Activity of the antimicrobial peptide P34 against bovine alphaherpesvirus type 1

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ABSTRACT: *Previous studies have demonstrated the antimicrobial activity of the peptide P34. In this study, the antiviral potential of P34 and the* in vitro *mechanism of action were investigated against bovine alphaherpesvirus type 1 (BoHV1). P34 exhibited low toxicity, a high selectivity index (22.9) and a percentage of inhibition of up to 100% in MDBK cells. Results from antiviral assays indicated that P34 did not interact with cell receptors, but it was able to inhibit the viral penetration immediately after pre-adsorption. In addition, BoHV1 growth curve in MDBK cells in the presence of P34 revealed a significant reduction in virus titer only 8h post-infection, also suggesting an important role at late stages of the replicative cycle. Virucidal effect was observed only in cytotoxic concentrations of the peptide. These findings showed that the antimicrobial peptide P34 may be considered as a potential novel inhibitor of* in vitro *herpesviruses and must encourage further investigation of its antiherpetic activity in animal models as well as against a wide spectrum of viruses.*
Key words: *antiviral, herpesvirus, peptide.*

Atividade do peptídeo antimicrobiano P34 contra o alfaherpesvírus bovino tipo 1

RESUMO: *A atividade antimicrobiana do peptídeo P34 já foi previamente demonstrada. Neste estudo, o potencial antiviral do P34 e o mecanismo de ação* in vitro *contra o alfaherpesvírus bovino tipo 1 (BoHV1) foram investigados. O P34 exibiu baixa toxicidade, alto índice de seletividade (22.9) e percentagem de inibição viral de até 100% em células MDBK. Os resultados dos ensaios antivirais indicaram que não interage com receptores celulares, mas é capaz de inibir a penetração viral, imediatamente após a pré-adsorção. Além disso, a curva de crescimento do BoHV1 em células MDBK na presença do P34 revelou uma significativa redução no título somente após 8h de infecção, sugerindo também uma importante atividade do peptídeo nas fases finais do ciclo replicativo. Efeito virucida frente / BoHV1 foi observado apenas em concentrações citotóxicas do peptídeo. Os dados obtidos indicam que o peptídeo antimicrobiano P34 pode ser considerado um potencial composto inibidor de herpesvírus,* in vitro*, e estimulam posteriores investigações sobre sua atividade anti-herpética em modelos animais, bem como contra outros vírus.*
Palavras-chave: *antiviral, herpesvírus, peptídeo.*

INTRODUCTION

Bovine herpesvirus 1 (BoHV1), is an alphaherpesvirus responsible for significant losses incurred by disease and trading restriction in the cattle industry. Primary infection is accompanied by various clinical manifestations such as infectious bovine rhinotracheitis, abortion, vulvovaginitis, and systemic infection in neonates (MUYLKENS et al., 2007).

P34 is a peptide produced by *Bacillus* sp. strain P34, isolated from the fish Piau-com-pinta (*Leporinus* sp.) reported in the Brazilian Amazon basin (MOTTA et al., 2007a). Many peptides are naturally produced in many organisms including bacteria, insects, plants, and vertebrates, and have been extensively studied with respect to their antimicrobial activity. These antimicrobial peptides (AMPs) are conserved components of the innate immune response (BULET et al., 2004).

The antimicrobial activity of P34 was characterized as a fengycin-like substance (MOTTA et al., 2007a) with a broad inhibitory effect against Gram-negative and Gram-positive bacteria such as *Listeria monocytogenes* and *Bacillus cereus* (MOTTA et al., 2007b). Further characterization of P34 by infrared spectroscopy and mass spectra demonstrated the hydrophobic nature of the peptide. This antimicrobial peptide has a molecular mass of 1,456Da and it is relatively heat-stable and sensitive to proteolytic enzymes (MOTTA et al., 2007b).

Additionally, a screening aimed to identify its activity against viruses of veterinary importance demonstrated that P34 exhibited antiviral activity against feline herpesvirus type 1 (FHV-1) and equine arteritis virus (EAV) (SILVA et al., 2014).

Despite the massive research efforts for the development of new compounds or even the enhancement of efficacy of the traditional chemical molecules, there are only a few drugs currently licensed for the treatment of herpesviruses. Resistance of human herpesviruses (HHV) to acyclovir has been widely reported and cidofovir and foscarnet - the other two drugs usually used for HHV therapy - are both nephrotoxic and their use restricted to confirmed cases of acyclovir resistance (GALDIERO et al., 2013).

A promising alternative for the development of new compounds with anti-herpetic activity and to overcome the side effects and resistance to the current therapies would be the search for antiviral peptides. Particularly, antimicrobial peptides can exert a broad spectrum of activity on infectious agents; they can be highly specific and effective and even biodegraded by peptidases, which limit their accumulation and results in lower toxicity (GALDIERO et al., 2013). Several reports have shown the antiviral potential of synthetic and naturally occurring peptides against different viruses such as HHV-1 and HHV-2, human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV), influenza A virus, cytomegalovirus, adenovirus, rotavirus and other viruses (DAHER et al., 1986; CARRIEL-GOMES et al., 2007; BASU et al., 2009). The objective of this study was to investigate the antiviral potential and the *in vitro* mechanism of action of the antimicrobial peptide P34 against bovine alphaherpesvirus type 1 (BoHV1).

MATERIALS AND METHODS

Peptide P34, virus and cells

The antimicrobial peptide P34 was produced as previously described (MOTTA el al., 2007b) and protein concentration measured by the Lowry method (Total Protein Kit, Micro Lowry, Sigma Aldrich, USA). P34 was stored in small aliquots at -20ºC until use.

The Los Angeles (LA) strain of BoHV1 was used in all assays. The virus was propagated and titrated in Madin Darby bovine kidney cells (MDBK, NBL-1– ATCC® Number: CCL22™, USA) as previously described (SPEARMAN, 1908; KARBER, 1931). Cells were cultured in Eagle's Essential Medium (E-MEM; Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 10mg mL^{-1} enrofloxacin (Bayer, Brazil), 0.025ug mL^{-1}amphotericin B (Cristália, Brazil), penicillin 200UI mL^{-1} (Sigma Aldrich Corporation, USA) and 0.2ug mL^{-1} streptomycin (Vetec, Brazil) and maintained at 37ºC and 5%CO$_2$ atmosphere.

Cytotoxicity assays

Different concentrations of P34 (1.0 to 5.5μgmL^{-1}) were added onto confluent monolayers of MDBK cells and the cellular viability was assessed after 72h of incubation by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method and neutral red assay as described elsewhere (MOSMANN, 1983; BORENFREUND & PUERNER, 1985). The absorbance at 540nm was measured in a microplate reader (TP-READER NM, Thermo Plate). The percentage of cell viability was calculated by AT/AC x 100; where AT and AC indicate the absorbance of treated and control cells, respectively (VAUCHER et al., 2010). The 50% cytotoxic concentration (CC$_{50}$) was defined as the concentration that caused a reduction of 50% in dye content. All cytotoxic assays were performed in triplicate.

Antiviral assays

Antiviral activity was analysed by comparing the virus titres reached on P34 treated with non-toxic concentration (1.2μg/mL)and non-treated MDBK cells and results were expressed in terms of percentage of inhibition (PI) using antilogarithm values of 50% tissue culture infectious dose per 100μL (TCID$_{50}$100μL^{-1}), as follows: PI = $[1-(T/C)]\times100$, where T and C are the antilogarithms of the titers observed in the P34 treated and control (without peptide) cells respectively. The P34 concentration which inhibited the cytopathic effect (CPE) by 50% was defined as the 50% effective concentration (EC$_{50}$) and the selectivity index (SI) was determined as the ratio between CC$_{50\,and}$ EC$_{50}$. Each one of the antiviral assays was performed in triplicate and virus titers were expressed as TCID$_{50}$100μL^{-1}.

Cytopathic effect (CPE) inhibition assay

BoHV1 was inoculated onto confluent monolayers of MDBK cells in the presence or absence of P34 (1.2μg mL^{-1}) and viral titers were determined 48h and/or 72h post infection (hpi).

Virus yield reduction assay

Confluent monolayers of MDBK cells were inoculated with 100 TCID$_{50}$ of BoHV1 and incubated for 1h at 37ºC in a 5% CO$_2$ atmosphere. After a washing step to remove non-adsorbed viral particles, cells were incubated or not with the non-toxic concentration of P34, as previously described by LUGANINI et al. (2010). After incubation for 24 and 48h, microplates were frozen, thawed, and the

supernatant submitted to virus titration.

Effect of P34 on pre-treated cells

       Monolayers of MDBK cells were incubated with a non-toxic concentration of P34 ($1.2\mu g mL^{-1}$) during 6 and 24h at 37ºC. After this period, treated cells were washed with E-MEM and a titration with BoHV1 was performed. Viral titer was determined after 72h and compared with controls (BoHV1 titration on non-treated cells).

Effect of P34 on cell receptors for BoHV1

       P34 ($1.2\mu g mL^{-1}$) was added onto confluent monolayers of MDBK cells for 1h at 37°C. After washing with phosphate buffered saline (PBS), 100 $TCID_{50}$ of BoHV1 was inoculated onto cells treated with P34 followed by incubation for 1h at 37°C. E-MEM was added onto cells after a new washing step.The experiment was also carried out in absence of P34. After 48h of incubation, all microplates were submitted to freeze-thaw cycles with subsequent titration of the supernatant.

Viral penetration assay

       Confluent monolayers of MDBK cells grown in 96-well plates were inoculated with 100 $TCID_{50}$ of BoHV1 and adsorbed for 2h at 4ºC, as previously described by HU &HSIUNG (1989) with modifications. After removing the medium containing unbound virus, $1.2\mu g mL^{-1}$ of P34 was added or not (control) during viral entry to the cells, at 37°C for 10min. Cells were then washed with neutralization buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, 0.5 M glycine, pH 3.0) for 1min to inactivate non-penetrated viruses. After washing twice with PBS, E-MEM was added onto cells and after 72h of incubation at 37°C the microplates were submitted to freeze-thaw cycles and the supernatant was titrated.

Effect of P34 on BoHV1 growth curve in MDBK cells

       Confluent monolayers of MDBK cells were infected with BoHV1 with a multiplicity of infection (MOI) of 1. After adsorption for 1h at 37ºC, cells were then washed with E-MEM and treated or not with P34 ($1.2\mu g mL^{-1}$) for 1, 8, 12 and 18h. Supernatants of infected cells, treated or not with P34 at different time points were collected and kept at -70ºC for titration.

P34 virucidal effect

       BoHV1 was incubated with P34 ($1.2\mu g mL^{-1}$) for 6, 12 and 24h at 4°C, 20°C and 37°C. The presence of residual infectious virus was evaluated by titration on confluent monolayers of MDBK cells. A viral suspension with E-MEM without P34 was used as positive control.

Statistical analysis

       Statistical analysis were performed using a two-tailed Student's t-test and values were considered significant when $P<0.01$.
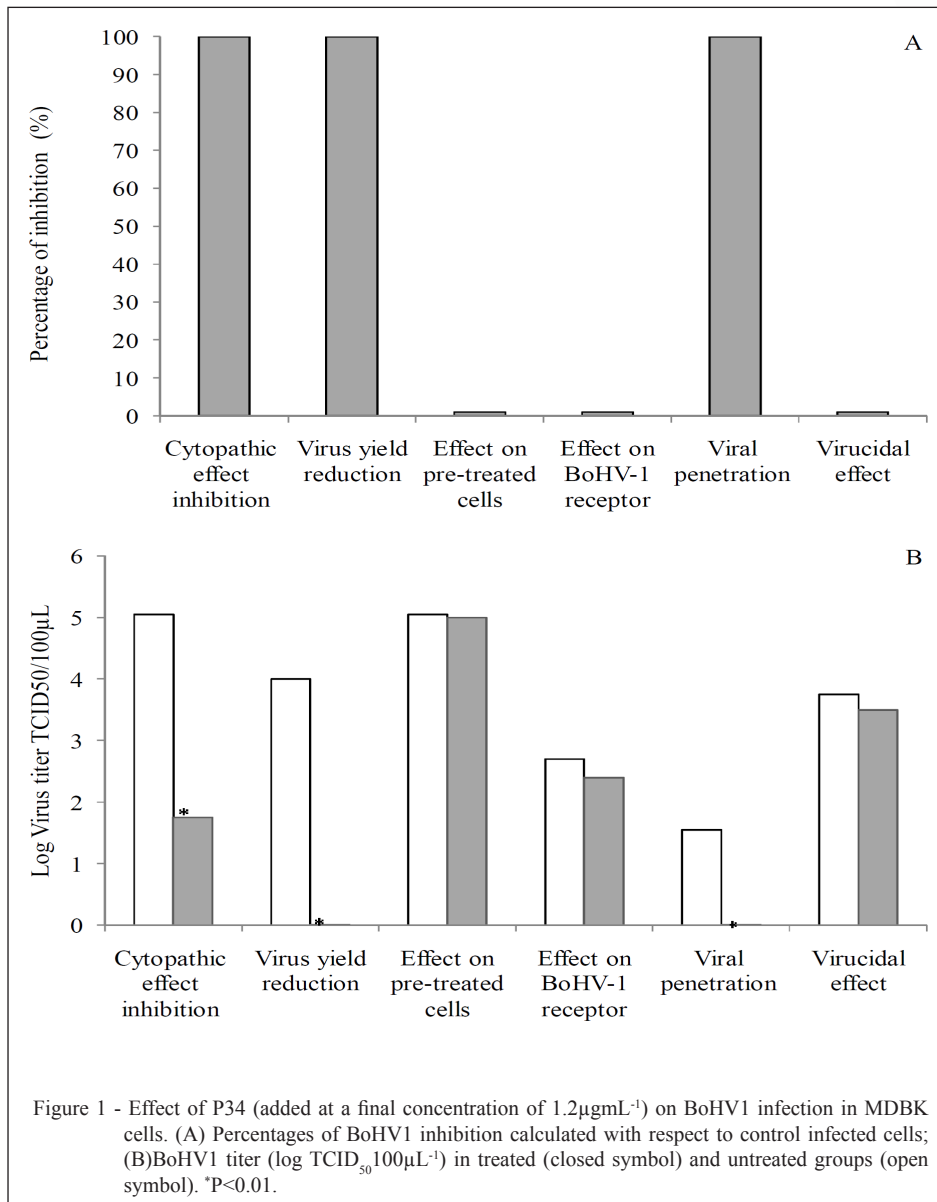
RESULTS AND DISCUSSION

       Antimicrobial peptides have been gaining attention as important therapeutic intervention alternatives in the field of disease prevention and care against a variety of microorganisms (OYSTON et al., 2009). There are about 100 peptide-based drugs in the market, constituting about 10% of the entire drug market (CRAIK et al., 2013).

       Antiviral activity exerted by peptides often appears to be a result of a direct effect on the viral envelope or by interference with different stages of the viral replication cycle (WACHSMAN et al., 2003; JENSSEN et al., 2006). In this study, the antiviral potential and the *in vitro* mechanism of action of the antimicrobial peptide P34 against BoHV1 was investigated. Toxicity of the peptide on MDBK cells was not observed at concentrations $\leq 1.2\mu g$ mL^{-1}, and it was used in all the antiviral assays. The CC_{50} of the peptide P34 was $2.29\mu g$ mL^{-1} and the average EC_{50} was $0.1\mu g$ mL^{-1}.

       Percentages of viral inhibition up to 100% were observed using P34 in the different antiviral assays (summarized in figure 1 A), confirming the antiviral potential of P34 *in vitro*. The CPE inhibition assay allowed us to observe a significant reduction on virus titers between untreated infected cultures ($10^{5.05}$ $TCID_{50}$) and those cultures treated with P34 ($10^{1.75}$ $TCID_{50}$), resulting in a PI of 99.94%. In the virus yield reduction assay, the BoHV1 titers observed were $10^4 TCID_{50}$ after 48h of incubation and in the presence of P34 no viral titer was detected, as demonstrated by the absence of CPE (PI of 100%).

       Considering the low toxicity of the P34 for MDBK cells and the significant inhibitory effect of BoHV1 *in vitro*, we investigated the step of BoHV1 replication cycle targeted by the P34. To better understand the mechanism of action, a viral growth curve was performed in the presence or absence of P34 in order to address whether the peptide would still inhibit the virus after the binding step or in a post-infection stages of the BoHV1 replication cycle in MDBK cells. Analyzing the BoHV1 growth curve in MDBK cells in the presence of P34 (Figure 2), it was possible to observe a significant reduction in the titer 8h post-BoHV1 infection (PI=90%). After 12 and 18h of treatment, PIs of 99% and 99.9% were detected,
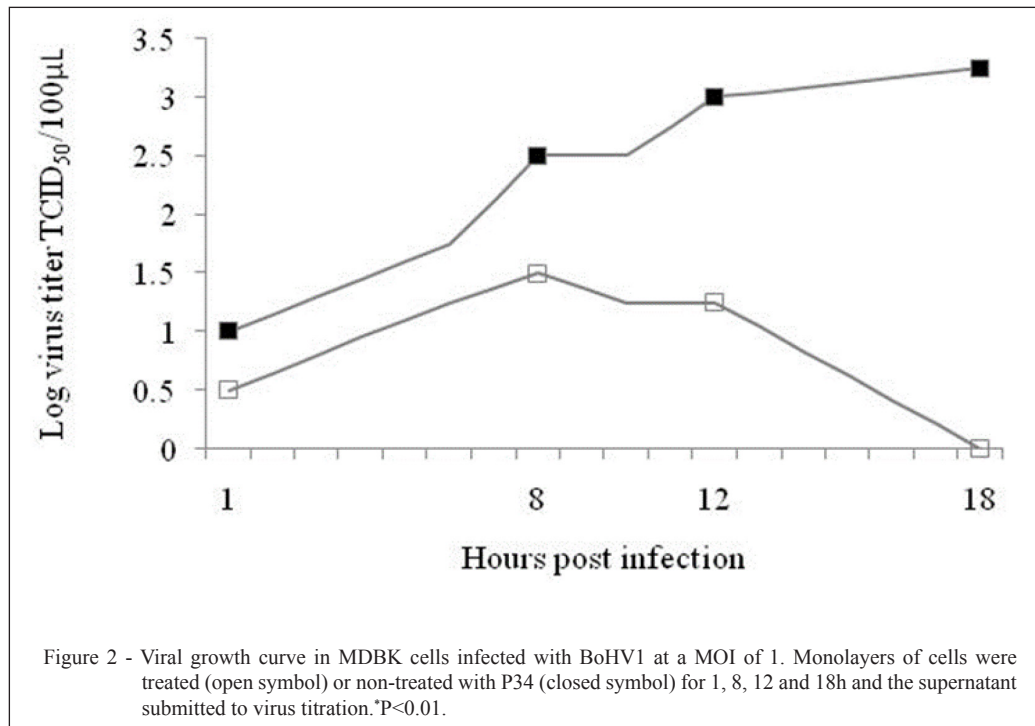
Figure 1 - Effect of P34 (added at a final concentration of 1.2µgmL^{-1}) on BoHV1 infection in MDBK cells. (A) Percentages of BoHV1 inhibition calculated with respect to control infected cells; (B)BoHV1 titer (log TCID$_{50}$100µL^{-1}) in treated (closed symbol) and untreated groups (open symbol). *P<0.01.

respectively (P<0.01). Based on these preliminary results, P34 was added only 8hpi to verify the activity of P34 at late stages of the BoHV1 replication cycle. Viral titration performed after 24h incubation at 37°C showed no detectable virus in the supernatant of treated cells. Conversely, 10^3 TCID$_{50}$ of BoHV1 was detected in the positive controls (data not shown).

Pre-treatment of MDBK cells with P34 for 6 or 24h did not result in reduction of the viral titer. Similarly, the assay performed in order to verify the possibility of interaction with receptors on MDBK cells, indicated the peptide was not capable of

blocking or interact with BoHV1 receptors. Addition of 100TCID$_{50}$ of the virus on MDBK cells resulted in a titer of 10$^{2.70}$ TCID$_{50}$ after 48h of incubation. When the peptide was incubated for 1h before the inoculation of 100 TCID$_{50}$ of the virus a similar titer (10$^{2.4}$TCID$_{50}$) was observed (Figure 1B).

Results obtained from the viral penetration assay have indicated that peptide P34 may inhibit virus penetration. No detectable virus was observed when P34 was added on cell monolayers immediately after pre-adsorption at 4°C whereas 10$^{1.5}$ TCID$_{50}$ of BoHV1 was detected

Figure 2 - Viral growth curve in MDBK cells infected with BoHV1 at a MOI of 1. Monolayers of cells were treated (open symbol) or non-treated with P34 (closed symbol) for 1, 8, 12 and 18h and the supernatant submitted to virus titration.*P<0.01.

in the absence of the peptide (Figure 1). Due to the lipophilic and hydrophobic features of P34, the mechanism of action may be explained by interaction of the peptide with the viral envelope and subsequent interference or even blockage of the viral binding to host cell receptors. Additionally, in order to determine whether P34 might directly inactivate BoHV1 virions, a viral suspension was incubated along with the peptide under different conditions of temperature and time. Results of these experiments showed the virus titer was not reduced after treatment with $1.2\mu g\ mL^{-1}$ up to 24h.

Overall, P34 presented a SI of 22.9, indicating a great potential to be used as a therapeutic drug, once its security margin has been determined (AL-KHAYAT & AHMAD, 2012). Taken together, the results showed that peptide P34 acts in different steps of the BoHV1 replication in MDBK cells. According to the obtained data, P34 exerts activity in the adsorption/penetration and assembly/egress stages of the viral particle, possibly through interactions with phospholipids and/or viral proteins. Even though the results presented here are promising, further investigations must be performed to unravel the molecular basis for the exact anti-BoHV1 mechanism of action of P34.

CONCLUSION

Taken together, the findings of this study showed that the peptide P34 may be considered as a potential novel inhibitor of herpesvirus infection and may encourage additional investigation of its potential antiherpetic activity in animal models.

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