscher et al. 2009). The three other branched analogues, LFchimera-R, Di-LFcin and Di-LFampin, were made likewise. The remaining peptides in Table 1 were all linear, including the twin sequences LFcin-LFampin and LFampin-LFcin, and were built on preloaded Fmoc-Arg(Pbf)-NovaSyn® TGA (0.23 mmol/g) or Fmoc-Gly-Wang resin LL (0.33 mmol/g) resins.

On-resin labelling of an aliquot of each of the LFcin17–30, LFampin265–284 and LFchimera peptides was carried out with a 20-fold excess of fluorescein-5-isothiocyanate (FITC; Invitrogen, Breda, The Netherlands) on peptides N-terminally elongated with a γ-aminobutyric acid residue, to prevent Edman-type rearrangements during the cleavage reaction. Peptides were purified to at least 95 % purity by semipreparative RP-HPLC (Jasco Corporation, Tokyo, Japan) on a Vydac C18-column (218MS510; Vydac, Hesperia, CA, USA) and their identity was confirmed by MALDI-TOF mass spectrometry on a Microflex LRF mass spectrometer equipped with an additional gridless reflectron (Bruker Daltonik, Bremen, Germany) as described previously (Bolscher et al. 2011).

Parasite strains and culture conditions

L. donovani promastigotes of MHOM/SD/00/1S-2D strain (kindly provided by S. Turco, School of Medicine, University of Kentucky, Lexington) and 3-Luc strain,

Table 1 Properties of synthetic LF-peptides

Peptide [a]	Sequence	#AA	Mr	Charge [b]
LFcin17-30	FKCRRWQWRMKKLG	14	1923	+6
LFampin265-284	DLIWKLLSKAQEKFGKNKSR	20	2390	+4
LFchimera [c]	FKCRRWQWRMKKLG – K | DLIWKLLSKAQEKFGKNKSR	35	4422	+12
LFchimera-R [c]	DLIWKLLSKAQEKFGKNKSR – K | FKCRRWQWRMKKLG	35	4422	+12
Di-LFcin [c]	FKCRRWQWRMKKLG – K | FKCRRWQWRMKKLG	29	3956	+14
Di-LFampin [c]	DLIWKLLSKAQEKFGKNKSR – K | DLIWKLLSKAQEKFGKNKSR	41	4889	+10
LFcin-LFampin	FKCRRWQWRMKKLG-DLIWKLLSKAQEKFGKNKSR	34	4295	+10
LFampin-LFcin	DLIWKLLSKAQEKFGKNKSR-FKCRRWQWRMKKLG	34	4295	+10

[a] The purity of the peptides was at least 95 % and the authenticity of the peptides was confirmed by MALDI-TOF mass spectrometry (Bolscher et al. 2011)

[b] Calculated overall charge at pH = 7.0

[c] The carboxyl group of the linking lysine (C-terminal) is in carboxamide form

derived from the first one, which express a cytoplasmic form of *Photinus pyralis* luciferase (Luque-Ortega et al. 2003), were grown at 26 °C; *L. pifanoi* axenic amastigote strain MHOM/VE/60/Ltrod (kindly provided by A. A. Pan) was grown at 32 °C and harvested as described (Luque-Ortega and Rivas 2010; Luque-Ortega et al. 2003).

Leishmanicidal activity of LF-peptides

Parasites were harvested at the late exponential growth phase, washed twice with Hank's balanced salt solution (HBSS: 137 mM NaCl, 5.3 mM KCl, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$ and 0.4 mM Na_2HPO_4, pH = 7.2) supplemented with 10 mM D-glucose (HBSS + Glc), and resuspended in the same medium at a final parasite density of 2×10^7 parasites/mL, as standard conditions.

The short- and long-term leishmanicidal activities were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, as described previously (Luque-Ortega and Rivas 2010). They are represented by IC_{50} (peptide concentration causing 50 % inhibition of MTT reduction at 4 h incubation) and LC_{50} (peptide concentration causing 50 % inhibition of *Leishmania* proliferation) parameters, respectively. Briefly, parasites were transferred into a 96-microwell plate (120 µL/well) and incubated with peptides (concentration range 0–50 µM) under their respective standard conditions. Afterwards, a 20 µL aliquot from each well was transferred into a new 96-well microplate with 130 µL/well of its respective complete medium and incubated for 72 h at 26 °C or 96 h at 32 °C to allow proliferation of the surviving promastigotes or amastigotes, respectively (long-term assay). At the end of incubation, growth was assessed by incubating the parasites with 0.5 mg/mL MTT solution (final concentration) at their respective temperature until colour development. The resulting blue formazan crystals were solubilized with 10 % SDS and read in a microplate reader (680 Bio-Rad microplate ELISA reader) fitted with a 595 nm filter. For the short-term assay, immediately after 4 h of incubation of the parasites with the peptides, MTT was added to each well and proceeded as described before (Luque-Ortega and Rivas 2010). The results represented by IC_{50} and LC_{50} parameters are obtained through the GraphPad Prism 5 programme that also provides the standard error of the fitted data. The final values reported correspond to the average of at least two independent experiments (conditions tested in triplicates), and the uncertainty is the propagated error, i.e. $(1/n)\sqrt{\sum_n e_i^2}$, based on the standard errors of each experiment, e_i.

Real-time monitoring of in vivo changes in intracellular levels of ATP in *L. donovani* promastigotes

Intracellular levels of ATP in living *L. donovani* promastigotes were monitored using a previously described protocol (Luque-Ortega et al. 2003). Briefly, *L. donovani* promastigotes of the 3-Luc strain were resuspended at 2.2×10^7 parasites/mL in HBSS + Glc with the free-membrane-permeable luciferase substrate DMNPE-D-luciferin [D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester] (25 µM, final concentration), and 90 µL of the suspension was distributed into a black 96-well microplate.

When luminescence reached a plateau, 10 μL of the peptide was added ($t = 0$) and the decay on the luminescence monitored in a BMG Polarstar Galaxy microwell reader (Öffenburg, Germany) equipped with luminescence setting. Luminescence data were referred as percentage of luminescence corrected with the corresponding values of untreated promastigotes.

Plasma membrane permeabilization

The entrance of the membrane-impermeable vital dye SYTOX Green into the parasite cytoplasm was evaluated in two different experiments. First, *L. donovani* promastigotes (90 μL at 2.2×10^7 parasites/mL) were incubated with SYTOX Green (1 μM, final concentration) in HBSS + Glc for 10 min. Once the basal fluorescence, measured at $\lambda_{ex} = 504$ nm and $\lambda_{em} = 524$ nm in a BMG Polarstar Galaxy microwell reader (Öffenburg, Germany), become stable, 10 μL of the corresponding peptide solution ($t = 0$) was added and the increase in fluorescence monitored, and expressed as percentage relative to full permeabilized cells achieved by the addition of 0.1 % Triton X-100 (Luque-Ortega and Rivas 2010). Second, to assess dye incorporation into individual parasites, *L. donovani* promastigotes were incubated with the peptides at the standard conditions described above (4 h, 26 °C), and the vital dye was added at the end of the incubation. The fluorescence was measured by cytofluorometry in a Beckman-Coulter FC-500 Analyzer. To achieve maximal permeation while preserving parasite morphology, parasites were incubated with 15 μM digitonin.

Confocal microscopy

L. donovani promastigotes were incubated with the peptides labelled with FITC under standard conditions. After 4 h incubation, propidium iodide (PI) (50 μg/mL, final concentration) was added to the cells. A 15 μL drop of each sample was placed in microscope slides (previously coated with polylysine) and parasites were observed and photographed in a Confocal Laser Scanning Microscope Leica TCS-SP2 ABOS.

Electron microscopy

A suspension of *L. donovani* promastigotes was incubated with the peptides under standard conditions. Afterwards, parasites were washed with PBS and fixed in a 3 % (w/v) glutaraldehyde solution in PBS (1–2 h), included with 2.5 % (w/v) osmium tetroxide (OsO_4) (1–2 h), gradually dehydrated in ethanol, included with propylene oxide (1 h), embedded in Epon 812 resin and finally observed and photographed in a JEOL-1230 transmission electron microscope (Luque-Ortega and Rivas 2010).

Results

The LFchimera peptide shows the highest leishmanicidal activity among the different LF-peptides

The leishmanicidal activity of LFcin17–30, LFampin265–284 and LFchimera was determined by MTT reduction. All these LF-peptides were leishmanicidal against *L. donovani* promastigotes below 50 μM (Table 2). LFchimera was the most active, with IC_{50} and LC_{50} values close to 4 μM (Table 2). Axenic amastigotes were much less susceptible, regardless of the peptide assayed—LFcin17–30 and LFampin265–284 did not reach IC_{50} or LC_{50} at the highest concentration tested (50 μM), while the LFchimera showed a significant activity below 10 μM (Table 2).

A straightforward interpretation of the similarity between IC_{50} and LC_{50} values (Table 2) is that the parasites are unable to recover from the damage produced by the peptides in the first 4 h of incubation.

In order to evaluate a possible synergistic effect, we compared the activity of the LFchimera on *L. donovani* promastigotes with that of its two moieties, LFcin17–30 and LFampin265–284, added in equimolar ratio at identical concentrations than for LFchimera (0–10 μM). As seen in Fig. 2, an additive rather than synergistic effect was observed for the non-conjugated peptides. Conversely, the inhibitory activity of LFchimera was significantly higher than the sum of its separated components, for the whole range of studied concentrations (Fig. 2).

LF-peptides caused plasma membrane permeabilization of *Leishmania* promastigotes

The fast action of the peptides is consistent with a lethal activity based on membrane permeabilization. To probe this, we used several complementary approaches, as described in detail below.

First, the intracellular levels of free ATP in living parasites were monitored in real time. Promastigotes of the 3-Luc strain, expressing a cytoplasmic form of luciferase, were incubated with caged-free permeable DMNPE-luciferin, with free ATP as the limiting factor for luminescence (Luque-Ortega et al. 2003). Previously, an inhibitory effect of the peptide on purified luciferase had been discarded for all peptides tested (<2 %). Immediately after peptide addition ($t = 0$), a fast and dose-dependent decrease of luminescence was observed (Fig. 3), consistent with the bioenergetic collapse of the parasites. This means that the

Table 2 Leishmanicidal activity of LFcin17–30, LFampin265–284, LFchimera and LFchimera analogues against *L. donovani* promastigotes and *L. pifanoi* amastigotes

Peptide	Charge[a]	*L. donovani* promastigotes		*L. pifanoi* amastigotes	
		$IC_{50}^{b,c}$ (μM)	$LC_{50}^{b,d}$ (μM)	$IC_{50}^{b,c}$ (μM)	$LC_{50}^{b,d}$ (μM)
LFcin17–30	+6	21.9 ± 1.1 (4)	25.3 ± 1.6 (4)	>50 (3)	>50 (3)
LFampin265–284	+4	30.9 ± 1.0(4)	28.8 ± 0.9 (4)	>50 (3)	>50 (3)
LFchimera	+12	3.7 ± 0.2 (7)	3.0 ± 0.2 (7)	6.3 ± 0.2 (5)	7.3 ± 0.8 (5)
LFchimera-R	+12	3.5 ± 0.1 (2)	3.5 ± 0.2 (2)	4.5 ± 0.2 (2)	6.0 ± 1.1 (2)
Di-LFcin	+14	2.4 ± 0.1 (2)	2.1 ± 0.2 (2)	3.8 ± 0.4 (2)	4.3 ± 0.6 (2)
Di-LFampin	+10	4.6 ± 0.1 (2)	4.2 ± 0.2 (2)	5.4 ± 0.5 (2)	5.9 ± 0.4 (2)
LFcin–LFampin	+10	4.1 ± 0.1 (2)	3.6 ± 0.1 (2)	5.4 ± 0.3 (2)	6.3 ± 0.4 (2)
LFampin–LFcin	+10	1.7 ± 0.1 (2)	1.5 ± 0.1 (2)	1.6 ± 0.2 (2)	0.8 ± 0.1 (2)

[a] Calculated overall charge at pH = 7.0

[b] Each value represents the average of the independent experiments (number indicated in parenthesis next to each value) and the error reported is the propagated error (based on the standard errors of each experiment, see text)

[c] IC_{50} is the peptide concentration causing 50 % inhibition of MTT reduction at 4 h incubation

[d] LC_{50} is the peptide concentration causing 50 % inhibition of *Leishmania* proliferation

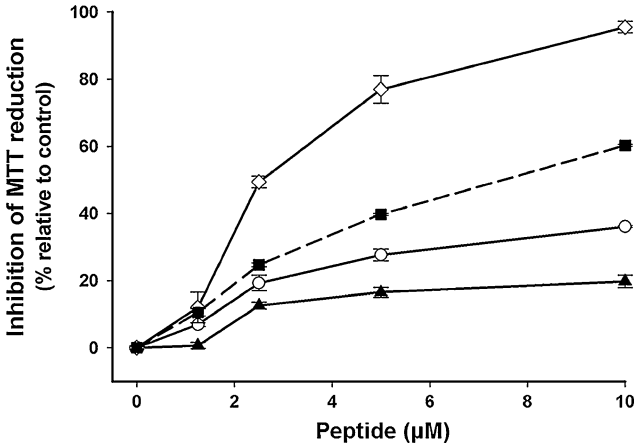

Fig. 2 Effect of LF-peptides on the viability of *L. donovani* promastigotes. Parasites were incubated with different concentrations of LFcin17–30 (*open circle*), LFampin265–284 (*filled triangle*), LFchimera (*open diamond*) and its two constituent peptides together (*filled square*) for 4 h in HBSS + Glc. The results of one representative experiment out of three (conditions tested in triplicate) are presented as percentage of inhibition of MTT reduction (reflecting the inhibition of parasite viability) relative to control

presence of the peptide disturbs the membrane, leading to impaired ATP production by either loss of ionic gradient or—for large enough lesions—by ATP leakage, in any event culminating in parasite death. In this assay, LFchimera turned out to be the most active peptide at concentrations in the 1.25–10 μM range, while the other two peptides achieved similar effects only at concentrations in the 10–50 μM range (Fig. 3).

Based on the above results, we evidenced that these peptides induce membrane damage. In order to ascertain the severity of the damages, the entrance of SYTOX Green, a vital dye that binds exclusively to nucleic acids of permeabilized cells enhancing its fluorescence, was monitored after adding the peptides. A fast and dose-dependent accumulation of the dye (particularly for LFampin265–284 and LFchimera) (Fig. 4b, c, upper panel) was observed, reaching saturation minutes after peptide addition. Again, LFchimera was more active than its separated components reaching 100 % of dye entrance (all cells are dead) at 10 μM, whereas the other two peptides at this concentration only cause ~20 % of dye entrance (Fig. 4, upper panel). These results reinforced the previous conclusion from luminescence experiments about membrane damage and its severity, as the entrance of a large molecule is allowed. LFcin17–30 showed a different kinetics when compared with the other two peptides (Fig. 4a, upper panel), with a much slower rate of dye entrance than the fast bioenergetic collapse observed for *L. donovani* 3-Luc promastigotes (Fig. 3a). As the binding of SYTOX Green to nucleic acids is practically irreversible, even if the cell recovers from the inflicted damage, this assay was complemented with the addition of the dye only at the end of the 4 h incubation and analyzed by cytofluorometry. In Fig. 4, lower panel, one can see that the lesion was permanent and the parasites were unable to recover from the damage suffered in the first few minutes (both assays of uptake of the vital dye are highly concordant), and thus dye accumulation was not due to transitory disruption of the membrane. The results from cytofluorometry also unveiled a different behaviour in membrane permeabilization. The most relevant feature is presented by LFcin17–30 that shows a new peak at significantly lower fluorescence intensity (Fig. 4a, lower panel). This result suggests that this peptide induces aggregation. It is important to note that

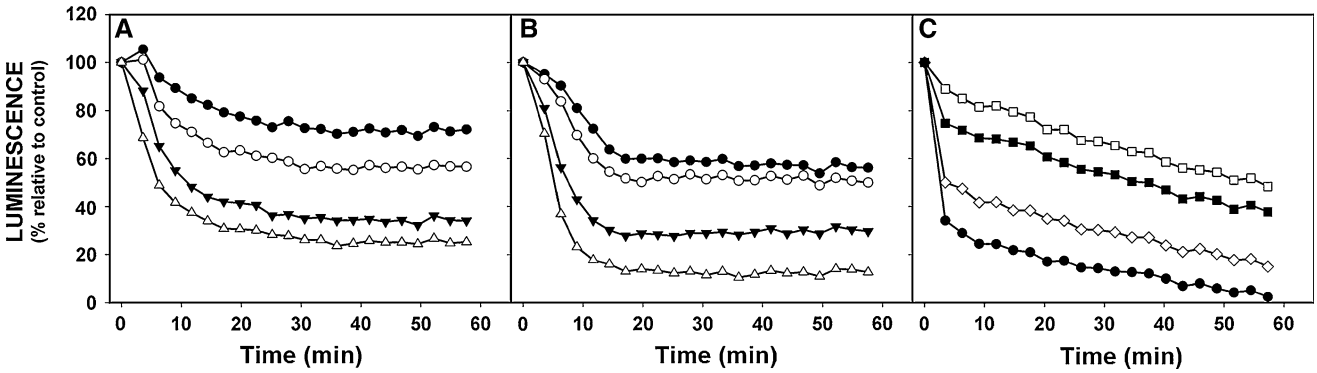

Fig. 3 Real-time decrease of luminescence in living *L. donovani* 3-Luc promastigotes induced by LF-peptides. Parasites expressing a cytoplasmic form of firefly luciferase were incubated with the free-membrane-permeable luciferase caged substrate DMNPE-D-luciferin (25 μM). Under these experimental conditions, free intracellular ATP is the limiting step for luminescence. Peptide was added at $t = 0$ (luminescence = 100 %). **a** LFcin17–30, **b** LFampin265–284 and **c** LFchimera. The results presented are of one representative experiment (out of two), and are shown as percentage relative to control parasites. Peptide concentrations (μM): 50 (*open triangle*); 35 (*filled inverted triangle*); 20 (*open circle*); 10 (*filled circle*); 5(*open diamond*); 2.5 (*filled square*); 1.25 (*open square*)

LFchimera (Fig. 4c, lower panel) showed a shift towards lower fluorescence at high peptide concentration, which may be due to displacement of the SYTOX Green previously bound to DNA by the polycationic peptide.

The accumulation of the peptides labelled with FITC (green) into parasites was monitored by confocal microscopy (Fig. 5). *L. donovani* promastigotes were incubated for 4 h with LFcin17–30-FITC, LFampin265–284-FITC and LFchimera-FITC, and PI (red) was added at the end of the experiment. For all peptides an identical result was observed, namely that all peptide-labelled cells were dead (red) (Fig. 5). Hence the peptides only enter cells when the plasma membrane is severely damaged. For LFcin17–30, cell aggregates were clearly seen (Fig. 5a) reinforcing the hypothesis raised above, based on cytofluorometry results (Fig. 4a, lower panel), that this peptide induces cellular aggregation.

Transmission electron microscopy (TEM) was used to identify morphological damages on the parasites after their incubation with LFchimera. Most of the *L. donovani* promastigotes incubated with 5 μM LFchimera (Fig. 6) showed cytoplasmic vacuolation, loss of definition of the organelles and a massive leakage of intracellular material (Fig. 6, dotted arrows). These results reinforce the idea that membrane damages are significant components of the peptide mechanism of action.

Leishmanicidal activity of LFchimera analogues can change significantly against *L. donovani* promastigotes and *L. pifanoi* amastigotes

Finally, we tested a set of LFchimera analogues in order to identify major traits for their activity. In these peptides, we varied the order of the constituent peptides (LFchimera-R), the total charge (Di-LFcin and Di-LFampin) and the linkage between the moieties (Table 1). Most analogues had an activity similar to that of LFchimera, but two of them, Di-LFcin and LFampin-LFcin, were more active, the latter against both forms of the parasite below 2 μM (Table 2). An interesting fact was that all peptides had similar activities on promastigotes and amastigotes, in contrast with LFchimera, which had a slightly lower activity against the amastigote form (Table 2).

Some preliminary conclusions can be drawn from this set of analogues: (1) dimerization of lactoferricin (Di-LFcin) improves the activity of the peptide; (2) linking LFcin17–30 and LFampin265–284 through an additional lysine is not crucial for the antimicrobial activity of the parental hybrid peptide, as the two analogues linked by a standard peptide bond showed similar (LFcin–LFampin) or even higher (LFampin–LFcin) activity than LFchimera (Table 2).

Discussion

One of the most successful strategies for the optimization of natural AMPs has been the design of hybrid analogues that adequately combine the active sequences of preexisting AMPs. Hybrid peptides derived from cecropins (Fink et al. 1989), cecropin and melittin (Boman et al. 1989; Merrifield et al. 1994), magainin (Guerrero et al. 2004; Shin et al. 1998) or temporin (Wade et al. 2002) have shown higher activity and/or specificity over the parental versions (Tachi et al. 2002). Less frequently, the notion has been extended to hybrids combining α- and β-peptide sequences (Schmitt et al. 2007), or even to highly structured peptides such as cysteine-stabilized hybrid defensins (Landon et al. 2008).

In LFchimera, a different strategy has been pursued: the topology of a cationic area in the N1 domain of bovine

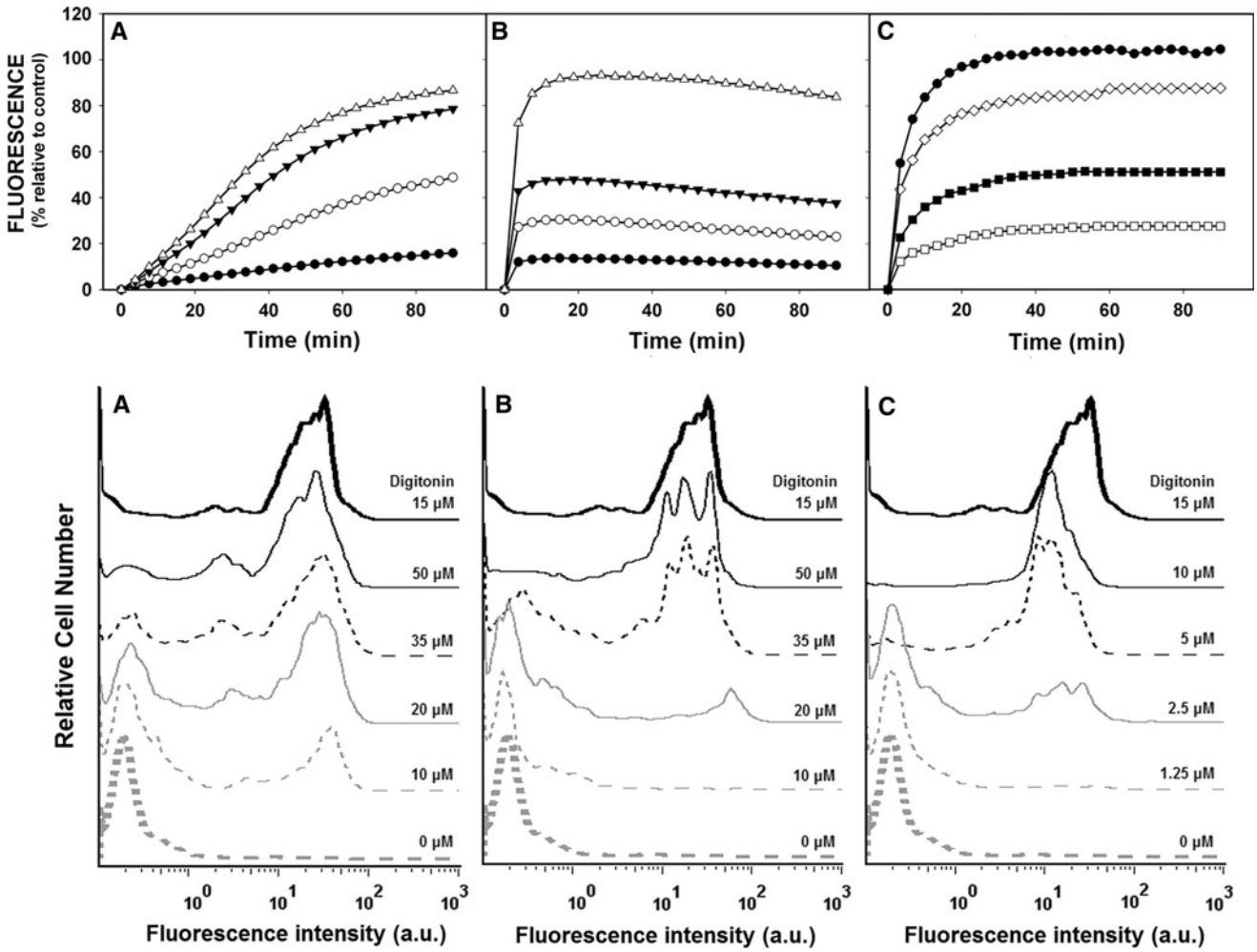

Fig. 4 Entrance of the vital dye SYTOX Green in *L. donovani* promastigotes induced by LF-peptides. *Upper panel* Kinetics of permeabilization after peptide addition ($t = 0$; fluorescence $= 0$) was measured by the increase in fluorescence due to dye binding to intracellular nucleic acids ($\lambda_{ex} = 504$ nm; $\lambda_{em} = 524$ nm). **a** LFcin17–30, **b** LFampin265–284 and **c** LFchimera. The results of one representative experiment (out of two) are shown, and expressed as percentage relative to fully permeabilized cells (with 0.1 % Triton X-100). Peptide concentrations (µM): 50 (*open triangle*); 35 (*filled inverted triangle*); 20 (*open circle*); 10 (*filled circle*); 5 (*open diamond*); 2.5 (*filled square*); 1.25 (*open square*). *Lower panel* Cell incorporation of SYTOX Green as assessed by cytofluorometry. Dye was added after 4 h incubation of the parasites with different concentrations of the peptides. Full permeabilization was achieved with 15 µM digitonin (*heavy solid black line*). Peptide concentration is shown above the corresponding line. **a** LFcin17–30, **b** LFampin265–284 and **c** LFchimera. The results of one representative experiment (out of three) are shown

lactoferrin was simulated by linking the active shortened sequences of lactoferricin (LFcin17–30) and lactoferrampin (LFampin265–284) through an additional lysine (Bolscher et al. 2009). The activity of this hybrid peptide on *Leishmania* largely surpasses that of its two component moieties, even when added simultaneously, similarly to what was found against other pathogens (Bolscher et al. 2009, 2012; Flores–Villaseñor et al. 2010; León-Sicairos et al. 2009; López-Soto et al. 2010). This effect is especially relevant for the amastigote, the pathological form of the parasite in vertebrates, against which LFampin265–284 and LFcin17–30 were inactive at the highest concentration tested (50 µM). The higher resistance of amastigotes to these three peptides compared to promastigotes is a trend followed by various AMPs in *Leishmania* (Fernández-Reyes et al. 2010; Guerrero et al. 2004).

To be an effective AMP, a peptide with the lowest possible cytotoxicity is desirable. Using haemolysis as a readout of cytotoxicity, LFcin17–30 and LFampin265–284 were shown to be non-toxic up to 100 µM in PBS, whereas LFchimera exhibited mild toxicity at 20 µM, a much higher concentration than its IC_{50} on *Leishmania* (León-Sicairos et al. 2009; van der Kraan et al. 2004, 2005a).

Our study of the mechanism of action has shown that disruption of the promastigote plasma membrane by the peptides can be clearly inferred from: (1) entrance of vital dyes such as SYTOX Green and PI into the parasites promoted by the peptides at the same range of

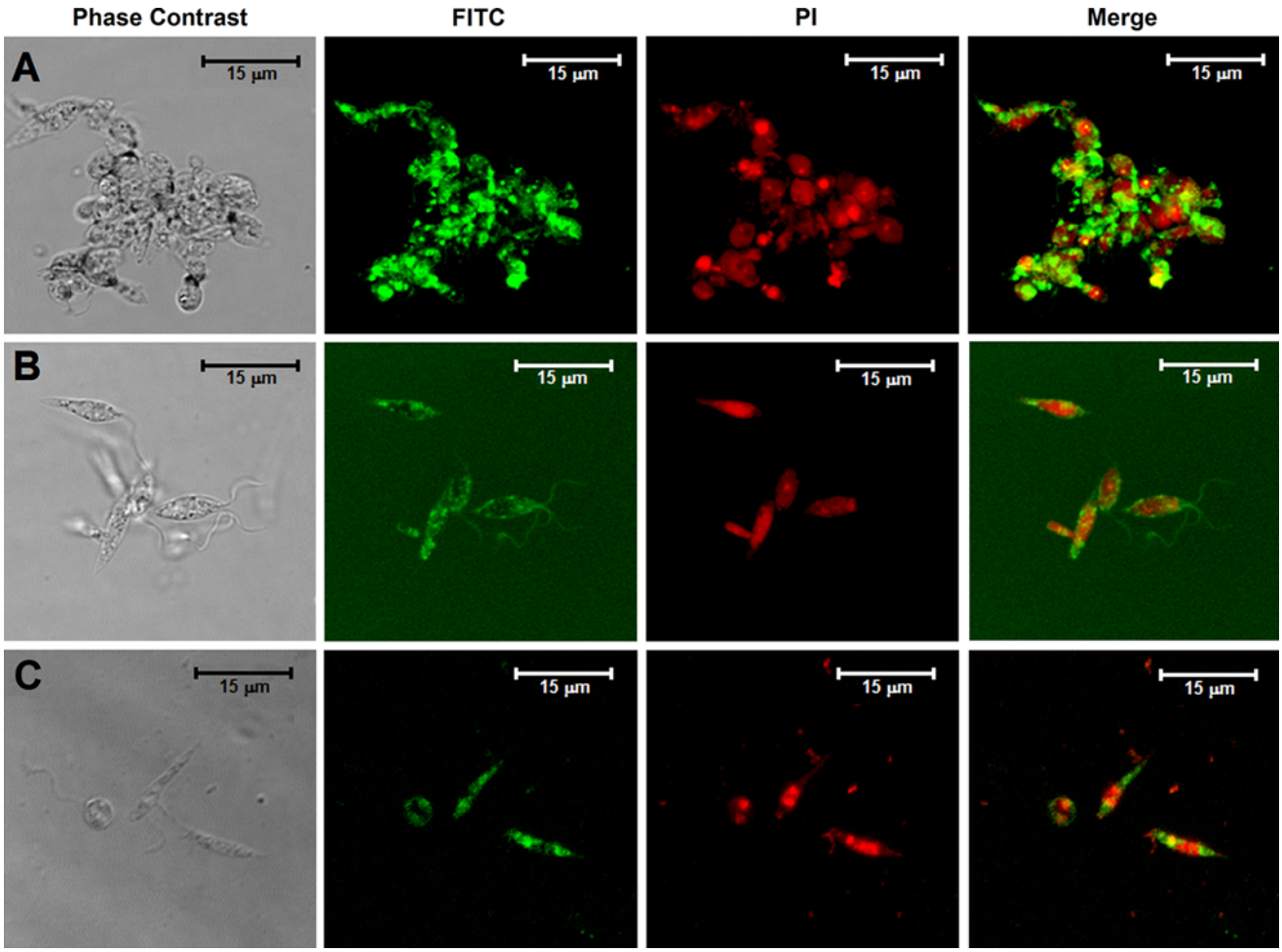

Fig. 5 Permeabilization of *L. donovani* promastigote plasma membrane by LF-peptides assessed by fluorescence microscopy. *L. donovani* promastigotes were incubated for 4 h with FITC-labelled LF-peptides (*green*) and PI (*red*) was added at the end of the incubation. Cells were photographed in a Leica TCS-SP2 ABOS confocal laser scanning microscope with final magnification of ×630, and representative pictures are shown. **a** 10 µM LFcin17–30-FITC, **b** 20 µM LFampin265–284-FITC and **c** 2.5 µM LFchimera-FITC (colour figure online)

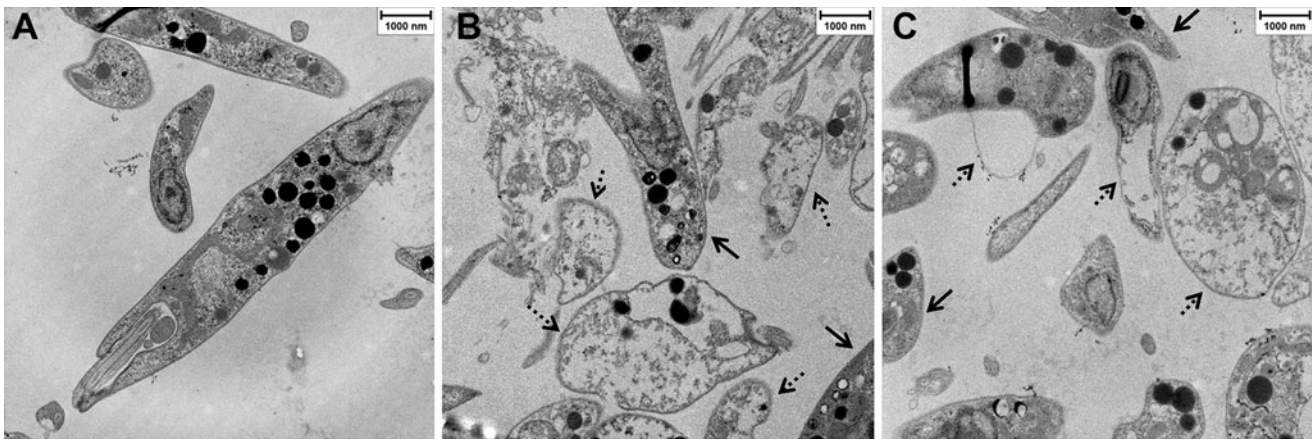

Fig. 6 Electron microscopy of *L. donovani* promastigotes treated with LFchimera. Parasites were incubated with the peptide for 4 h and were observed and photographed in a transmission electron microscope. Representative pictures are shown. **a** Control, **b**, **c** 5 µM LFchimera, two different views. *Solid arrows* intact cells, *dotted arrows* damaged cells

concentration where inhibition of proliferation is achieved; and (2) a fast drop in the intracellular levels of free ATP after peptide addition, assessed in real time and in vivo on 3-Luc promastigotes. For LFcin17–30, SYTOX Green uptake was considerably slower than luminescence decrease, whereas for LFampin265–284 and LFchimera both processes had similar rates. This may be rationalized if the bioenergetic collapse is due initially to loss of the ionic gradient leading to membrane depolarization, described for LFcin peptides on bacteria (Aguilera et al. 1999; Ulvatne et al. 2001), and only later to larger membrane damage, as required for SYTOX Green entrance. Besides membrane damage, the lethal mechanism for LFcin17–30 may also include intracellular targets, most likely mitochondria, the main ATP source in *Leishmania*, as found for histatin 5, an AMP inhibiting oxidative phosphorylation in this parasite (Luque-Ortega et al. 2008). LFcin was found to localize in the cytoplasm of *Staphylococcus aureus* and *Escherichia coli* (Haukland et al. 2001), and mitochondria was pointed out for the LFcin-induced apoptosis in tumoral cells; furthermore, the peptide was described as capable of permeabilizing not only the plasma membrane, but also the mitochondrial one in Jurkat cells in a later step (Mader et al. 2007). Confocal microscopy did not help us to fully elucidate the mechanism, as parasites treated with any of the three peptides showed a heavy incorporation of fluoresceinated peptide, and also propidium iodide labelling. Thus, although membrane permeabilization was proven, the possibility of intracellular targets cannot be discarded. On the other hand, intracellular presence of our LF-peptides in *Leishmania* could be just a consequence of membrane permeabilization, without implying the existence of internal targets.

Membrane-disrupting activity for this set of LF-peptides has been thoroughly addressed in negatively charged liposomes (Bolscher et al. 2009). Although caveats must be raised when comparing model and native membranes, the biophysical studies served to confirm the permeabilization capacity, with LFchimera as the most active peptide (Bolscher et al. 2009). Electron microscopy results suggest the possibility of an all or none permeabilization mechanism for LFchimera, as intact parasites coexist with severely damaged ones. This behaviour has also been described for magainins in *Leishmania* (Guerrero et al. 2004) and may be feasibly explained by a cooperative process of peptide insertion and/or pore formation once the membrane-bound peptide reaches a certain threshold value, as shown for other peptides (Bastos et al. 2008; Teixeira et al. 2010).

To elucidate which structural features of LFchimera make this peptide so active, as well as to optimize its leishmanicidal profile, a set of analogues were synthesized and assayed. Some preliminary conclusions can be drawn from our experiments. Switching the α- and ε-attachment of the moieties of the original LFchimera, as in the LFchimera-R analogue, did not produce a significant change in activity, while dimerization of lactoferricin (Di-LFcin) improved activity, most likely by the increase in positive charge (+14 vs. +12 of LFchimera and +10 of Di-LFampin, see Tables 1, 2). The attachment of both sequence moieties to the α and ε ends of a lysine residue, postulated to mimic the topology of such peptides in native LF, is not mandatory—linking both sequences by a simple α-peptide bond produced similar (LFcin–LFampin) or even higher (LFampin–LFcin) leishmanicidal activities than LFchimera. In recent NMR studies in chloroform/methanol/water mixed solvent, the special linking of both sequence stretches through the lysine residue was recently reported to increase the helical content of LFchimera versus the component sequences; yet, the study did not show that linkage through the lysine residue of LFchimera was able to maintain the spatial orientation of native LF (Haney et al. 2012). The higher activity of LFampin–LFcin, the most active analogue studied here, may similarly arise from an even stronger enhancement of secondary structure, a feature proven to be crucial for activity (Adao et al. 2011; Haney et al. 2009; van der Kraan et al. 2005a, 2006). Further research is needed to establish the secondary structure of these LFchimera analogues, as well as to uncover their mechanism of action and cytotoxicity.

The strong activity found for lactoferricin on different protozoan parasites (Omata et al. 2001; Turchany et al. 1995), as well as for LFchimera on *E. histolytica* (López-Soto et al. 2010), has been extended for LFchimera to *Leishmania* in the present work. Of particular note is the fact that both component moieties of LFchimera can be juxtaposed into a linear sequence with a strong improvement in activity over both amastigotes and promastigotes. Hence, the joint presence of both cryptopeptides into a single covalent structure, rather than their linkage through a lysine residue as initially assumed, appears to be crucial for the extremely high activity of the hybrids. This finding may open the way to potential therapeutical application of these peptides, since its structural simplicity would make large-scale production feasible by recombinant means.

Acknowledgments This work was supported by FCT through CIQ(UP) to MB and TS; the Spanish–Portugal cooperative project "Ação Integrada n° E-40/90" to TS, MB, LR, and DA; the ISCIII-Red de Investigación Cooperativa en Enfermedades Tropicales (RICET)-FEDER grants, RD 06/0021/0006, European Union grants HEALTH-2007-223414 to LR and DA, and FIS PS09-01928 to LR; by a grant from the University of Amsterdam for research into the focal point "Oral Infections and Inflammation" to JB and KN; and by MICINN grant SAF2011-24899 to DA.

Conflict of interest The authors declare that they have no conflict of interest.

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