Activity of antimicrobial peptides and synergy with enrofloxacin against Mycoplasma pulmonis

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ABSTRACT

We have shown in a previous study that associations of antimicrobial peptides (AMPs), which are key components of the innate immune system of all living species, with the fluoroquinolone enrofloxacin can successfully cure HeLa cell cultures of \textit{Mycoplasma fermentans} and \textit{M. hyorhinis} contaminations. In the present work, the in vitro susceptibility of \textit{M. pulmonis}, a murine pathogen, to enrofloxacin and four AMPs (alamethicin, globomycin, gramicidin S and surfactin) was investigated with special reference to synergistic associations and the effect of mycoplasma cell concentration. Enrofloxacin and globomycin, displayed the lower MICs (0.4 \(\mu\)M) followed by gramicidin S (3.12 \(\mu\)M), alamethicin (6.25 \(\mu\)M) and surfactin (25 \(\mu\)M). When varying the mycoplasma cell concentration from \(10^4\) to \(10^8\) CFU/ml, the MICs of enrofloxacin and globomycin increased whilst those of the three other molecules remained essentially constant. The MBC of enrofloxacin (0.8 \(\mu\)M) was also lower than those of the peptides (6.25-100 \(\mu\)M) but the latter killed the mycoplasma cells much faster than enrofloxacin (2 hours vs. one day). The use of the AMPs in association with enrofloxacin revealed synergistic effects with alamethicin and surfactin. Interestingly, the mycoplasma-killing activities of the two combinations enrofloxacin (MIC/2) + alamethicin (MIC/4) and enrofloxacin (MIC/2) + surfactin (MIC/16) were about two orders of magnitude higher than those of these three molecules used separately. These results support the interest devoted to AMPs as a novel class of antimicrobial agents and pinpoint their ability of potentiating the activity of conventional antibiotics such as fluoroquinolones.
INTRODUCTION

The continuous emergence of clinical bacterial strains resistant to one or several conventional antibiotics has become a major medical and veterinary problem in the last decade and has urged the search of novel anti-infective agents. Among the compounds currently under investigation for their therapeutic potential are several antimicrobial peptides (AMPs), with a number of them reaching the phase of clinical evaluation and marketing (for review see 1, 6, 19, and 27). Some of these AMPs are natural components of the defence system of living organisms, from bacteria to plants and animals (6, 8, and 16). These peptides are usually amphipathic and cationic, and their size ranges generally from 6 to 40 amino acid residues. Although they are very diverse in structure, origin and biosynthesis mode, they share a high affinity for membranes that are, for most of them, the main cell target (for review, see 7 and 27). In spite of the growing wealth of information on AMPs, many issues are still unanswered, particularly those concerning their mechanism of action and the factors responsible for their efficacy and antimicrobial spectrum. In vitro, the action of an antibiotic against a given bacterium depends on many factors including (i) the chemical nature of the molecule, (ii) the antibiotic concentration, (iii) the bacterial density, (iv) the time of incubation, and (v) the presence or absence of another antibiotic. In this study, the influence of these factors on the antibacterial activity of selected AMPs was first investigated and compared to the activity of a conventional antibiotic, enrofloxacin (EFX). Moreover, as combinations of drugs may enhance antibacterial activity and hamper resistance development, the association of AMPs with EFX was also analyzed.

This work was focused on mycoplasmas (class Mollicutes) which include pathogens causing persistent and chronic respiratory, arthritic and urogenital diseases in humans and many animal hosts (5, 15, 17). These minimalist bacteria (9) are characterized by the simplicity of their cell envelope that lacks a cell wall and other components (LPS, outer
membrane, teichoic acids) found in Gram-positive or in Gram-negative bacteria. This simplicity explains why mycoplasmas are attractive organisms for the study of antibiotics such as AMPs which primarily target the plasma membrane. It also explains why the number of antibiotics that can be used for treating mycoplasmal infections is limited to tetracyclines, the macrolide-lincosamide-streptogramine-ketolide group, fluoroquinolones and phenicols (2). The present work focuses on *Mycoplasma pulmonis* which is the etiologic agent of respiratory mycoplasmosis in rodents. This pathogen is considered a very useful model for studying mycoplasmal respiratory infections, including those commonly caused by *Mycoplasma pneumoniae* in humans (17).

More specifically, we investigated (i) the influence of antibiotic concentration, bacterial density, and incubation time on the antimycoplasmal activities of four AMPs (alamethicin, gramicidin S, globomycin, and surfactin) and (ii) the benefits of combining the latter with enrofloxacin (EFX), a fluoroquinolone widely used in veterinary medicine and having a potent activity against mycoplasmas (for review see 2). Among the four AMPs mentioned above, alamethicin is a 20-residue amphipathic peptide produced by the fungus *Trichoderma viride* and member of the petaibol family (for review see 25). Alamethicin helices form transmembrane, voltage-dependent pores by aggregation in lipid bilayers (for review see 12, 18). The other AMPs are of bacterial origin and share a cyclic structure. While gramicidin S is a decapeptide (for review see 24) surfactin is a lipooctapeptide acting as a tensioactive on bacterial membranes (for review see 22) and globomycin a lipopentapeptide that specifically inhibits the bacterial signal peptidase II (11, 22) These four AMPs were chosen for their structural diversity, their known antimycoplasmal activity (4, 14) and because additive effects with EFX on mycoplasmas have already been observed in a tissue cell culture model system (20).
MATERIALS AND METHODS

Bacterial strain and culture medium

The *M. pulmonis* MpUR1.1 clone was obtained by randomly picking a colony from a culture of *M. pulmonis* UAB CTIP isolate as previously described (14). The mycoplasmas were grown in Hayflick medium containing 5% horse serum. For agar plates, noble agar (DIFCO) at a final concentration of 1% (wt/vol) was added.

Antimicrobial agents

Alamethicin, gramicidin S, surfactin, chloramphenicol, tylosine, mitomycin C and tetracycline were of commercial origin (Sigma), whereas globomycin was a gift from Dr. S. Miyakoshi (Sankyo Co., Japan). Streptomycin, and enrofloxacin (EFX) were obtained from Laboratoires Diamant (Paris, France) and Bayer AG (Leverkusen, Germany) respectively.

Determination of the MICs and the MBCs

The antimycoplasmal activities of antibiotics were determined as described previously (4, 14). Briefly, mycoplasmas at an initial concentration of $10^6$ CFU/ml were grown in Hayflick medium containing 5% horse serum in 96-well plates in the presence of twofold serial dilutions of the antibiotics. The drug concentrations ranged from 0.4 to 100 µM. A change in the colour of the phenol red added to the medium as a pH indicator was used to monitor bacterial growth. The MIC was defined as the lowest antibiotic concentration that completely inhibited the growth of mycoplasmas after 48 h. For MBC determinations, cells were incubated in the presence of AMPs for 2 hours and EFX for 24 hours and then plated on solid culture medium. The plates were incubated at 37°C under anaerobic conditions and examined after 4-5 days for the appearance of colonies. The MBC was defined as the lowest antibiotic concentration that killed $\geq 99.9\%$ of the cells. Experiments were performed in triplicate.
**Checkerboard titration for antibiotic combination studies.**

Antibiotic combinations were tested by the checkerboard titration method using 96-well microtiter plates. The drug concentrations ranged 0.4 to 50 µM, 0.1 to 1.25 µM, 0.05 to 6.25 µM, 0.012 to 1.6 µM and 0.006 to 0.8 µM for surfactin, alamethicin, gramicidin S, globomycin and EFX, respectively. The fractional inhibitory concentration (FIC) index for combinations of two antimicrobials was calculated according to the equation: FIC index = \( \text{FIC}_A + \text{FIC}_B = (A/\text{MIC}_A) + (B/\text{MIC}_B) \), where A and B are the MICs of drug A and drug B in the combination, \( \text{MIC}_A \) and \( \text{MIC}_B \) are the MICs of drug A and drug B alone. The FIC indexes were interpreted as follows: \( \leq 0.5 \), synergy; \( > 0.5-4 \), indifference; \( > 4 \), antagonism (13).

Experiments were performed in duplicate.

**Time killing assay**

Aliquots of exponentially growing mycoplasmas (2.5 x 10^8 CFU/ml in Hayflick medium) were resuspended in fresh Hayflick broth at approximately 10^8, 10^6 and 10^4 CFU/ml and exposed to antimicrobial agents, alone at 1x MIC for 1, 2 or 24 hours at 37°C. Then, 0.1 ml samples were serially diluted in Hayflick medium and plated onto agar plates to obtain viable colonies. The control experiment consisted in plating cultures of MpUR1.1 without antibiotics. For combinations studies, antimicrobial agents were used at their MIC or at concentrations equal to MIC/2 for EFX, MIC/4 for alamethicin and MIC/16 for surfactin according to checkerboard titration results. Experiments were performed in duplicate.
RESULTS

Effect of mycoplasma concentration on antibacterial activity of AMPs

The in vitro efficacy of EFX and the AMPs against *M. pulmonis* was first evaluated by measuring their growth inhibition and killing activity. MICs of AMPs and of EFX, determined at standard mycoplasma concentrations (10^6 CFU/ml), ranged from 0.4 to 25 µM (Table 1). Specifically, the following order of decreasing activity was recorded: EFX and globomycin (0.4 µM) > gramicidin S (3.12 µM) > alamethicin (6.25 µM) > surfactin (25 µM). The MBCs were determined in the same conditions (Table 1). For the selected antibiotics, the ratio MBC/MIC was in the range from 2 to 4, except for globomycin for which it was higher (MBC/MIC = 31).

Having defined the susceptibility of *M. pulmonis* for these antibiotics in standard conditions, different factors likely to affect this efficacy were changed. The first that was evaluated is the initial bacterial concentration in the assay. When this concentration varied from 10^4 CFU/ml to 10^8 CFU/ml, the MICs of EFX and globomycin were eight-fold increased (Figure 1). Similar results were obtained with other conventional antibiotics such as chloramphenicol, streptomycin, tylosin, mitomycin C, and tetracycline (data not shown). In contrast, within the same range of mycoplasma concentrations, the MICs remained the same for gramicidin S and surfactin and increased, but only two-fold, for alamethicin (Figure 1). The same question was addressed by a time killing assay using the selected antimicrobial agents at their MIC at three different cellular concentrations: 10^4, 10^6 and 10^8 CFU/ml (Figure 2). When used alone, EFX and globomycin killed 3-times and 2-times less bacterial cells, respectively, in presence of 10^8 CFU/ml than in presence of 10^4 CFU/ml whereas the percentage of killing by gramicidin S, surfactin and alamethicin did not change (Figure 2). These results indicate that the AMPs
retained the same efficacy even when the concentration of the bacterial inoculum increased, with the exception of globomycin that was similar in this regard to conventional antibiotics.

**Time course of anti-mycoplasma activity of AMPs and EFX.**

Due to their unspecific action on cell membranes, AMPs kill bacteria more rapidly than conventional antibiotics. With the aim to verify this on *M. pulmonis*, we followed the time-course of cell killing by the selected AMPs and by EFX. Antibiotics were added at a final concentration equal to their MIC to a log-phase *M. pulmonis* culture (10⁶ CFU/ml) and the counting of viable cells was realized after 1, 2 and 24 h of incubation. Results were expressed as the percentage of surviving cells (Figure 3). After one hour of incubation, the decreasing order of viable cells was 98% for gramicidin S, 89% for EFX, 68% for globomycin, 43% for surfactin and 31% for alamethicin. After two hours, the bactericidal activity of all antimicrobial agents was enhanced but without modifying their order of efficacy (Figure 3). In contrast, after 24 hours of incubation, the order of efficacy was altered since the percentages of viable cells were 0.22 for EFX, 0.16 for gramicidin S, 0.08 for surfactin, 0.05 for globomycin, and virtually 0 for alamethicin (Figure 3). These data show that the highest antibacterial activity against *M. pulmonis* was obtained after 24 hours of incubation for both EFX and AMPs, and that the most active AMP used alone was alamethicin.

**Effects of the combination of EFX and AMPs on *M. pulmonis***

Combinations of the four AMPs with EFX were tested in order to enhance mycoplasma killing. The efficacy of these combinations was assessed using the checkerboard method and a time-course killing assay.
In the checkerboard method, FIC indexes were equal to 0.56 for the EFX-surfactin association, 0.75 for EFX-alamethicin and 1 for both EFX-globomycin and EFX-gramicidin S associations (Figure 4). Actually, the FIC of the EFX-surfactin combination suggested a synergy of action of two compounds since it proved to be quite close to the threshold (FIC ≤ 0.5) defined by current international standards for a synergistic action (21).

Two different experiments were performed using the time-course killing method: (i) with concentrations of antimicrobial agents equal to the MIC and (ii) with concentrations equal to MIC/2, MIC/4 and MIC/16 for EFX, alamethicin, and surfactin, respectively. These concentrations were chosen according to the results obtained with the checkerboard method: *M. pulmonis* growth was inhibited using combinations of EFX at a MIC/2 concentration and alamethicin at a MIC/4 concentration (Figure 4A). The EFX-surfactin combination inhibited the mycoplasma growth at concentrations equal to MIC/2 for EFX and MIC/16 for surfactin (Figure 4B).

A time-course killing assay using EFX and AMPs at their MIC indicated that whatever the time of incubation (1, 2 or 24 h), the number of viable cells detected after the action of EFX-gramicidin S or EFX-globomycin combinations on *M. pulmonis*, was similar to that obtained after the action of each of the AMPs alone (Figure 5A). In contrast, for the EFX-surfactin combination, the number of viable cells dropped by almost two orders of magnitude (∼ 90-fold) after 24 hours, as compared to the action of surfactin alone (Figure 5A). The EFX-alamethicin combination showed the highest efficacy, even after one hour (2.4% of viable cells vs. 31% with the AMP alone). After 2h of incubation, cell killing was close to 99.9% meaning that the number of viable cells was 100 times lower than with the AMP alone (Figure 5A).

We then assessed the killing activities after 2 and 24 hours of these two combinations using this time concentrations inferior to the MIC (Figure 5B). The mycoplasma-killing
activities of the two combinations enrofloxacin (MIC/2) + alamethicin (MIC/4) and enrofloxacin (MIC/2) + surfactin (MIC/16) were almost two orders of magnitude higher than those of these three molecules used separately, indicating synergistic effects between EFX and the two AMPs.
DISCUSSION

After determining the MICs and MBCs of alamethicin, globomycin, gramicidin S and surfactin against *M. pulmonis* (Table 1), we assessed the importance of the bacterial cell concentration on the activities of these AMPs. In our standard conditions (10⁶ CFU/ml) and on a molar basis, the MIC of globomycin was equal to that of EFX whilst the MICs of the three other AMPs were from 7.8 to 62.5 higher than that of EFX; in the same conditions, the MBCs of the peptides were 7.8 to 125 times higher than that of EFX, suggesting a better efficacy of the fluoroquinoline compared with the AMPs. However, the increase of the mycoplasmal initial concentration from 10⁴ to 10⁸ CFU/ml increased the MIC values of EFX and globomycin, but very little or not at all those of the three other AMPs. Although the mechanism underlying the lack of sensitivity of AMPs to the variation of the bacterial cell density remains unknown, one can hypothesize that at the MIC and above, a large fraction of AMP molecules remains in solution whilst only a small fraction probably acts on the membrane, affecting its integrity. Hence, increasing bacterial density raises the recruitment of new peptides monomers from the solution that in turn destabilize the mycoplasmal membrane without significantly modifying the overall active concentration of AMP able to inhibit bacterial growth. Whatever the rationale underlying this phenomenon, the independence of AMPs activities with respect to the target cell concentration is a significant advantage over the mode of action of conventional antibiotics that are dependent of cell concentration. In contrast to membrane-active AMPs, the main and specific target of globomycin is the signal peptidase II (SPase II), an enzyme involved in lipoprotein maturation. Indeed, as demonstrated in two previous studies performed on the mollicute *Spiroplasma melliferum*, the action of globomycin on the cell membrane is quite marginal, even when used at high concentrations (3, 4). Hence, as for conventional antibiotics acting with a high affinity on a
specific protein target, the concentration of globomycin required for inhibiting or killing bacterial cells is proportional to their concentration.

This study was also aimed at identifying possible synergistic interactions between EFX and the four AMPs. The checkerboard method and the time-course killing assay showed indifferent interactions between the fluoroquinolone EFX and globomycin, or gramicidin S. In contrast, this method revealed that combining EFX with alamethicin or surfactin led to FIC indexes < 1, indicating possible additive effects between these molecules. These results were confirmed by the time-course killing assay as the EFX-alamethicin and EFX-surfactin combinations. When in these associations the three molecules were used at their respective MICs, it was possible kill virtually all the mycoplasma cells after 2 hours with the EFX + alamethicin combination and after 24 hours with the EFX + surfactin combination. Furthermore, the mycoplasma-killing activities of the two combinations enrofloxacin (MIC/2) + alamethicin (MIC/4) and enrofloxacin (MIC/2) + surfactin (MIC/16) were almost two orders of magnitude higher than those of these three molecules used separately. Consistent the fact that the association of EFX with alamethicin was capable of curing a HeLa cell culture of \textit{M. fermentans} in 30 min and of \textit{M. hyorhinis} in 24 hours (20), this result indicate synergistic effects between EFX and alamethicin or surfactin against \textit{M. pulmonis}.

Hence, the two salient results of our study are that in vitro, (i) AMPs retain the same level of activity over a large range of \textit{M. pulmonis} concentrations and (ii) there is a synergistic action between the fluoroquinolone enrofloxacin and two AMPs (alamethicin and surfactin). Associating EFX with either of these two AMPs allowed lower concentrations of EFX and AMPs to be used as compared to the concentrations of each molecule taken alone. This is important for although AMPs are broad-spectrum antimicrobials displaying rapid bactericidal activity, in the perspective of therapeutic applications these advantages are counterbalanced by a limited in vivo stability (notably for AMPs composed of L-\alpha-amino acid residues) and
possible toxicity towards mammalian cells (see 19 for a recent review on this subject). Toxicity remains a major concern because the MICs of AMPs are usually one or two orders of magnitude higher than those of many conventional antibiotics. This was confirmed in this study when comparing the MIC and MBC of EFX with those of the AMPs (Table 1). Furthermore, the activity of AMPs can be hampered in vivo by different biological factors such as enzymatic cleavage or absorption by serum lipoproteins. Fortunately, a better knowledge of the structure-activity relationships of AMPs makes it feasible to design molecules with a better therapeutic index. For example, Dartois et al. (10) have recently designed cationic cyclic peptides displaying a broad antimicrobial spectrum and a prolonged systemic activity in mouse. Additionally, although the pharmaceutical usefulness of AMPs has still to be fully established, it is noticeable that several molecules are presently under clinical development (see e.g. 1, 19, and 27) and one may expect still better achievements in the future by associating conventional antibiotics with engineered AMPs. We believe that this track should be explored in the case of mycoplasmas.
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REFERENCES


**Figure legends**

FIG. 1. Effect of variations in the *M. pulmonis* concentration on the MIC of AMPs and enrofloxacin. Various concentrations (10^4 to 10^8 CFU/ml) of exponential phase growing cells of MpUR1.1 were incubated with twofold dilutions of antibiotics. Inhibitory concentrations of antibiotics were determined by monitoring changes of colour of phenol red after 24 and 48 hours. For each cellular concentration, the ratio inhibitory concentration / MIC was calculated. At the standard concentration (10^6 CFU/ml), the ratio is equal to 1. The experiment was performed 3 times and the results did not vary from one experiment to the next.

FIG. 2. Effect of variations in the *M. pulmonis* concentration on the bactericidal activity of AMPs and EFX. Cultures of *M. pulmonis* at three different concentrations (10^4, 10^6, 10^8 CFU/ml) were incubated with antibiotics (concentration equal to the MIC). Aliquots were withdrawn after 24 hours and diluted for viable count expressed in percentage of the viable count of the control culture (without antibiotic). Average values from two independent experiments are shown on the figure. The values did not significantly vary from one experiment to the next (at the most, there was a 10% variation in CFU/ml).

FIG. 3. Rate of killing of *M. pulmonis* by AMPs and enrofloxacin. Log-phase bacteria (about 10^6 CFU/ml) were obtained in Hayflick medium and concentrations equal to the MIC of antibiotics (see Table 1) were added at time zero. Aliquots were withdrawn at the indicated times (1, 2 and 24 hours), and diluted to count viable cells. The percentage of viable cells is equal to 100% for the control (*i.e.* MpUR1.1 without antibiotic). Average values from two independent experiments are shown. The values did not significantly vary from one experiment to the next (at the most, there was a 6.4% variation in CFU/ml).
FIG. 4. Effects of combinations of enrofloxacin and AMPs on *M. pulmonis* growth by the checkerboard system. A culture of *M. pulmonis* (10^6 CFU/ml) was incubated with twofold dilutions of antibiotics associations starting from 2 x MIC to MIC/32. The inhibition of growth was monitored by the colour change of Hayflick medium. FIC indexes were subsequently calculated (see Materials and Methods) for each of the combinations: EFX with alamethicin, (A), EFX with surfactin (B), with globomycin (C) and with gramicidin S (D). Experiments were performed in duplicate. No significant changes were observed from one experiment to another.

FIG. 5. Effect of the combination of enrofloxacin and AMPs on *M. pulmonis* growth by the viable cell count technique. (A) Log-phase bacteria (about 10^6 CFU/ml) were obtained in Hayflick medium. Viable cell count was assessed by incubating mycoplasma with each antibiotic alone (white for EFX and grey for AMPs) or with a combination of concentrations of enrofloxacin with globomycin, gramicidin S, surfactin and alamethicin equal to the MIC (black). Aliquots were withdrawn after 1, 2 and 24 hours and diluted for viable count. Average values from two independent experiments are shown. The values did not significantly vary from one experiment to the next (at the most, there was a 6.2% variation).

(B) The same combinations experiments were performed using EFX at its MIC/2, alamethicin at its MIC/4 and surfactin at its MIC/16. Viable cell counts were performed after 2 and 24 hours. Average values from two independent experiments are shown. The values did not significantly vary from one experiment to the next (at the most, there was a 5% variation in CFU/ml).
TABLE 1. MICs and MBCs of AMPs and enrofloxacin for *M. pulmonis* UR1.1

<table>
<thead>
<tr>
<th></th>
<th>MIC (µM)/(µg/ml)</th>
<th>MIC/MIC EFX&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MBC (µM)/(µg/ml)</th>
<th>MBC/MBC EFX&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alamethicin</td>
<td>6.25/12.3</td>
<td>15.6</td>
<td>25/49.1</td>
<td>31.2</td>
</tr>
<tr>
<td>Globomycin</td>
<td>0.4/0.26</td>
<td>1</td>
<td>12.5/8.2</td>
<td>15.6</td>
</tr>
<tr>
<td>Gramicidin S</td>
<td>3.12/3.6</td>
<td>7.8</td>
<td>6.25/7.1</td>
<td>7.8</td>
</tr>
<tr>
<td>Surfactin</td>
<td>25/25.9</td>
<td>62.5</td>
<td>100/103.6</td>
<td>125</td>
</tr>
<tr>
<td>Enrofloxacin (EFX)</td>
<td>0.4/0.14</td>
<td>1</td>
<td>0.8/0.29</td>
<td>1</td>
</tr>
</tbody>
</table>

MIC determinations were realized in Hayflick medium containing 5% horse serum with the standard concentration (10<sup>6</sup> CFU/ml) of exponential growth phase cells of MpUR1.1. For MBC determinations, 0.1 ml aliquots were withdrawn after 2 hours for AMPs and 24 hours for enrofloxacin and then diluted and plated for viable cell count. Experiments were performed in triplicate. <sup>a</sup>The comparison of AMP efficacy with that of EFX is indicated using ratio of MIC or MBC values (µM).
Percentage of viable cells (%)

Antibiotics

Gramicidin S
Enrofloxacin
Globomycin
Surfactin
Alamethicin

10^4 CFU/ml
10^6 CFU/ml
10^8 CFU/ml