Killing of *Mycobacterium avium* by Lactoferricin Peptides: Improved Activity of Arginine- and d-Amino-Acid-Containing Molecules

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*Mycobacterium avium* causes respiratory disease in susceptible individuals, as well as disseminated infections in immunocompromised hosts, being an important cause of morbidity and mortality among these populations. Current therapies consist of a combination of antibiotics taken for at least 6 months, with no more than 60% overall clinical success. Furthermore, mycobacterial antibiotic resistance is increasing worldwide, urging the need to develop novel classes of antimicrobial drugs. One potential and interesting alternative strategy is the use of antimicrobial peptides (AMP). These are present in almost all living organisms as part of their immune system, acting as a first barrier against invading pathogens. In this context, we investigated the effect of several lactoferrin-derived AMP against *M. avium*. Short peptide sequences from both human and bovine lactoferricins, namely, hLFcin1-11 and LFcin17-30, as well as variants obtained by specific amino acid substitutions, were evaluated. All tested peptides significantly inhibited the axenic growth of *M. avium*, the bovine peptides being more active than the human. Arginine residues were found to be crucial for the display of antimycobacterial activity, whereas the all-d-amino-acid analogue of the bovine sequence displayed the highest mycobactericidal activity. These findings reveal the promising potential of lactoferricins against mycobacteria, thus opening the way for further research on their development and use as a new weapon against mycobacterial infections.

The genus *Mycobacterium* includes several species capable of causing disease in humans and other animals. *Mycobacterium tuberculosis* is one of the most deadly human pathogens, killing 1 million to 2 million people every year (1), while *Mycobacterium avium* and *Mycobacterium intracellulare* are frequent opportunistic pathogens of immunosuppressed individuals and people with chronic respiratory distress (2, 3). Equipped with a complex lipid-rich cell envelope and adapted to proliferate inside the host’s macrophages, mycobacteria tend to cause persistent infections, which are difficult to cure, requiring long treatment regimens that rely on the combination of several drugs. The increasing emergence of drug-resistant strains of mycobacteria makes the treatment of these diseases even more challenging (1).

The vast array of antimicrobial peptides (AMP) produced by virtually all living organisms as natural barriers against infection is a new and promising source of potential antimicrobial weapons. Although they have little sequence homology, the vast majority of AMP contain a high proportion of hydrophobic and cationic amino acids, and when in contact with bacterial membranes, they adopt amphipathic structures (4). The fact that AMP are active against a wide variety of microorganisms, combined with the observation that both the L and D enantiomers are active, supports the notion that the target of these peptides is a general structure conserved across different bacterial species, such as the cytoplasmic membrane (5, 6). Thus, it is thought that electrostatic attraction is responsible for the initial approach between the cationic peptide and the negatively charged phospholipids usually present at the pathogen’s surface (4). Downstream events that lead to bacterial killing are not yet fully understood (4, 6). Acting in such a fundamental and conserved biological target as the bacterial membrane, AMP are believed to be less prone to the development of secondary microbial resistance than other types of antibiotics (4, 7). Therefore, a deeper understanding of the mechanisms of action of AMP, along with the establishment of structure-activity relationships, is an essential step toward the identification and development of more active molecules with improved therapeutic characteristics and possibly higher specificity toward pathogens of interest.

Lactoferricins are naturally occurring peptides, formed by the cleavage of the highly cationic N1 terminal domain of the iron-binding protein lactoferrin. Bovine lactoferricin is composed by 25 amino acids, corresponding to residues 17 to 41 in the native protein (5, 8), and has broad-spectrum antimicrobial activity (5, 9–15). A shorter version of bovine lactoferricin containing amino acids 17 to 30 (LFcin17-30) was found to have even higher antimicrobial activity (16). A human peptide with the first 11 amino acids from human lactoferrin (hLFcin1-11) was also demonstrated to be active against a great variety of bacteria, including antibiotic-resistant strains, and fungi, especially *Candida albicans* (17–21), and was already used in different preclinical and clinical trials, where its overall safety was proved (20, 22).

In the present work, we investigated the activity against *M. avium* of hLFcin1-11 and LFcin17-30, as well as peptides obtained...
from these by specific amino acid substitutions, to test the importance of various factors on the potency of AMP against this pathogen.

**MATERIALS AND METHODS**

**Peptides.** Human lactoferricins (hLFcin1-11 and hLFcin1-11 all K) (Table 1) were prepared as C-terminal amidates by standard Fmoc/ Bu [1-(9H-fluoren-9-yl)-methoxy carbonyl]/tert-butylation chemistry] solid-phase peptide synthesis on a Liberty1 microwave (MW) peptide synthesizer (CEM). Briefly, Fmoc-Rink-amide resin (NovaBiochem, Switzerland) was preswelled for 15 min in N,N-dimethylformamide (DMF) (VWR International, Portugal) and then transferred into the MW reaction vessel. The initial Fmoc deprotection step was carried out using 20% piperidine in DMF containing 0.1 M 1-hydroxybenzotriazole (HOBt) (Sigma-Aldrich, Germany) in two MW irradiation pulses: 30 s at 24 W plus 3 min at 28 W, with the temperature in both cases being no higher than 75°C. The Fmoc-protected C-terminal amino acid (Bachem, Switzerland) was then coupled to the resin, using 5 molar equivalents (eq) of the Fmoc-protected amino acid in DMF (0.2 M), 5 eq of 0.5 M N,N,N’,N’-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate/HOBt in DMF, and 10 eq of 2 M N-ethyl-N,N-disopropylamino (DIPEA) (Sigma-Aldrich, Germany) in N-methylpyrrolidone (NMP) (VWR International, Portugal); the coupling step was carried out for 5 min at 35–40°C MW irradiation, with a maximum temperature reaching 75°C. The remaining amino acids were sequentially coupled in the C → N direction by means of similar deprotection and coupling cycles, except for incorporation of the following: (i) Fmoc-Arg(Pbf)-OH, whose coupling was done in two steps, 25 min with no MW irradiation (room temperature), followed by 5 min of coupling at 25 W, and (ii) Fmoc-Cys(Tmt)-OH and Fmoc-Trp(Boc)-OH, both also coupled in two steps, first 2 min of coupling without MW irradiation (room temperature) and then 4 min of coupling at 25 W, with the maximum temperature reaching 50°C. Following completion of sequence assembly, the resin were released from the resin with the concomitant removal of side chain protecting groups, by a 3-h acidolysis at room temperature using a trifluoroacetic acid (TFA) (VWR International, Portugal)-based cocktail containing triisopropylsilane (TIS) (Sigma-Aldrich, Germany) and water as scavengers (TFA-TIS-H2O, 95:2.5:2.5 [vol/ vol/ vol]). Crude products were purified by reverse-phase (RP) liquid chromatography on a Vydac C18 column (238TPB1520; Vydac, Hesperia, CA, USA) to a purity of at least 95%, as confirmed by high-performance liquid chromatography (HPLC) analysis on a Hitachi-Merck LaChrom Elite system equipped with a quaternary pump, a thermostated (Peltier effect)-automated sampler, and a diode-array detector (DAD). Pure peptides were quantified by UV absorption spectroscopy (Helios Gama, Spectronic Unicam), and their molecular weights were confirmed to be as expected by electrospray ionization/ion trap mass spectroscopy (ESI/IT MS) (LCQ-DecaXP LC-MS system; ThermoFinnigan).

Bovine lactoferricins (LFcin17-30, D-LFcin17-30, LFcin17-30 all R, and LFcin17-30 all K) (Table 1) were synthesized by solid-phase peptide synthesis using Fmoc-protected amino acids (Oregan Pharma GmbH, Heidelberg, Germany) in a Syro II synthesizer (Biotage, Uppsala, Sweden) as described previously (24). Peptides were purified to a purity of at least 95% by semipreparative RP-HPLC (Isco Corporation Tokyo, Japan) on a Vydac C8 column (218SM10; Vydac, Hesperia, CA, USA), and the authenticity of the peptides was confirmed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry on a Microflex LRF mass spectrometer equipped with an additional gridless reflectron (Bruker Daltonik, Bremen, Germany) as described previously (25).

All purified peptides were freeze-dried, after which peptide stock solutions were prepared in phosphate-buffered saline (PBS) (pH = 7.4) and stored at −20°C until use.

**Bacteria.** The strains of Mycobacterium avium used in this study were clinical isolates. Strain 2447 smooth transparent variant (SmT) was provided by F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium); strains 2-151, either SmT or SmD (smooth-domed), was provided by J. Belisle (Colorado State University, Fort Collins, CO, USA).

Mycobacteria were grown to mid-log phase in Middlebrook 7H9 medium (Difco, Sparks, MD) containing 0.05% of Tween 80 (Sigma, St. Louis, MO) and 10% of ADC supplement (albumin-dextrose-catalase) at 37°C. Bacteria were harvested by centrifugation, washed twice with saline containing 0.05% Tween 80, resuspended in the same solution, and briefly sonicated in order to disrupt bacterial clumps. The suspension was stored in aliquots at −80°C until use. Just before use, an aliquot was quickly thawed and diluted to the appropriate concentration.

**Effect of antimicrobial peptides on the viability of M. avium in axenic cultures.** M. avium was grown in Middlebrook 7H9 medium to exponential phase (bacterial growth was monitored by measuring the optical density at 600 nm). Bacteria were seeded at 10^6 CFU per well into 96-well flat bottom plates and incubated with the peptides in a final volume of 200 μl. Each condition was tested in triplicate. The plates were incubated at 37°C in a humid atmosphere. After 1, 2, 4, or 7 days of incubation, bacterial viability was measured by a CFU assay. A bacterial suspension from each well was serially diluted in water containing 0.05% Tween 80. The dilutions were plated in Middlebrook 7H10 agar medium (Difco, Sparks, MD) and supplemented with OADC (oleic acid-albumin-dextrose-catalase), and the colonies were counted after 7 days at 37°C.

**TABLE 1 Antimicrobial activities of lactoferricin peptides against Mycobacterium avium**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Mol wt (kDa)</th>
<th>Charge</th>
<th>2447 SmT IC90</th>
<th>2447 SmT IC50</th>
<th>2-151 SmT IC90</th>
<th>2-151 SmT IC50</th>
<th>2-151 SmD IC90</th>
<th>2-151 SmD IC50</th>
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<tbody>
<tr>
<td>hLFcin1-11</td>
<td>GRRRRSVQVCA</td>
<td>1,384</td>
<td>+5</td>
<td>15.8 ± 4.5</td>
<td>34.6 ± 22.4</td>
<td>11.0 ± 4.1</td>
<td>65.8 ± 19.3</td>
<td>15.2 ± 2.9</td>
<td>37.9 ± 15.9</td>
</tr>
<tr>
<td>hLFcin1-11 all K</td>
<td>GKKKKSQVQCA</td>
<td>1,262</td>
<td>+5</td>
<td>39.1 ± 6.9*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LFcin17-30</td>
<td>FKKRRQWQRMKKLG</td>
<td>1,923</td>
<td>+6</td>
<td>14.2 ± 1.5</td>
<td>18.9 ± 4.0</td>
<td>8.0 ± 1.5</td>
<td>22.8 ± 9.1</td>
<td>12.4 ± 0.3</td>
<td>21.5 ± 4.0</td>
</tr>
<tr>
<td>D-LFcin17-30</td>
<td>FKKRRQWQRMKKLG</td>
<td>1,923</td>
<td>+6</td>
<td>10.7 ± 0.9*</td>
<td>14.4 ± 1.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LFcin17-30 all K</td>
<td>FKKKQWQKMKKLG</td>
<td>1,839</td>
<td>+6</td>
<td>18.0 ± 2.1**</td>
<td>34.4 ± 8.2**</td>
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<tr>
<td>LFcin17-30 all R</td>
<td>FRRQQWQRMRLG</td>
<td>2,007</td>
<td>+6</td>
<td>10.8 ± 1.6*</td>
<td>19.3 ± 4.8</td>
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</tbody>
</table>

* Molar weight.
* Calculated overall charge at pH 7.0.
* Each value represents the average for at least three independent experiments, with the corresponding SD indicated. IC50 is the peptide concentration that inhibits 50% of mycobacterial viability at 7 days of incubation. IC90 is the peptide concentration that inhibits 90% of mycobacterial viability at 7 days of incubation.

<table>
<thead>
<tr>
<th>Charge</th>
<th>2447 SmT IC90</th>
<th>2447 SmT IC50</th>
<th>2-151 SmT IC90</th>
<th>2-151 SmT IC50</th>
<th>2-151 SmD IC90</th>
<th>2-151 SmD IC50</th>
</tr>
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<tr>
<td>4.5</td>
<td>34.6 ± 22.4</td>
<td>11.0 ± 4.1</td>
<td>65.8 ± 19.3</td>
<td>15.2 ± 2.9</td>
<td>37.9 ± 15.9</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>18.9 ± 4.0</td>
<td>8.0 ± 1.5</td>
<td>22.8 ± 9.1</td>
<td>12.4 ± 0.3</td>
<td>21.5 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>10.7 ± 0.9*</td>
<td>14.4 ± 1.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.9</td>
<td>18.0 ± 2.1**</td>
<td>34.4 ± 8.2**</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>10.8 ± 1.6*</td>
<td>19.3 ± 4.8</td>
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</table>

SD) against M. avium strain.

DIC, 0.05 (compared to results for LFcin17-30 all R, by cross-comparison within the bovine peptides).
difference, in terms of log_{10} CFU/ml, between the first and the last days of incubation was designated “log increase.”

IC_{50} and IC_{90} (peptide concentration that inhibits 50% or 90% of mycobacterial growth after 7 days of incubation) values were obtained by fitting CFU data through a four-parameter dose-response sigmoidal curve using the GraphPad Prism software program (GraphPad Software Inc., La Jolla, CA). The reported final values correspond to the average of at least three independent experiments, with the corresponding standard deviation (SD) indicated.

Statistical analysis was performed with GraphPad Prism software using one-way analysis of variance (ANOVA) with adjustment for multiple comparisons in two ways: (i) comparing each value to a control (the corresponding parental peptide) and (ii) performing cross-comparisons among all peptides (within each family). The statistical significance, adjusted P value, and confidence intervals were obtained through the Bonferroni method for a chosen 95% significance.

SEM. *M. avium* 2447 SmT was grown in Middlebrook 7H9 medium to exponential phase. Approximately 10^6 CFU of bacteria were incubated with the peptides (25 μM) for 2 days at 37°C. After incubation, cytopsin centrifugation (1,000 rpm, 2 min) (Cytopsin 4; Thermo Scientific) was used to concentrate the bacteria in glass coverslips previously coated with APES (3-aminopropyltriethoxysilane) to promote bacterial adherence. Bacteria were fixed with 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (supplemented with 5 mM MgCl2 and 5 mM CaCl2; pH = 7.2) for 2 h, followed by postfixation with 1% osmium tetroxide (OsO4) in 0.1 M sodium cacodylate buffer (1 h). Bacteria were then dehydrated by successive incubations with increasing concentrations of ethanol (10 to 100%), dried in a critical point dryer (CPD7501; Polaron), and sputter coated with a gold/palladium thin film (50 s with a 15-mA current) using SPI Module sputter coater equipment. The scanning electron microscopy (SEM) exam was performed using a high-resolution (Schottky) environmental scanning electron microscope with X-ray microanalysis and electron backscattered diffraction analysis: Quanta 400 FEG ESEM/EDAX Genesis X4M.

**RESULTS**

Antimycobacterial activity of lactoferricin peptides. Human and bovine lactoferricin peptides (hLFcin1-11 and LFcin17-30) were evaluated for their antimicrobial activities against three different strains of *M. avium* (2-151 SmT, 2447 SmT, and 2-151 SmD) (28). Both peptides were active against the three strains in axenic cultures, with an IC_{50} of ≤15 μM (Table 1). The bovine peptide tended to be more active than the human one, exhibiting lower IC_{50} values for all *M. avium* strains. The activities of each peptide were similar between the *M. avium* strains (Table 1).

Arginine residues were reported to be critical for the antimicrobial activity of lactoferricins (5, 8, 29). hLFcin1-11 has four arginine residues, and LFcin17-30 has three (Table 1). In order to investigate whether these residues were important for the display of antimycobacterial activity, we synthesized and tested lactoferricin analogues in which arginine residues were replaced by lysine (all-K variants). Lysine was chosen because it has the same charge as arginine, and hence any differences in activity would not be attributable to the alteration of overall positive charge. Since bovine LFcin17-30 has three lysine residues, we also tested a variant of this peptide in which all lysines were replaced by arginines (all-R variant).

When tested against *M. avium* 2447 SmT in axenic culture, the all-K variants of each peptide were significantly less active than the corresponding original peptide (Table 1) (*P < 0.0001 for IC_{50} for human peptides; P = 0.0329 for IC_{50} and P = 0.0074 for IC_{90} for bovine peptides). Conversely, the all-R variant of LFcin17-30 was slightly more active than the original peptide (Table 1) (for IC_{50}, P = 0.0377). Of note, even in the all-K variants, the bovine peptide exhibited a stronger antimicrobial activity than the human peptide (*P < 0.0001) (Table 1).

Given that one of the limitations of therapeutic applications of peptides is their sensitivity to proteolysis, we thought it would be interesting to know whether replacement of all native amino acids in LFcin17-30 by their d enantiomers (less prone to degradation) would preserve the peptide’s activity against *M. avium*. Remarkably, the d-LFcin17-30 sequence (D-LFcin17-30) was found to be significantly more active than the corresponding native peptide (Table 1) (for IC_{50}, P = 0.0455).

We observed in all cases an abrupt decrease in mycobacterial viability at peptide concentrations around 12.5 μM, which translated into very close values for IC_{50} and IC_{90} (Table 1). This suggested that a critical concentration threshold may exist for the peptides to exert their activity. To test this hypothesis, we assessed the effect of the human and bovine parental peptides, hLFcin1-11 and LFcin17-30, on *M. avium* 2447 SmT using narrower concentration intervals in the 12.5 to 25 μM range. As shown in Fig. 1, this experiment confirmed the existence of a threshold concentration and further showed that the dose-effect correlation is different for the two peptides. For hLFcin1-11, there is a gradual decrease in the viability of *M. avium* as the peptide concentration increases, whereas for LFcin17-30, there is a critical inhibitory concentration above which the increase in the peptide concentration has no further effect on mycobacterial viability.

To assess in detail the time dependence of the action of lactoferricin peptides against *M. avium* 2447 SmT, the antimicrobial activity was determined by CFU at the end of 1, 2, and 4 days of incubation.
incubation. No significant effects were seen up to 1 day. All peptides showed significant inhibitory activity after 2 days of incubation at 12.5 μM (Fig. 2) and 25 μM (not shown). For the most active peptides (hLFcin1-11, hLFcin17-30, d-LFcin17-30, and LFcin17-30 all R), mycobacterial death was observed at 4 days (Fig. 2).

Similar to what was observed at 7 days of incubation, at shorter times the bovine peptides tended to be more active than the human ones, and the peptides with arginines (human or bovine) were more active than those with lysines.

**Surface and ultrastructural alterations in *M. avium* induced by lactoferricins.** To gain a better understanding of the mechanisms of antimicrobial action of lactoferricin peptides against *M. avium*, we performed scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to identify surface and ultrastructural alterations in the bacteria upon peptide treatment. After 2 days of incubation with lactoferricins at 25 μM, SEM analysis of *M. avium* 2447 SmT revealed overall shape deformations (Fig. 3, left panel, arrow), increased roughness of the bacterial surface, and the accumulation of biological material outside the cells (Fig. 3, left panel, arrowhead), this last suggesting leakage of intracellular content. Ultrastructural alterations were also seen using TEM, namely, condensation of cytoplasmic dense material, such as proteins (Fig. 3, right panel, empty arrow), once again with the suggestion of leakage of intracellular material, indicated by accumulation of material extracellularly (Fig. 3; right panel, arrowhead), as well as translucent cytoplasm and a high number of “ghost” cells (Fig. 3, right panel, asterisk). In agreement with the previous results for activity, the bovine peptides induced more severe structural alterations in the bacteria than the human ones, especially d-LFcin17-30 and LFcin17-30 all R (Fig. 3).

**DISCUSSION**

Considering the potential of AMP as new alternative therapies to fight infectious diseases and the urgent need to develop more efficient therapies for infections caused by mycobacteria, we tested several lactoferricin peptides against *Mycobacterium avium*. Aside from the antimicrobial activity, toxicity is a fundamental parameter in possible future use. hLFcin1-11 has already been used in different preclinical and clinical trials, where its overall safety was proved (20, 22), and LFcin17-30 has been shown to be nontoxic for erythrocytes and rat hepatocytes up to a 50 μM concentration (30). In the present work, we found that both the human and bovine lactoferricins (hLFcin1-11 and LFcin17-30, respectively) were active against *M. avium* strains of different virulences, the bovine peptide being more active than the human one.

AMP vary widely in many aspects, such as length, sequence, structure, and source, but they share important common traits, such as a positive charge, presumed to be fundamental for interaction with the negatively charged surface of pathogen cells (4, 6), and amphipathicity, which enables better interaction with the hydrophobic part of the microbial membrane, leading to its disruption (31, 32). Arginine residues were previously suggested to be important for the antimicrobial activity of lactoferricins (5, 8, 29) and also to potentiate the internalization of peptides (33–35). Therefore, we studied lactoferricin variants with arginines replaced by lysines and vice versa. As shown in Table 1 and Fig. 2, all peptides, human and bovine, with all arginines replaced by lysines were less active against *M. avium*, whereas when lysines were replaced by arginines, the peptides were more active. This shows that arginine residues are crucial for the antimicrobial activity of these peptides against *M. avium*. The contribution of arginine for activity is probably not charge related, since it bears the same charge as lysine (+1). The higher activities of peptides with arginine residues could be the result of the presence of the bulky guanidinium group on the side chain, enabling a better interaction with the membranes, since multiple hydrogen bonds can in this case be formed with the lipid headgroups around, whereas lysines can interact with only one (5, 8, 33, 35, 36). Arginine can also establish stronger cation–π interactions, which occur mainly between aromatic residues (e.g., tryptophan) and residues with positively charged side chains (e.g., arginines and lysines), that could allow the peptides to penetrate deeper into the membrane (8, 33, 35, 36). Moreover, arginines can form hydrogen bonds while establishing cation–π interactions, with tryptophan, for instance, whereas lysines cannot (8, 33, 35, 36). All these factors combined may have contributed to a more efficient activity against mycobacteria (5, 8, 29, 33, 36).

Due to the large diversity of AMP and of their properties, there is most probably no universal mechanism for their action. In this work, we have contributed to the elucidation of the mechanism of action of lactoferricins against *M. avium*. Our observation that the d enantiomer of LFcin17-30 is more active than the l form suggests that antimycobacterial activity is not dependent on chiral centers, such as specific protein receptors, which is in agreement with previous observations made for lactoferricin and other AMP, such as magainin, cecropin, melittin, and protegrin, against other pathogens (5, 6, 37–39). One of the major drawbacks of the use of AMP in the clinic is their susceptibility to proteases and other plasma components, resulting in low metabolic stability and bioavailability (40). One way to overcome this problem is to use d peptides, which are more resistant to protease activity (32, 41). This seems to be the case for LFcin17-30, which exhibited a higher activity against *M. avium* with all d-amino acids, indicating that
This can be used advantageously in comparison to the L enantiomer.

The concentration-effect curves obtained for the antimicrobial activities of lactoferricins did not show a gradual decrease. Around 12.5 μM, there was a sharp drop in mycobacterial viability, more pronounced in the case of the bovine peptide, reflected in very close values for IC_{50} and IC_{90}. This observation is in line with some models of AMP activity that predict the existence of a threshold concentration for their antimicrobial activity (4).

*M. avium* is a slow-growing mycobacterium, and thus we used 7 days of culture to determine lactoferricin activity. However, previous reports showed that LFcin17-30 is able to exert antimicrobial activity against other pathogens in about 1 h (24, 42–44). In order to evaluate the kinetics of the activity of lactoferricins against *M. avium*, we performed several assays with shorter culture times. We did not observe any significant decrease in the number of viable mycobacterial cells in less than 2 days of incubation, with peptide concentrations up to 25 μM (data not shown). This “delay” in activity against *M. avium* compared to that against other pathogens can be explained by the particular characteristics of the mycobacterial cell wall, as well as an intrinsic low rate of proliferation. *Mycobacterium* species are characterized by a complex hydrophobic envelope of extremely low fluidity and high impermeability, which contributes to the capacity of the pathogen to survive inside the host and resist chemotherapy (45, 46). Furthermore, the possibility of an internal target for the action of these peptides against *M. avium* cannot be discarded. In fact, there are numerous evidences in the literature pointing to the probable existence of an internal target for lactoferricins (5, 47–49).

Finally, electron microscopy studies showed that all lactoferricin peptides tested induced significant changes both in the surface and in the ultrastructural organization of the mycobacterial cells. In agreement with the bacterial viability assays, D-LFcin17-30 and the all-R variant showed the most drastic effects on the mycobacterial morphostructure. However, these studies did not allow us to obtain definitive information on what is the target (or targets) of the peptides. The observed accumulation of biological material outside the cells, seen by both SEM and TEM, and the presence of translucent cytoplasm and a high number of "ghost" cells (TEM) indicate leakage and are compatible with cell membrane permeabilization. Clearer evidences that the peptides act by permeabilization, such as the appearance of vesicular budding and disappearance of the cell wall or membrane, were not observed. A mechanism of action that includes crossing over the bacterial surface and acting on internal targets has been proposed for bovine lactoferricins. The higher activity of lactoferricins containing arginines also supports this hypothesis, since this amino acid is often correlated with peptides that exert their activity on internal targets (33–35), as opposed to lysines, which are usually described as crucial.

**FIG 3** Surface and ultrastructural alterations in *M. avium* induced by lactoferricins at 2 days of incubation. *M. avium* 2447 SmT was incubated with hLFcin1-11, hLFcin1-11 all K, LFcin17-30, D-LFcin17-30, LFcin17-30 all K, or LFcin17-30 all R at 25 μM for 2 days. Cells were observed and photographed in a scanning electron microscope (left panels) and in a transmission electron microscope (right panels). Representative pictures are shown. Bar = 1 μm (left panels) or 0.5 μm (right panels). Symbols: arrow, shape deformations; arrowhead, increased roughness and accumulation of intracellular material outside; empty arrow, condensation of cytoplasmic material; asterisk, translucent cytoplasm and ghost cells.
cial for membrane-lytic activities. In recent studies, we have shown that LFcin17–30 has a mild effect on model membranes, acting by lipid segregation instead of leading to full membrane disruption, as observed for other membrane-active peptides (50). Further, Haukland et al. (47) showed that lactoferricin is found in the cytoplasm of *Staphylococcus aureus* and *Escherichia coli*, and Ulvate et al. (49) showed that it inhibits the macromolecular synthesis (DNA, RNA, and protein synthesis) of *E. coli* and *Bacillus subtilis*. The alterations seen suggest that the mechanism of action of the studied lactoferricins against mycobacteria is not confined to the cell wall or membrane but probably also includes action on internal targets, leading to impairment of several intra-cellular processes, such as DNA replication, DNA, RNA, and protein synthesis, protein folding, etc., which eventually culminates in cell death.

In summary, key molecular features of lactoferricin-based peptides for the display of antimycobacterial activity were identified, contributing to the understanding of their mechanism of action against mycobacteria. Hence, this work demonstrates that lactoferricin-related AMP are promising molecules for development of clinically useful weapons to treat infections caused by mycobacteria.

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**REFERENCES**


