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Short Communication

Synthetic analogues of bovine bactenecin dodecapeptide reduce herpes simplex virus type 2 infectivity in mice

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ABSTRACT

We have evaluated the potential of four synthetic peptides (denoted HH-2, 1002, 1006, 1018) with a distant relationship to the host defense peptide bovine bactenecin dodecapeptide for their ability to prevent genital infections with herpes simplex virus type 2 (HSV-2) in mice. All four peptides showed antiviral properties *in vitro* and reduced HSV-2 infection of Vero cells in a dose-dependent manner. Detailed analysis showed that the peptides were able to interfere with both viral attachment and entry, but not with replication post-entry, and were effective antivirals also when HSV-2 was introduced in human semen. Two of the peptides proved especially effective in reducing HSV-2 infection also *in vivo*. When admixed with virus prior to inoculation, both HH-2 and 1018 reduced viral replication and disease development in a genital model of HSV-2 infection in mice, and also when using very high infectious doses of HSV-2. These data show that peptides HH-2 and 1018 have antiviral properties and can be used to prevent genital herpes infection in mice.

Herpes simplex virus type 2 (HSV-2) is one of the most common causes of genital ulcerative diseases worldwide (Halioua and Malkin, 1999). The infection is prevalent in Sweden, with approximately 25% of the adult population being infected. HSV-2 is transmitted through sexual contact and the virus initially infects and replicates in the vaginal epithelium. Thereafter the virus enters sensory nerve endings whereby it reaches the dorsal root ganglia where a life-long persistent infection is established. The outcome of HSV-2 infection varies, with some individuals developing severe and recurrent episodes of genital herpes while others remain asymptomatic. Virus is shed intermittently in the genital tract irrespective of disease status, and the infection can thus be transmitted also by those who lack any symptoms of disease (Fatahzadeh and Schwartz, 2007).

Genital herpes is a risk factor for later HIV infection (Strick et al., 2006; Freeman et al., 2006) which has emphasized the need of developing effective HSV-2 preventive treatments. To date, there exist no vaccines against HSV-2 or any eradicating cure. Viral replication can however be controlled by drugs that inhibit the viral DNA polymerase. The standard HSV-2 treatment drugs acyclovir and penciclovir are effective in most patients, but there are cases of treatment resistance, especially in immune-compromised patients (Duan et al., 2008; Frobert et al., 2008; Reyes et al., 2003).

Host defense (antimicrobial) peptides (HDPs) are part of the first line of innate immune defenses against infections. Several groups of natural HDPs have been identified (Wiesner and Vilcinskas, 2010) and the most predominant in mammals are the defensins and the cathelicidins (Yeung et al., 2011). These peptides are produced mainly by epithelial, phagocytic and bone-marrow derived cells (Jenssen et al., 2006; Selsted and Ouellette, 2005; Zanetti, 2004) and can be found in different body compartments, including mucosal surfaces and fluids, where they show important anti-infective and anti-inflammatory activities (Straus and Hancock, 2006; Wiesner and Vilcinskas, 2010). HDPs have a potent ability to alter cellular functions such as chemotaxis, apoptosis, wound healing, cellular differentiation, and inflammation through extensive modulation of gene transcription and cytokine/chemokine production, and are thus considered immunomodulatory (Allaker, 2008).

A number of mammalian HDPs have antiviral properties and inhibit the growth of many different human viral pathogens, in particular enveloped viruses (Bai et al., 2007; Buck et al., 2006; Daher

Abbreviations: AMPs, anti-microbial peptides; GAG, glycosaminoglycans; HS, heparan sulfate; HSV-2, herpes simplex virus type 2; HDPs, host defense peptides; HNP, human neutrophil peptides.

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Table 1
Heparan-binding capacity and immunomodulatory properties of Bac2a and its derivatives.

Name	Sequence	M.W.	Elution from HS (mM NaCl)[a]	Net charge	Chemokine-induction in human PBMC (pg/ml)[b]		Reference
					CCL2	CXCL1	
Bac2a	RLARIVVIRVAR-NH_2[c]	1421	ND[b]	+5	442	8	Wieczorek et al. (2010); Hilpert et al. (2005)
1002	VQRWLIVWRIRK-NH_2	1652	193	+5	5566	2117	Nijnik et al. (2010)
1006	VQLRIWVRR-NH_2	1225	276	+4	3004	1245	
1018	VRLIVAVRIWRR-NH_2	1536	214	+5	13,041	2692	Wieczorek et al. (2010)
HH-2	VQLRIRVAVIRA-NH_2	1393	274	+4	10,235	2693	

[a] Peptides (2 mg/ml in milli-Q water) were analyzed for their HS binding activity using fast protein binding chromatography. A column containing HS attached to CNBr-activated Sepharose was packed using milli-Q water as medium. The column was attached to an ÄKTA purifier (Amersham Bioscience), set up to perform cationic exchange with an increasing NaCl gradient starting at 0% and running to 50% over 20 min at a flow rate of 1 ml/min. Peptide samples of 100 μl were injected and the retention time of the eluted peptides were detected at $\lambda = 214$ nm (Jenssen et al., 2004b).

[b] Venous blood from healthy volunteers was collected in accordance with University of British Columbia ethical approval and guidelines. Blood was separated by centrifugation over a Ficoll-Paque Plus (Amersham Biosciences) density gradient. PBMC (5×10^5) were seeded into 24 well tissue culture dishes (Falcon; BD Biosciences) at 1×10^6 cells/ml at 37 °C in 5% CO_2, and rested for 1 h. The cells were then exposed to peptides (50 μg/ml). 24 h tissue culture supernatants were analyzed for their content of CXCL1 (Gro-α) and CCL2 (MCP-1) using sandwich ELISA kits (BioSource International and eBiosciences, respectively).

[c] The sequence of the original bactenecin dodecapeptide is RLCRIVVIRVCR.

et al., 1986; Leikina et al., 2005; Steinstraesser et al., 2005). They have a broad range of antiviral mechanisms ranging from direct effects on the viral envelope, through inhibition of viral adsorption and entry to inhibition of intracellular targets (Jenssen et al., 2006). Both cathelicidins and defensins have antiviral activity towards HSV. LL-37, the only human cathelicidin, has anti-HSV-1 and HSV-2 activity *in vitro* (Yasin et al., 2000; Howell et al., 2006) and can, when administered to mice, partly protect against HSV-1 infection in the cornea and conjunctiva (Gordon et al., 2005). Accordingly, mice that lack mCRAMP, the murine homologue of LL-37, have an impaired ability to control HSV-1 replication (Howell et al., 2006). The human α-defensins human neutrophil peptides (HNP) 1, 2, 3 as well as θ-defensins and the human β-defensin 3 can inhibit HSV-1 and HSV-2 *in vitro* (Brandt et al., 2007; Daher et al., 1986; Hazrati et al., 2006; Sinha et al., 2003; Yasin et al., 2000) and do so by interfering with viral adhesion, entry and cell-to-cell spread (Sinha et al., 2003; Yasin et al., 2004). Defensins are found in vaginal fluid samples from healthy women, and the concentration of defensins in these samples correlates with their anti-HSV activity, both in viral infectivity assays *in vitro* and in a mouse model of genital herpes (John et al., 2005; Shust et al., 2010).

Increasing efforts have been made in recent years to design and develop synthetic peptides with enhanced antimicrobial or, more recently, immunomodulatory activities using natural host defense peptides as templates. There are now several examples of synthetic peptides with antiviral potential (Jenssen et al., 2006). These peptides often rely on their amphiphatic structure for their antiviral activity (Jenssen et al., 2004a; Yasin et al., 2000). Examples of synthetic antiviral peptides include both microbe-derived and host-derived peptide analogues, and these peptides can interfere with several medically important enveloped viruses (Cho et al., 2009; Mohan et al., 2010). The potential of using synthetic anti-microbial peptides (AMPs) against HSV is underscored by studies showing that synthetic derivatives of both human beta-defensins and frog magainins as well as several different families of synthetic peptides have anti-HSV activity *in vitro* (Egal et al., 1999; Jenssen et al., 2004a; Krepstakies et al., 2012; Luganini et al., 2011; Scudiero et al., 2010).

In this study we investigated the ability of four synthetic cationic AMPs with a distant relationship to bovine bactenecin dodecapeptide (1002, 1006, 1018 and HH-2; Table 1) to block HSV-2 infection *in vivo*. These peptides were synthesized by GenScript USA Inc. and were selected from a large panel of peptides based on their immunomodulatory properties, and three of these, 1002, 1018 and HH-2, have previously been reported to have strong immunomodulatory and weak anti-bacterial activities (Kindrachuk et al., 2009; Nijnik et al., 2010; Wieczorek et al., 2010).

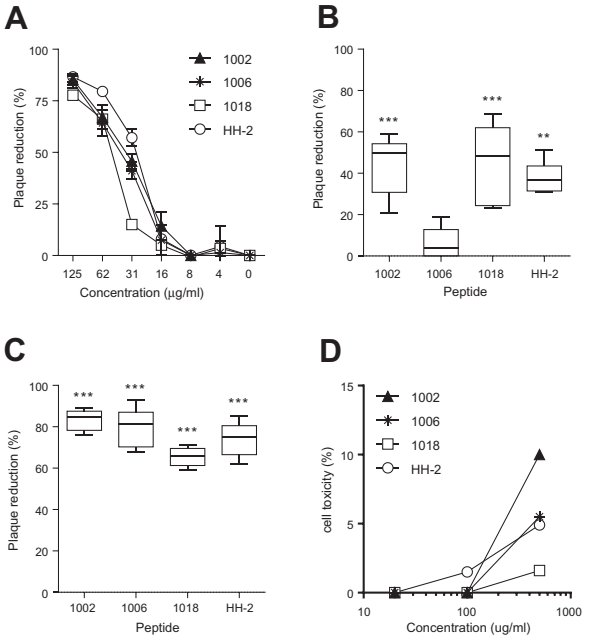

Fig. 1. Synthetic AMPs inhibit HSV-2 infection *in vitro*. Vero cells were exposed to HSV-2 (4×10^4 PFU) together with serial dilutions of peptides 1002, 1006, 1018 or HH-2 (A), following a 30 min pre-incubation with 1002, 1006, 1018 or HH-2 (100 μg/ml) (B), or together with 25% (v/v) human semen containing peptides 1002, 1006, 1018 or HH-2 (100 μg/ml) (C), Numbers of plaques were determined 3 days later, and the data are expressed as mean plaque reduction after infection in samples treated with either of the antimicrobials when compared to control samples (A) and as the median plaque reduction and the 25% and 75% percentile (the boxes) with the minimum and maximum responses (B and C). The cytotoxicity of peptides 1002, 1006, 1018 and HH-2 was determined after 24 h incubation on Vero cells using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) (D) and are expressed as percentage of specific cytotoxicity. $^{***}p < 0.001$ and $^{**}p < 0.01$ using ANOVA with Bonferroni's post-test.

When tested *in vitro*, all four peptides were able to reduce HSV-2 (strain 333) infection *in vitro* in a dose-dependent manner. Both peptides 1002 and 1018 reduced the number of plaques by >50% at a dose of 31 μg/ml (≈20 μM) whereas peptides 1006 and HH-2 gave a >50% plaque reduction at 62 μg/ml (≈40 μM). At 125 μg/ml (≈80 μM) peptides 1002 and 1018 reduced the number of plaques by >75% (Fig. 1A). The peptides were equally effective also

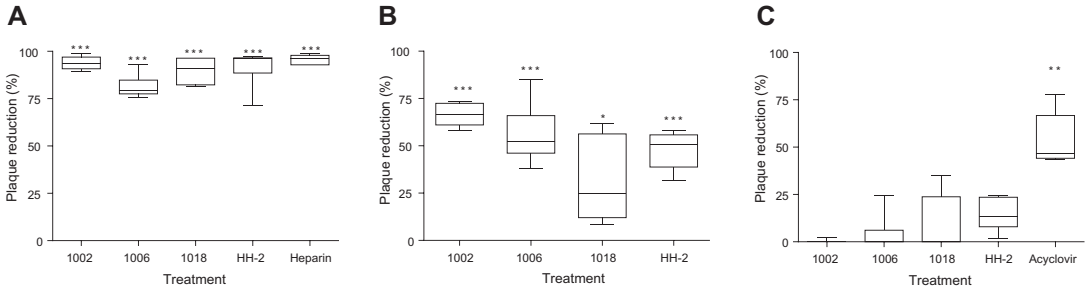

Fig. 2. Synthetic AMPs reduce viral attachment and entry. Vero cells were cooled to 4 °C and inoculated with 100 PFU/ml HSV-2 at 4 °C for 4 h. Unbound virus was removed by extensive washing, and the cultures were shifted to 37 °C for 30 min to permit viral penetration. Non-penetrant virus was inactivated by treating the cells with a pH 3.0 citrate buffer for 30 s followed by washing, and fresh medium was added. Synthetic peptides 1002, 1006, 1018 or HH-2 (100 μg/ml) were added either during the 4 °C incubation (viral attachment; A), at the time of temperature shift (penetration; B) or post-citrate treatment (post entry; C). Numbers of plaques were determined 3 days later, and the data are expressed as plaque reduction, depicted as medians and the 25% and 75% percentile (the boxes) with the minimum and maximum responses, after infection in samples treated with either of the antimicrobials when compared to control samples. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ using ANOVA with Bonferroni's post-test.

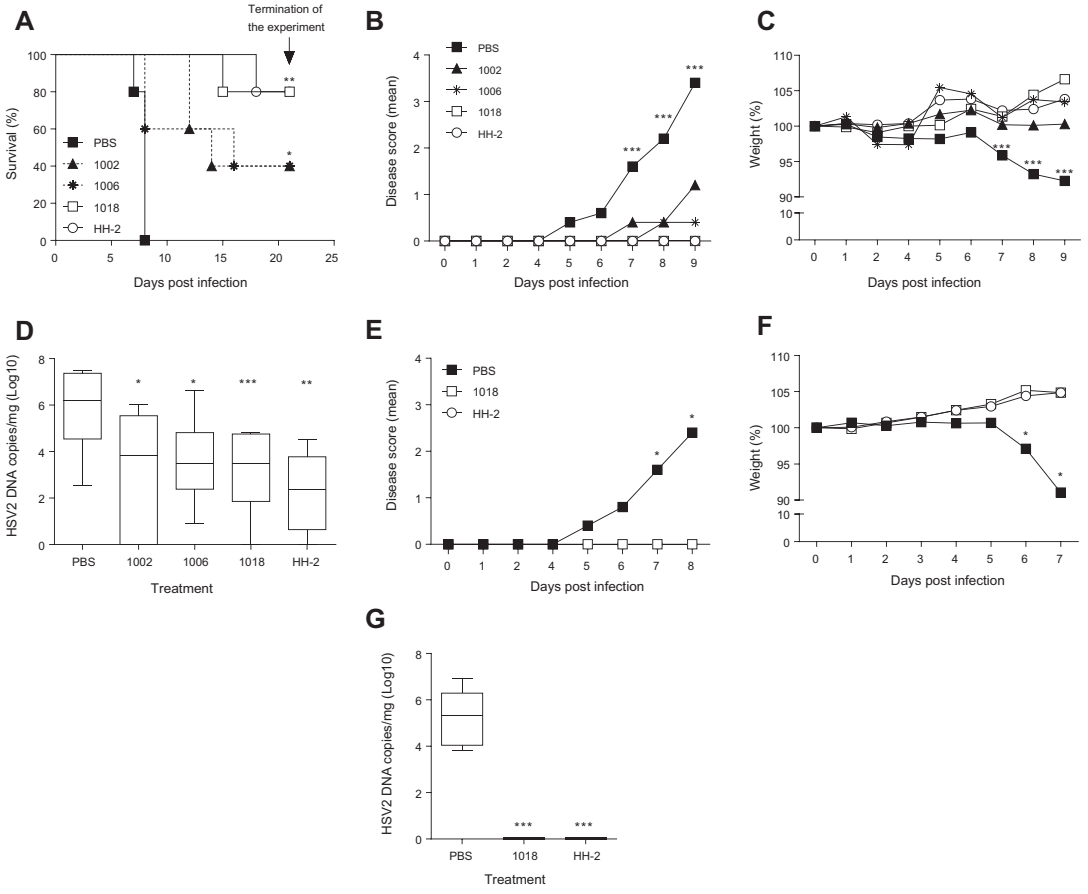

Fig. 3. 1018 and HH-2 reduce genital HSV-2-infection in mice. C57/BL6 mice were injected subcutaneously with 2 mg of Depo-Provera (Pharmacia) and 5 days later inoculated with 2×10^5 PFU (A), 4×10^4 PFU (B–D) or 8×10^3 PFU (E–G) of HSV-2 together with either 200 μg (A–D) or 100 μg (E–G) of 1002, 1006, 1018 or HH-2 in a total volume of 40 μl 0.9% NaCl. Disease development was followed daily and expressed either as survival (A; $n = 5$) or mean disease score graded from 0 to 5, i.e. healthy (0), genital erythema (1), moderate genital inflammation (2), genital lesion and/or generally bad condition (3), hind-limb paralysis (4), death or sacrifice due to paralysis (5) (Morrison et al., 1998) (B and E; $n = 10$). Weight loss was determined daily and is expressed in percentage of weight compared to day 0 of infection ($n = 10$ per group) (C and F). Numbers of HSV-2 DNA copies per mg spinal cord (day 9) were determined by quantitative PCR using TGCAGTTTACGTATAACCACATACAGC as forward primer and AGCTTGCGGGCCTCGTT as reverse primer to amplify a 118-nucleotide segment of the gB region as described {Namvar et al., 2005}. A plasmid (pUC57) containing the target sequence was constructed (GenScript) and amplified in *E. coli* XL-1 Blue, purified by HiSpeed Plasmid Maxi Kit (Qiagen) and quantified by spectrophotometer analysis. A standard curve was included in each run and based on 6 fivefold dilutions of the plasmid using an initial concentration of 1×10^6 HSV-2 genome copy numbers per reaction. Data are expressed as HSV-2 copy number per mg spinal cord depicted as medians and the 25% and 75% percentile (the boxes) with the minimum and maximum responses for $n = 10$ (D and G). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ using log-rank (Mantel–Cox) test (A) or ANOVA with Bonferroni's post-test (B–G). The studies were approved by the ethical committee for animal experiments at the University of Gothenburg.

against another strain of HSV-2, strain Lyons (Andrei et al., 1997) (not shown). Since HSV-2 infection is initiated through the binding of virus to cell surface glycosaminoglycans (GAG) such as heparan sulfate (HS) (WuDunn and Spear, 1989), we tested if the antimicrobials could bind to HS. All four peptides bound to HS, a representative GAG, with a relatively high binding activity (Table 1). Given

that the peptides could bind HS, we also tested if the peptides could block HSV-2 infection *in vitro* when used prophylactically. 1002, 1018 and HH-2 pre-treatment of Vero cells prior to HSV-2 exposure led to a significant inhibition of HSV-2 plaque formation (Fig. 1B). 1006 pre-treatment of Vero cells did however not interfere with viral infectivity (Fig. 1B). We also investigated to what extent the peptides retained their antiviral activity when HSV-2 was introduced in semen. All four peptides were still able to reduce HSV-2 infection when virus and peptides were admixed with 25% (v:v) human semen prior to administration (Fig. 1C). Lastly, 1002 showed a weak cytotoxic effect on Vero cells, whereas the other three synthetic AMPs had no/little cytopathic effects (Fig. 1D).

To identify at what specific stage of the viral infection that the AMPs exerted their effects, we conducted the synchronized infection assay. This assay, described in detail by Hazrati et al. (2006) allows us to evaluate separately the entry, penetration and post-entry stages of HSV-2 infection. All four synthetic peptides almost completely blocked infection when they were added during viral attachment (Fig. 2A). All four peptides were also capable of interfering with the viral penetration and reduced the number of plaques by approximately 50% (Fig. 2B). None of the peptides were capable of interfering with viral replication when added post-entry (Fig. 2C).

1002, 1006, 1018 and HH-2 were all capable of interfering with viral attachment and entry into Vero cells *in vitro* but none of the peptides could affect viral replication once viral entry was achieved. This pattern is similar to that of the human β-defensin 3, which also reduces viral attachment and entry but not post-entry events (Hazrati et al., 2006) and contrasts to the human α-defensins HNP-1, HNP-2, HNP-3 and HD5 which can reduce HSV-2 replication also post entry, and the human α-defensin HNP-4 and HD6 which only reduce viral attachment (Hazrati et al., 2006). Thus, the mode of action of our synthetic AMPs appears similar to that of human β-defensin 3.

Even though 1006 could reduce viral attachment *in vitro*, it was considerably weaker in this respect compared to the other peptides, and in particular when it was used to pre-treat cells at 37 °C. This implies that 1006 is less efficient at binding to Vero cells and/or that it is less stable at 37 °C compared to the other peptides.

To study the ability of the synthetic antimicrobial peptides to block HSV-2 infectivity *in vivo*, we used a well-known mouse model of genital HSV-2 infection (Parr et al., 1994). Female C57/BL6 mice obtained from ScanBur, Sweden were inoculated intra-vaginally with HSV-2 alone or with a mixture of HSV-2 and synthetic peptides. In this experimental setting, HH-2 and 1018 demonstrated strong antiviral activity *in vivo* (Fig. 3). First, we tested an extremely high dose of HSV-2 (2×10^5 PFU corresponding to $500 \times LD_{50}$). Animals receiving this dose of virus but no synthetic AMP were all dead within 8 days of viral exposure (Fig. 3A). Four out of five mice given virus admixed with either 1018 or HH-2 (200 μg) survived the viral challenge (Fig. 3A). Peptides 1002 and 1006 were less effective and three out of five mice in each group died from the infection (Fig. 3A).

Mice given an intermediate dose of HSV-2 (4×10^4 PFU corresponding to $100 \times LD_{50}$) admixed with 1018 or HH-2 showed almost no signs of disease (Fig. 3B) or weight loss (Fig. 3C) and 1018- and HH-2-treated animals had a median of only 2800 and 240 viral DNA copies per mg spinal cord, respectively, (Fig. 3D) compared to mice inoculated with virus alone where the average viral DNA content per mg tissue was above 10^6 ($p < 0.001$ by ANOVA with Bonferroni's post-test). Peptides 1002 and 1006 were again less effective, even though only two out of ten mice treated with peptide 1006 and five out of ten mice treated with peptide 1002 developed signs of disease (Fig. 3B), and the viral load in the CNS was reduced compared to PBS-treated animals (Fig. 3D).

Both 1018 and HH-2 completely blocked disease progression and viral replication in the CNS when mice were infected with a low dose of virus (8×10^2 PFU corresponding to $20 \times LD_{50}$). Furthermore, both AMPs retained this effect also at lower doses (100 μg) (Fig. 3E–G). In summary, HH-2 and 1018 appear to be particularly effective at preventing HSV-2-infection of mice.

The vast majority of studies showing antiviral activities of both natural and synthetic HDPs have been performed in tissue culture systems *in vitro*. An exception is the study by Hazrati et al. (2006) where it is shown that the human α-defensin HD5 is effective at reducing HSV-2-infection both *in vitro* and *in vivo*. We show that although HH-2, 1002, 1006 and 1018 were all highly effective as antivirals when tested used *in vitro*, only HH-2 and 1018 were capable of preventing viral dissemination as well as disease development *in vivo*. This shows that data from *ex vivo* systems must be treated with caution and highlight the importance of performing appropriate *in vivo* experimental tests before a synthetic AMP is extrapolated as being a potential microbicide.

Jenssen et al. (2004b) have previously shown that neither HS affinity nor the positive net charge of a synthetic peptide can alone predict the antiviral activity of a potential synthetic peptide mimic of AMPs or HDPs. We confirm this notion as the HS-binding activity did not correlate with either the prophylactic capacity of the peptides *in vitro* or the protective efficacy of the peptides *in vivo*. Thus, even though 1006 and HH-2 has a similar molecular weight and identical positive net charge and HS-binding activity, pre-treatment of Vero cells with HH-2 but not with 1006 protected the cells from a later HSV-2 infection and HH-2 was also superior at protecting mice from vaginal HSV-2 infection. Similarly, 1002 and 1018 were the largest of the four peptides, had identical net positive charge and both peptides had a lower affinity for heparin sulfate compared to 1006 and HH-2. Yet, both 1002 and 1018 were effective at preventing HSV-2 infection of Vero cells when used prophylactically, and 1018, but not 1002, was highly effective as an antiviral agent when used topically in mice. Thus, other factors than size, charge and HS affinity must influence the efficacy of AMPs both *in vitro* and *in vivo*.

In summary, we found that the four synthetic analogues of bovine bactenecin dodecapeptide were able to reduce HSV-2 infection in vitro in a dose-dependent manner by reducing viral attachment and entry. HH-2 and 1018 proved especially effective in preventing HSV-2 disease development in mice when the peptides were admixed with virus prior to administration. These data identify HH-2 and 1018 as potent HSV-2 virucides.

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