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In vitro activity of PR-39, a proline-arginine-rich peptide, against susceptible and multi-drug-resistant *Mycobacterium tuberculosis*

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We have investigated the *in vitro* activity of antimicrobial peptides against *Mycobacterium tuberculosis* using a radiometric method and cfu determinations. PR-39, a proline-arginine-rich antibacterial peptide from porcine leucocytes, was found to be active against drug-susceptible as well as multi-drug-resistant (MDR) clinical isolates of *M. tuberculosis*. The activity of PR-39 was concentration dependent, with 80% growth inhibition of *M. tuberculosis* H37Rv at 50 mg/L. The MDR *M. tuberculosis* strains E1380/94 and P34/95 were less susceptible to PR-39, with 39 and 49% growth inhibition at 50 mg/L peptide, respectively, suggesting a lower susceptibility than strain H37Rv and drug-susceptible clinical isolates. Reduction of counts of *M. tuberculosis* H37Rv and the MDR *M. tuberculosis* strain E1380/94 by PR-39 indicated that the growth inhibition seen in the radiometric assay is due to a mycobactericidal effect of the peptide. These observations suggest that antimicrobial peptides may play an important role in host defence against MDR *M. tuberculosis*.

Introduction

Mycobacterium tuberculosis, the cause of human tuberculosis, is a major killer among the infectious organisms. Although the incidence of tuberculosis in developed countries has declined during the past decades, the emergence of HIV and multi-drug-resistant *M. tuberculosis* strains (MDR-TB) has reversed the trend and worsened the outcome of treating the disease. Directly observed therapy with standard anti-tuberculosis chemotherapy is an important component of tuberculosis control. The presence of MDR-TB strains has drastically reduced the number of effective antibiotics and has focused attention on alternative bactericidal agents. To counteract the emerging MDR-TB threat it is necessary to enhance our knowledge of the microorganism and the innate host defences, and to find new approaches to anti-tuberculosis therapy.

Endogenous antimicrobial peptides are well recognized components of the innate immune defence.[1,2] PR-39 is a proline-arginine-rich antibacterial peptide that was isolated from pig intestine in 1991.[3] PR-39 has a broad antibacterial spectrum and is active against Gram-positive and Gram-negative bacteria. Synthetic D- and L-PR-39 peptides have non-identical antibacterial spectra, suggesting that stereo-specificity is important, at least against some bacteria such as *Pseudomonas* spp.[4] The antibacterial domain in PR-39 resides in residues 1–26, whereas shorter internal or end fragments do not possess such activity.[5] PR-39 binds to negatively charged membranes but appears not to form discrete pores in artificial membranes.[6] Unlike many other antibacterial peptides, PR-39 does not lyse *Escherichia coli* but rather inhibits DNA and protein synthesis.[7]

PR-39 is a multi-functional peptide with activities that also include involvement in wound healing through inhibition of syndecan expression,[8] anti-inflammatory properties through inhibition of NADPH oxidase[9] and chemoattractant activity for neutrophil leucocytes.[10] PR-39 is lethal against certain tumour cells, although with different potency against different malignant cells; >0.5 μM reduces the viability of kidney cells whereas >50 μM is required to reduce the viability of epithelial cells.[9] However, the antibacterial domain (1–26) of PR-39 does not appear to have cidal activity against tumour cells.[9]

Two other antibiotic peptides, defensin and protegrin-1 (PG-1), have been shown to be active against *Mycobacterium avium* complex and *M. tuberculosis*.[11,12] Defensin acts on the mycobacterial cell envelope and disrupts the membrane architecture.[13] A similar permeability increasing activity of defensin has also been seen against *E. coli*.[14]

We have previously used the radiometric system to

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analyse drug resistance patterns of *M. tuberculosis*.[15] In this study, we investigated the activity of PR-39 and two other antimicrobial peptides (AMPs), cecropin P1 and LL-37, against various mycobacteria.

Materials and methods

Bacteria

M. tuberculosis H37Rv ATCC 25618, *M. avium* ATCC 26518 and *Mycobacterium smegmatis* ATCC 19420 were purchased from the American Type Culture Collection (Rockville, MD, USA). *M. tuberculosis* E1380/94 and P34/95 were clinical isolates from patients with pulmonary MDR-TB, received from Tartu University Lung Hospital, Estonia. Strain E1380/94 was resistant to all of the first-line drugs (isoniazid, rifampicin, streptomycin, ethambutol), as well as some second-line drugs, and strain P34/95 was resistant to isoniazid and rifampicin.[15] *M. tuberculosis* #894-D11, a well-characterized, low-level, streptomycin-resistant strain, was received from Dr E. C. Böttger (Medizinische Hochschule, Hannover, Germany).[16] BTB 98-492 was a drug-susceptible Swedish clinical isolate. All bacteria were stored at –70°C in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) containing 10% glycerol. Thawed aliquots were cultured on Loewenstein–Jensen medium before use.

Peptides

PR-39 and vasoactive intestinal polypeptide (VIP) were isolated from porcine intestine.[3] Cecropin P1,[17] LL-37 (kindly donated by B. Agerberth, Karolinska Institutet, Stockholm, Sweden), human defensin and protegrin (kindly donated by R. Lehrer, UCLA, CA, USA) were all synthetic peptides.

Methods

Anti-mycobacterial activity was assessed by means of a BACTEC radiometric assay (Becton Dickinson, MD, USA).[18,19] Peptides were dissolved in sterile distilled water, and 100 μL aliquots were inoculated into BACTEC 12B vials containing 2 mL 7H12 Middlebrook medium (Becton Dickinson) before inoculation with bacteria. The bacteria were suspended in sterile phosphate-buffered saline (PBS) to a density of 1.0 McFarland standard (3×10^8 cfu/mL). These suspensions were further diluted 1:10, and 100 μL aliquots were inoculated into the test vials to give a final concentration corresponding to 1.5×10^6 cfu/mL. The growth index (GI) values were monitored for 8 days. A 10-fold dilution of the control bacterial suspension was inoculated into new vials, the GI values of which were considered equivalent to 90% inhibition of growth. The effect of AMPs was evaluated by comparing the GI values of the control vials with those of peptide-treated cultures [$(\Delta GI_{sample}/\Delta GI_{control}) \times 100$ = % relative growth]. Growth rates were estimated from the calculated doubling time of exponential increasing metabolic activity. MIC_{50} was defined as the concentration giving 50% reduction in GI.

Standard colony count assays were performed to assess the bactericidal activity of PR-39. PR-39 was added at 50 or 100 mg/L to BACTEC vials containing 7H12 medium inoculated with *M. tuberculosis* H37Rv or MDR strain E1380/94, respectively. After incubation for 24 h at 37°C, 100 μL aliquots of each suspension were plated on 7H11 agar and incubated at 37°C for 21 days. The *M. smegmatis* isolate was resuspended in Middlebrook 7H9 medium (Difco Laboratories) and treated with 50 mg/L PR-39 or cecropin P1 for 4 h at 37°C in 5% CO_2. After incubation, a cfu count was determined by diluting the samples and plating them on Luria–Bertani agar plates. Plates were incubated at 37°C and colonies were counted every day for 5 days.

Statistical analyses were performed by Mann–Whitney and Kruskal–Wallis tests using the GraphPad Prism program (Intuitive Software for Science, CA, USA).

Results

Mycobactericidal activity of PR-39

GIs of untreated and PR-39-treated *M. tuberculosis* H37Rv are shown in Figure 1a. Treatment of *M. tuberculosis* H37Rv with 50 mg/L PR-39 caused a delay of measurable growth followed by a growth rate that paralleled control suspensions and 10-fold diluted suspensions. VIP had no effect. Figure 1b illustrates the relative growth of bacteria incubated with PR-39 calculated for each day. The relative growth was higher on days 1 and 2, but levelled out on days 4–5. Consequently, mean relative growth on days 3–5 was used to present relative growth in further experiments. The mycobactericidal effect of PR-39 was examined by incubating the peptide (at 50 and 100 mg/L) with *M. tuberculosis* strain H37Rv or MDR strain E1380/94 suspended at 3×10^6 cfu/mL in Middlebrook medium. Cfus were determined after 24 h incubation as described in Materials and methods. PR-39 caused a decrease in the cfus of *M. tuberculosis* in a dose-dependent manner. This did not differ significantly from the relative growth reduction observed in the BACTEC system (Figure 1c). The fast growing mycobacterial species *M. smegmatis* was treated with 50 mg/L PR-39 or cecropin P1. In these experiments, PR-39 reduced the cfu by >90% and cecropin P1 by 5% after 4 h incubation at 37°C.

Concentration-dependent activity of PR-39 against M. tuberculosis

PR-39 reduced the growth of *M. tuberculosis* H37Rv and the clinical MDR isolate E1380/94 in a concentration-

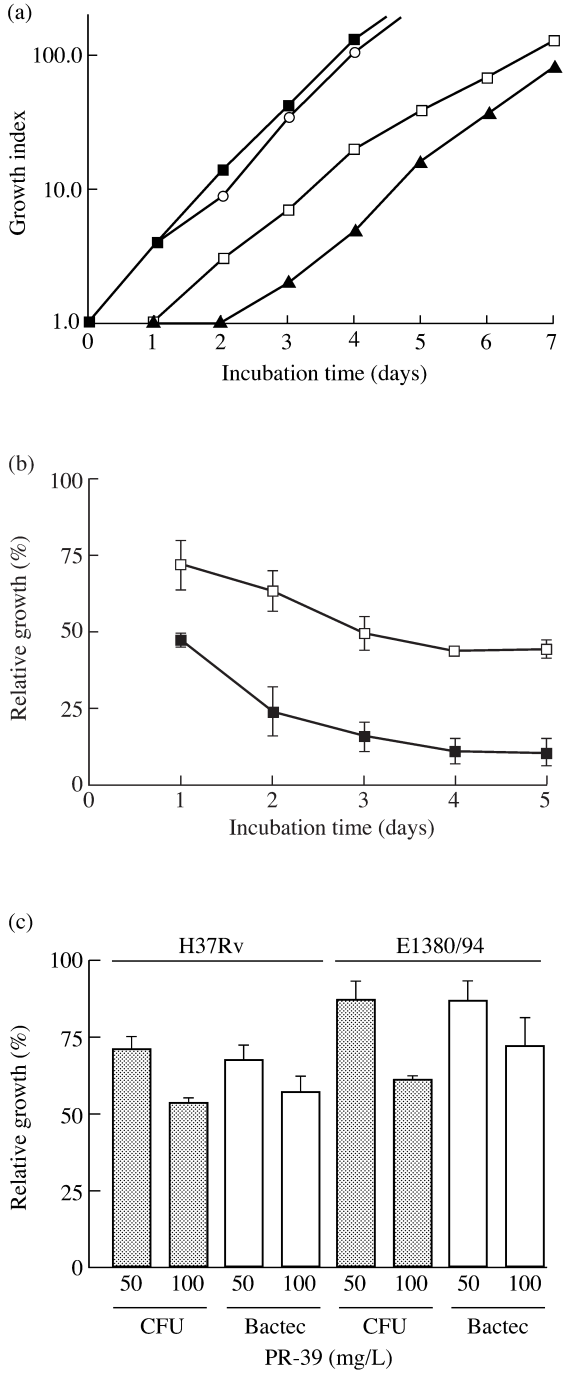

Figure 1. Radiometric and cfu assay analysis of PR-39 activity against *M. tuberculosis*. (a) BACTEC vials containing 7H12 medium were inoculated with *M. tuberculosis* H37Rv. PR-39 at 50 mg/L (□) or VIP at 50 mg/L (○) was added, and the GI was followed together with that of undiluted control suspension (■) and 1:10 diluted inoculum (▲). (b) PR-39 at 100 mg/L was added to strain H37Rv (■) or MDR strain E1380/94 (□). The difference in GI between treated and control bacteria was calculated and presented as percentage relative growth. (c) PR-39 was added at 50 or 100 mg/L to strain H37Rv or MDR strain E1380/94, incubated for 24 h at 37°C, and the activity of PR-39 was evaluated by cfu (shaded bar) or BACTEC (white bar). Data are expressed as the means ± S.D. of three independent experiments.

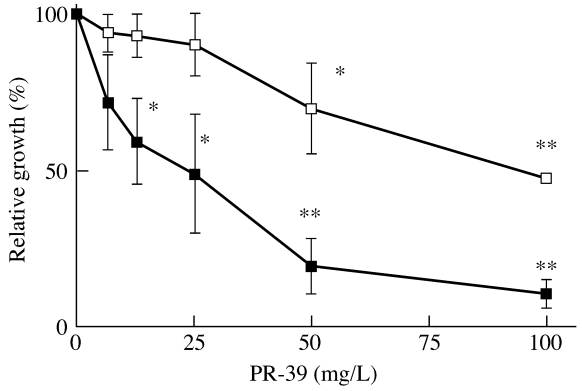

Figure 2. Concentration-dependent inhibition of *M. tuberculosis* by PR-39. PR-39 was added at different concentrations to strain H37Rv (■) and MDR strain E1380/94 (□). The average difference in GI between treated and control bacteria was calculated at days 3–5 and presented as relative growth. Data are mean ± S.D. of duplicate or triplicate experiments. $*P < 0.05$, $**P < 0.01$, compared with control.

dependent manner (Figure 2). The effect was most pronounced against *M. tuberculosis* H37Rv, with close to 30% growth inhibition at 6.25 mg/L peptide and 80% GI inhibition at 50 mg/L. The MDR strain E1380/94 showed about 50% growth inhibition when treated with PR-39 at 100 mg/L. The MIC_{50} values calculated were 17 ± 9 mg/L for H37Rv and 93 ± 12 mg/L for E1380/94, suggesting that the latter had a five-fold lower susceptibility to the peptide than strain H37Rv.

Activity of PR-39 against clinical isolates of susceptible and MDR M. tuberculosis and other mycobacteria

Two drug-susceptible clinical isolates of *M. tuberculosis* and the *M. smegmatis* strain were as susceptible as *M. tuberculosis* H37Rv, while the two MDR *M. tuberculosis* strains were less susceptible to PR-39 at 50 mg/L (Figure 3) when tested in the BACTEC system. The Kruskal–Wallis test showed a significant difference in relative growth between drug-susceptible clinical isolates and the MDR strains. PR-39 had low activity against *M. avium.* The growth rates of H37Rv and E1380/94 at 1.29 ± 0.03 and 1.19 ± 0.24, respectively, were not significantly different, while *M. avium* grew faster.

Comparison of activity against mycobacteria with other AMPs

We have compared the activity of the AMPs defensin HNP-1, LL-37, protegrin PG-1 and cecropin P1 with that of PR-39. HNP-1 and PG-1 have internal disulphide bonds that constrain their three-dimensional structure, while the

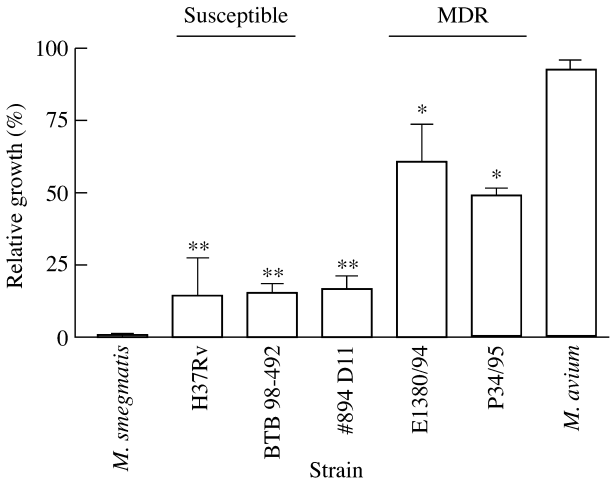

Figure 3. Activity of PR-39 against clinical drug-susceptible and MDR *M. tuberculosis*. PR-39 was added at 50 mg/L to antibiotic-susceptible and -resistant isolates of *M. tuberculosis* as well as other *Mycobacterium* strains. *M. tuberculosis* #894-D11 demonstrates low-level streptomycin resistance. The average difference in GI between treated and control bacteria was calculated at days 3–5 and presented as relative growth. $*P < 0.05$; $**P < 0.01$.

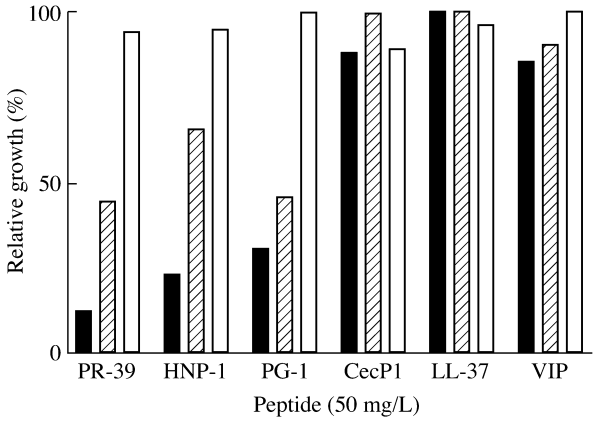

Figure 4. Activity of AMPs against *M. tuberculosis* and *M. avium*. PR-39, HNP-1, PG-1, LL-37, cecropin P1 (CecP1) and VIP were added at 50 mg/L to strain H37Rv (black bar), MDR strain E1380/94 (striped bar) or *M. avium* (open bar). The average difference in GI between treated and control bacteria was calculated at days 3–5 and presented as relative growth.

others are linear extended peptides with amphipathic α-helices. The linear peptides LL-37, cecropin P1 and VIP were inactive against the three strains of mycobacteria tested, whereas HNP-1 and PG-1 showed an activity pattern against mycobacteria similar to that of PR-39 (Figure 4). *M. avium* was resistant, *M. tuberculosis* H37Rv susceptible and the MDR strain *M. tuberculosis* E1380/94 moderately susceptible to PR-39.

Discussion

In this study, we demonstrated that the antibacterial peptide PR-39 inhibits growth of both drug-susceptible and MDR clinical strains of *M. tuberculosis*. To our knowledge, this is the first report of an antimicrobial peptide (AMP) active against MDR-TB defined as resistant to both isoniazid and rifampicin.

The effect of PR-39 was assayed by the BACTEC radiometric method, which detected growth of mycobacteria by measurement of $^{14}CO_2$ released as a consequence of bacterial catabolism. After 5 days of incubation in PR-39 at 50 mg/L, *c.* 80–90% inhibition of bacterial growth of drug-susceptible or low-level streptomycin-resistant strains was observed. The MDR-TB strains E1380/94 and P34/95 were inhibited by 39–49%. PR-39 reduced the cfus of H37Rv and MDR-TB strain E1380/94 at a level comparable to its bacteriostatic activity, showing that the inhibition of growth involved killing of the bacteria. It has been reported that the radiometric method and cfu measurements correlate well.[20,21] Our data on *M. smegmatis* suggest further that killing is rapid, since a reduction of >90% cfu was obtained within 4 h of incubation.

The unusually lipid-rich cell wall structure of mycobacteria make the cell surface hydrophobic, which causes aggregation of the cells and reduces its permeability to various molecules. Since *M. tuberculosis* differs from the majority of bacteria in that it may survive and multiply within phagocytic cells, it is likely that its unique cell envelope helps it to evade and escape intracellular host antibacterial and bactericidal mechanisms, as well as affording protection against some antibiotics. This compact and hydrophobic membrane structure could be one reason for the low activity observed for the LL-37 and cecropin P1 against the mycobacteria isolates tested.

The role of AMPs in host defence against mycobacteria is still unclear. Human or rabbit defensin as well as porcine PG-1 has been shown to be active against *M. avium* complex and *M. tuberculosis*.[11,12] Mikiyama *et al.*[11] reported similar inhibition of *M. tuberculosis* H37Ra growth by PG-1 to that found in our study with *M. tuberculosis* H37Rv. Ogata *et al.*[12] showed PG-1 activity against *M. avium* complex, although we could not reproduce this result against our own strain of *M. avium*. The reason for this is not clear but could be due to strain differences or the different experimental methods used.

After initiation of infection by mycobacteria, an inflammatory response follows that results in an accumulation of macrophages and neutrophils. Mycobacteria grow primarily within macrophages, but once activated these cells have the capacity to kill mycobacteria. To date, LL-37 but not defensin has been detected in human lung macrophages.[22] Neutrophils may play a much more important role in tuberculosis than previously believed.[23] They are continuously recruited to foci of infection where they may phagocytose mycobacteria.[24] Whether ingestion of the

bacteria is followed by killing has been questioned.[25,26] Others have demonstrated killing,[24,27] and Jones *et al*.[28] showed that killing is independent of the oxygen metabolic burst. This raises the question of whether AMPs are involved in a mycobactericidal mechanism present in neutrophils. Defensin, PG-1 and PR-39 are produced and stored in phagocytes, mainly neutrophils, where they are believed to play a part in the non-oxidative defence mechanisms of these cells. Defensins[29] and PR-39[30] are found in neutrophils at mycobactericidal concentrations, but can also be released during phagocytosis and infection.[31,32] Released peptides may act as chemotactic agents, defensin for monocytes and PR-39 for neutrophils.[10,33] Thus, AMPs may be contributing to intracellular and extracellular defence against *M. tuberculosis*.

The observation that MDR-TB strains are more resistant to PR-39 than drug-susceptible strains is interesting. We did notice a statistical difference between the groups, but further analysis of other MDR strains is required to conclude if this is a general phenomenon. We did not find any difference in the metabolic doubling time, thus the activity does not seem to correlate with growth rates. The mechanism of action of PR-39 is not yet fully defined. The peptide does not lyse *E. coli* and acts through a mechanism that may involve stereo-specificity.[4,7] An initial membrane uptake is postulated that is similar to that seen with defensin.[6,13,14] This would not be sufficient for PR-39 to exert a bactericidal effect, but rather additional internalization and interaction with intracellular proteins are postulated as killing mechanisms.[5,34] Recent findings with *Salmonella*, *Staphylococcus aureus* and *Neisseria gonorrhoeae* show that surface lipid modification as well as alteration in efflux pumps may influence the susceptibility of bacteria to AMPs,[35–37] also suggesting that different AMPs may have different mechanisms of action. Target structures in both drug-susceptible and MDR mycobacteria need to be investigated further to determine whether MDR *M. tuberculosis* has altered physiological properties that allow it to evade peptide-based innate immune defences.

In conclusion, studies of the mechanism of action of AMPs against mycobacteria will increase our understanding of the host immune response in general as well as of the defence mechanisms of bacteria. It will be of interest to explore the possible role of PR-39 in the innate host defence against tuberculosis.

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