Antiviral activity of a Bacillus sp. P34 peptide against pathogenic viruses of domestic animals

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Abstract

P34 is an antimicrobial peptide produced by a Bacillus sp. strain isolated from the intestinal contents of a fish in the Brazilian Amazon basin with reported antibacterial activity. The aim of this work was to evaluate the peptide P34 for its in vitro antiviral properties against canine adenovirus type 2 (CAV-2), canine coronavirus (CCoV), canine distemper virus (CDV), canine parvovirus type 2 (CPV-2), equine arteritis virus (EAV), equine influenza virus (EIV), feline calicivirus (FCV) and feline herpesvirus type 1 (FHV-1). The results showed that the peptide P34 exhibited antiviral activity against EAV and FHV-1. The peptide P34 inhibited the replication of EAV by 99.9% and FHV-1 by 94.4%. Virucidal activity was detected only against EAV. When P34 and EAV were incubated for 6 h at 37 °C the viral titer reduced from 10^4.5 TCID50 to 10^2.75 TCID50, showing a percent of inhibition of 98.6%. In conclusion, our results demonstrated that P34 inhibited EAV and FHV-1 replication in infected cell cultures and it showed virucidal activity against EAV. Since there is documented resistance to the current drugs used against herpesviruses and there is no treatment for equine viral arteritis, it is advisable to search for new antiviral compounds to overcome these infections.

Key words: antimicrobial peptides, antiviral activity, herpesvirus, equine viral arteritis.

Introduction

The impact of the increasing resistance of microorganisms to drugs and specific antimicrobial substances has motivated several research groups. Since their discovery, the antimicrobial peptides (AMPs) are conquering special attention as important therapeutic alternatives for the prevention and treatment of infections caused by a large number of microorganisms (Oyston et al., 2009). AMPs are universal features of the defense systems of all forms of life, with representatives found in organisms ranging from bacteria, plants, invertebrate and vertebrate species, including mammals (Jenssen et al., 2006). Studies about antiviral compounds date from 1950 (Felipe et al., 2006), but for several reasons such as serious side effects, just a few drugs were approved for clinical use (De Clercq, 2004).

Antimicrobial activity was reported among several bacteria isolated from the aquatic environments of Brazilian Amazon basin (Motta et al., 2004). Among them, a species of Bacillus producing an antimicrobial peptide was isolated from the intestinal contents of the fish Piau-com-
pinta (*Leporinus* sp.) (Motta et al., 2007b). This peptide was purified and named P34 and its antimicrobial activity was characterized as a fengycin-like substance (Motta et al., 2007a). Its inhibitory activity was detected against Gram-positive bacteria, like *Listeria monocytogenes* and *Bacillus cereus* (Motta et al., 2007b), and Gram-negative bacteria like *Escherichia coli* and *Salmonella enteritidis* (Motta et al., 2008). While some studies on P34 have shown its importance as a potential food preservative (Motta et al., 2007a), little attention has been addressed to its application as an antimicrobial substance in clinical studies.

Since there is no data regarding the antiviral activity of this peptide, the aim of the present work was to evaluate the activity exerted by the peptide P34 against canine adenovirus (CAV-2), canine coronavirus (CCoV), canine distemper virus (CDV), canine parvovirus type 2 (CPV-2), equine arteritis virus (EAV), equine influenza virus (EIV), feline calicivirus (FCV) and feline herpesvirus type 1 (FHV-1).

Materials and Methods

**Antimicrobial peptide (P34), cells and viruses**

The peptide P34 was produced as described elsewhere (Motta et al., 2007b). After purification, total protein concentration was measured in triplicate by the Lowry method according to the manufacturer’s protocol (Total Protein Kit, Micro Lowry, Peterson’s Modification - Sigma Aldrich, USA). The purified peptide was analyzed by mass spectrometry (Stein, 2008) (Ettan MALDI-TOF ProSystem, Amersham Biosciences, Sweden) operating in reflectron mode with positive ionization at 20 kV and using a matrix of α-ciano-4-hydroxycinnamic acid (Sigma-Aldrich, USA). The peptide was stored at -20 °C until used for antiviral assays.

Madin-Darby Canine Kidney (MDCK - ATCC® Number: CCL-34™, USA), Crandell-Rees Feline Kidney (CRFK - ATCC® Number: CCL-94™, USA) and Rabbit Kidney (RK13 - ATCC® Number: CCL-37™, USA) cells were cultivated in Eagle’s minimum essential medium (E-MEM - Sigma Aldrich, USA) supplemented with 10% of bovine fetal serum (BFS, Gibco, USA), penicillin (Sigma-Aldrich, USA), streptomycin (Vetec, Brasil), amphotericin B (Cristália, Brasil) and enrofloxacin (Bayer, Brasil), in an incubator at 37 °C.

The antiviral activity of the AMP P34 was evaluated against viruses with different phenotypic and genotypic features. FCV (Weiblen et al., 1998), CCoV (MAV 795 strain), EAV (Bucyrus strain) and EIV (local isolate) were kindly provided by the Virology Laboratory of the Federal University of Santa Maria (UFSM). CAV-2 (Toronto A26/61 strain), CDV (Lederle VR128 strain), CPV-2 (Cornell strain) and FHV-1 (B927 strain) were kindly provided by Desidério Finamor Veterinary Research Institute (IPVDF). These viruses were propagated on MDCK, CRFK or RK13 cell cultures.

**Cytotoxicity assays**

MDCK, CRFK and RK13 cells grown in microplates (TPP, Switzerland) were incubated with different concentrations of P34 (from 0.23 μg/mL to 6.87 μg/mL) for 72 h at 37 °C and 5% CO2. Cell viability was measured by the neutral red dye uptake (NRU, Vetec, Brasil) assay (Borenfreund and Puerner, 1984) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) procedure (Mosmann, 1983). The percentage of cell viability (CV) was calculated as: CV = AT/ AC x 100, where AT and AC were the absorbances of treated and control cells, respectively (Vaucher et al., 2010). The cytotoxicity of P34 was expressed as the concentration at which 50% cytotoxicity was observed (CC50).

**Antiviral assays**

**Cytopathic effect inhibition (CPE) assay**

The inhibition of CPE assays were performed on confluent MDCK, CRFK and RK13 cell monolayers, in the presence or absence of P34 in its non-cytotoxic concentration for each cell lineage, described in the results. Endpoint titrations were performed as described elsewhere (Mahy and Kangro, 1996) and titers were expressed in tissue culture infective dose 50% (TCID50/100 µL). The cells were kept in an incubator at 37 °C and observed for CPE after 72 h.

The viral percents of inhibition (PI) were calculated by PI = [1- (Titer of treated/ Titer of controls)] x 100, adapted from Felipe et al. (2006).

**P34 virucidal effect**

Virus strains were incubated at 37 °C for 6 h with E-MEM in the presence or absence of P34 (in non-cytotoxic concentrations for each cell lineage). After the incubation period, the infectivity was immediately determined by virus titrations on cell cultures.

**Statistical analysis**

All assays were performed in triplicate. Statistical analysis were performed using a two-tailed Student’s t-test and values were considered significant when p < 0.05.

**Results**

**Peptide P34**

The mass spectrum of the purified peptide P34 revealed typical m/z peaks of the lipopeptide fengycin (Figure 1). The m/z peaks at 1449.8, 1463.8 and 1477.8 differed by 14 Da, equivalent to a CH2 group. These peaks were assigned to C15, C16 and C17 forms of Ala-6-fengycin. Other peaks corresponding to Na+ and K+ adducts of fengycin were also observed.
P34 cytotoxicity

In order to distinguish selective antiviral activity from cytotoxicity, the peptide was evaluated on MDCK, CRFK and RK13 cells by the NRU and MTT assays. CC50 was quite similar in both NRU and MTT tests for each cell lineage. CC50 values were 2.11 μg/mL, 2.5 μg/mL and 3.92 μg/mL for MDCK, CRFK and RK13 cells, respectively. Cytotoxicity was not observed at 1.37 μg/mL of the peptide P34 for MDCK, 0.92 μg/mL for CRFK and 2.29 μg/mL for RK13 cell cultures. These concentrations were then used in all the subsequent assays.

Antiviral assays

Titrations showed that the presence of the peptide P34 had no statistically significant effect (p > 0.05) against the production of viral particles of CAV-2, CCoV, CDV, CPV-2, EIV and FCV. However, a significant reduction on viral titers occurred when P34 (2.29 μg/mL and 0.92 μg/mL, respectively) was incubated with EAV and FHV-1 (Table 1). The titer of EAV was expressively reduced from 10⁷ TCID₅₀ to 10¹.⁷₅ TCID₅₀ in the presence of P34, presenting a PI of 99.9%. The titer of FHV-1 was 10⁴.₅ TCID₅₀ in the presence of P34 and 10².⁷₅ TCID₅₀ in its absence, resulting in a PI of 94.4%. The peptide P34 had only a direct inactivating effect against EAV infectious particles. A potent virucidal effect was observed and EAV infectivity was reduced by 98.6%. After 6 h of incubation, EAV titer was reduced from 10⁴.₅ TCID₅₀ to 10¹.⁷₅ TCID₅₀ in the presence of P34 (p < 0.05).

Discussion

A great number of peptides isolated from different sources have been studied for antiviral activities (Andreu and Rivas, 1998; Antimicrobial Peptide Database/ APD: http://aps.unmc.edu/AP/main.html). Several AMPs have been tested, but just a few of them have reached the clinical routine (Oevermann et al., 2003, Wachsman et al., 2003).

Ideally, to be the most useful, any antimicrobial agent has to exhibit a broad-spectrum antimicrobial activity (Mohan et al., 2010). P34 is an anionic, thermostable, hydrophobic, lipidic, bacteriocin-like substance produced by a Bacillus sp. with antimicrobial properties against bacteria (Motta et al., 2007b, 2008) and viruses, according to the present study. However, anionic antimicrobial peptides are...
explain the activity of P34 against FHV-1 would be its interaction with the viral particles for the cellular receptors used in penetration or viral replication, or even exerts a competitive effect. A possible mechanism proposed to explain the activity of P34 against FHV-1 would be its interaction with cellular receptors like heparan sulfate, or even the blocking of certain viral glycoproteins. Heparan sulfate is the most important glycosaminoglycan molecule associated with to herpesvirus attachment to host cells (Spillmann, 2001; Luganini et al., 2010), consequently, any interference with heparan sulfate can reduce the viral infection (Shieh et al., 1992).

The virucidal activity of P34 may be due to a physicochemical interaction of the membrane-active surfactant with the virus lipid membrane, similarly to fengycin (Steller et al., 1999) or, alternatively the peptide P34 is EAV-specific, as no viral inactivation was detected against all the other enveloped viruses tested. We hypothesize that this peptide inactivates the virus through an interaction with a non-lipidic structural component.

EAV is a member of the family Arteriviridae and belongs to the order Nidovirales, along with porcine respiratory and reproductive syndrome virus, simian hemorrhagic fever virus and lactate dehydrogenase elevating virus (de Vries et al., 1997; Gorbalenya et al., 2006). Although equine viral arteritis causes severe economic losses to the equine industry, there is no specific treatment (Timoney and McCollum, 1993). Thus, there is a need for the development of antiviral drugs for the treatment of the disease. Herpesviruses are cosmopolitan agents causing several infections to humans and animals, especially in immunocompromised individuals (Felipe et al., 2006). A remarkable feature of the members of this family is their ability to cause and reactivate latent infections in their hosts, and this is important for the control of the disease (Hübner et al., 2005). Among the drugs that possess inhibitory action against herpesvirus replication, the most used in the human medicine are the nucleoside analogues (De Clercq, 2012) and there is evidence of resistance to some of them (De Clercq, 2004). Likewise it is necessary to search for new compounds with alternative mechanisms of action.

Effective antiviral agents are lacking, specifically those which target RNA viruses (Li et al., 2011). The current antiviral drug armamentarium comprises about 40 compounds that have been officially approved for clinical use; however, most of the approved drugs are used for the treatment of human immunodeficiency virus infections (Felipe et al., 2006). The fast and increased pathogen dissemination and resistance to drugs have forced the scientists to consider alternative methods to overcome infections (Motta et al., 2007a), mainly the emerging and re-emerging viral infections (Li et al., 2011). Therefore, as many AMPs are produced in nature, they may become an alternative to control specific pathogen infections (Riley and Wertz, 2002) and, according to the present study, the peptide P34 may be an interesting therapeutic prospection for the treatment of horses and cats affected by EAV and FHV-1, respectively. However, more detailed studies in vitro and in vivo must be performed to elucidate the specific mechanism of action of this peptide against viruses.

In summary, our results have indicated that the peptide P34 showed antiviral activity against EAV and FHV-1, with virucidal properties only against EAV. Nevertheless no antiviral activity was detected against CAV-2, CCoV, CDV, CPV-2, EIV and FCV.

### Table 1 - Antiviral activity of the peptide P34 against some viruses with different genotypic and phenotypic features.

| Virus | Genotypic and phenotypic features | P34 antiviral activity |
|-------|----------------------------------|-------------------------|
| CAV-2 | Non-enveloped                    | Absent                  |
| CPV-2 | Non-enveloped                    | Absent                  |
| CCoV  | Enveloped                        | Present                 |
| CDV   | Enveloped                        | Absent                  |
| EAV   | Enveloped                        | Present                 |
| EIV   | Enveloped                        | Absent                  |
| FCV   | Non-enveloped                    | Absent                  |

very rare (Paulmann et al., 2002) and it is thought that these peptides were developed in response to the resistance mechanisms toward cationic antimicrobial peptides (Lai et al., 2002), which are found in all species and are potential broad-spectrum antiviral agents (Albiol-Matanic and Castilla, 2004).

In order to evaluate the peptide P34 as an antiviral substance in vitro, CAV-2, CPV-2 and FHV-1 were exposed to the AMP, being all DNA viruses, only FHV-1 having an envelope (Felipe et al., 2006; Decaro and Buonavoglia, 2012, San Martín, 2012). The RNA viruses tested were CCoV, CDV, EAV, EIV and FCV, all enveloped viruses except for FCV (Seki et al., 2003; Abd-Eldaim et al., 2005; Diel et al., 2006; Gorbalenya et al., 2006; Decaro et al., 2007). According to the assays performed it seems that the peptide P34 does not have a broad antiviral activity, since it only inhibited EAV and FHV-1.

Some peptides have demonstrated their ability to kill rapidly a broad range of microorganisms including multidrug resistant bacteria, fungi and viruses by their lytic membrane properties (Reddy et al., 2004). AMPs like surfactin, magainin, mellitin and cecropin are known for their ability to interact with lipid membranes resulting in destabilization, translocation, pore formation or lysis (Vollenbroich et al., 1997; Sitaram and Nagaraj, 1999). It is possible that the peptide P34 interferes with the adsorption, penetration or viral replication, or even exerts a competitive effect with the viral particles for the cellular receptors used for EAV and FHV-1 infections. Blocking viral entry may occur by specific interactions with cellular receptors or viral envelope compounds, apart from viral glycoproteins (Jenssen et al., 2006). A possible mechanism proposed to explain the activity of P34 against FHV-1 would be its interaction with cellular receptors like heparan sulfate, or even the blocking of certain viral glycoproteins. Heparan sulfate is the most important glycosaminoglycan molecule associated with herpesvirus attachment to host cells (Spillmann, 2001; Luganini et al., 2010), consequently, any
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List of Abbreviations

AMP - antimicrobial peptide
CAV-2 - canine adenovirus type 2
CC<sub>50</sub> - Cytotoxic concentration 50%
CCoV - canine coronavirus
CDV - canine distemper virus
CPE - cytopathic effect
CPV-2 - canine parvovirus type 2
CRFK - Crandell-Rees Feline Kidney
EAV - equine arteritis virus
EIV - equine influenza virus
E-MEM - Eagle's minimum essential medium
FCV - feline calicivirus
FHV-1 - feline herpesvirus type 1
MDCK - Madin-Darby Canine Kidney
MTT - diphenyltetrazolium bromide
NRU - neutral red uptake
P34 - peptide P34
PI - percent of inhibition
RK13 - Rabbit Kidney Cells
TCID<sub>50</sub> - tissue culture infective dose 50%

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