SUMMARY

"New perspectives on substances with leishmanicidal activity: developing of antimicrobial peptides with leishmanicidal activity on the external surface of plant virus nanoparticles"

Aims

Canine leishmaniasis, currently expanding in Italy and in the countries of the Mediterranean basin is caused by the protozoan *Leishmania*. It is a zoonotic disease transmitted by the bite of an infected sandfly belonging to the genus *Phlebotomus*. To date, no really effective vaccine formulation is available and so, in order to fight this disease and limit its spread, a promising strategy could be the use of AMPs for the control and killing of the parasite (Gradoni 2015). These AMPs could be displayed on the external surfaces of chimeric virus nanoparticles based on plant viruses *Tomato bushy stunt virus* (TBSV) and *Potato virus X* (PVX) and then tested in order to evaluate their leishmanicidal potentiality and their cytotoxicity against canine erythrocytes. The results could be useful for the formulation of new leishmanicidal products.

Materials and methods

This research project involved the display of different AMPs with microbicidal activity on the outer surface of the chimeric virus nanoparticles (CVNPs) based on plant viruses *Tomato bushy stunt virus* (TBSV) (Grasso *et al.*, 2013) and *Potato virus X* (PVX) (Lico *et al.*, 2006; Marconi *et al.*, 2006) and the evaluation of the antimicrobial activity of the characterized chimera against some bacterial strains and *Leishmania infantum* promastigotes. The use of plant virus nanoparticles (pVNPs) for the production of AMPs could have two benefits: i) the CVNPs, displaying the AMP in fusion with the capsidic protein (CP), would be directly synthesized by plants; this could reduce enormously the costs of production because the AMPs until now used were synthesized chemically and ii) the exposition of the AMP of interest with high density on these molecular cages could favor the bioavailability, the stability and the protection of the peptide from the proteolysis, compared to the naked AMPs extracted from the organism.

Results

Considering these conditions, a careful bibliographic study was performed in order to identify the AMPs with a low cytotoxic activity against erythrocytes and with a good leishmanicidal and bactericidal activity, tested *in vitro* against both replicative forms of the protozoan *Leishmania* and against other pathogenic bacteria, respectively.
Among all, the Temporin A, Temporin B, Bombinin H2 and Esculentin 1b (1,18) peptides were selected.
Using standard recombinant DNA techniques, the chimeras named TBSV-TempA, TBSV-TempB, TBSV-BombH2, PVX-Sma-TempA, PVX-Sma-TempB, PVX201-2A-TempA, PVX201-2A-TempB and PVX201-2A-Esc were realized.
The Temporin A and Temporin B peptides were fused to both plant viruses, because for the temporins the end having functionality is not known, while it is the C-terminal end for the Bombinin H2 peptide (Simmaco *et al.*, 2009) (the peptide was so fused to the C-terminal end of the CP of TBSV, the region displayed on the external surface) and the N-terminal end for Esculentin 1b (1,18) peptide (Mangoni

et al., 2003) (the peptide was so fused to the N-terminal end of the CP of PVX, the portion on the outer surface).
The test for infectivity performed inoculating *N. benthamiana* plants showed that the replicative and infectious chimeras were the CVNPs based on TBSV and the PVX201-2A-Esc chimera.
Only these ones were characterized at the protein and nucleic acid level.
At first, differently from expected results, the yield of CVNPs after small scale purification was low: 14,8 μg/g for TBSV-TempA and TBSV-TempB, 120 μg/g for TBSV-BombH2 and 16,8 μg/g for PVX201-2A-Esc. This was probably due to the presence of such heterologous sequences affecting the normal infectivity of chimeras.
The subsequent analyses of characterization (silver stained-SDS PAGE, Western Blotting and RT-PCR) showed that the chimeras TBSV-TempB, TBSV-BombH2 and PVX201-2A-Esc were the only ones displaying correctly the entire AMP on the outer surface.
In fact, especially the RT-PCR analysis, showed that in TBSV-TempA chimera there was a rearrangement at the genomic level causing the loss of 11 amino acids of the heterologous peptide, while the TBSV-BombH2 chimera, stable in the first generation of infection, in the second generation showed a genomic recombination with the loss of the first 15/20 amino acids of the peptide.
Therefore, for the next experiments, only the TBSV-TempB, TBSV-BombH2 I and PVX201-2A-Esc chimeras were used.
The preliminary *in vitro* assay was performed with the aim to analyze the microbicidal potentiality of the realized CVNPs against the Gram negative bacterium *E. coli* (XL1-Blue strain) and Gram-positive bacterium *Bacillus megaterium*.
The most promising data were obtained with the TBSV-TempB chimera causing, at the tested concentration (0.610 μM of peptide), the death of 20% of *B. megaterium* bacteria and the PVX201-2A-Esc chimera showing the 30% of mortality of the bacteria *E. coli* with 0.581 μM of peptide (Table 1 and 2).
Later, the MTT assay was optimized in order to test the leishmanicidal activity of CVNPs against *L. infantum* promastigotes. Unfortunately, at the tested concentrations, no mortality was showed. Probably this negative result is due to the very low concentration of the peptide used in this assay.

Discussion and conclusions

The realized chimera did not cause the mortality of *L. infantum* promastigotes at the tested concentrations. Considering the difficulty to obtain *in planta* high yield of CVNPs, the cause of non-activity could be the use of extremely low concentrations of the interested AMPs. Besides, in literature mainly different *Leishmania* strains were used and it is known that leishmanicidal agents could act differently with various strains and forms of the protozoan.
For example the Temporin B (Mangoni *et al.*, 2005) and Bombinin H2 (Mangoni *et al.*, 2006) peptides are able to provoke the death of 50% of *L. donovani* promastigotes (LC50) at 8,6 μM and 7,3 μM, respectively, while in literature indications of activity of Esculentin 1b (1,18) peptide against *Leishmania* are not present. The Operating Units, in future, want to perform other *in vitro* assays in order to test the leishmanicidal activity of CVNPs using greater amounts and the potential cytotoxicity against canine erythrocytes. Finally they want also to try the PVX201-wt virus, given the promising results against *E. coli* bacteria.