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Article

Evaluation of the Inactivation of Infectious Herpes Simplex Virus by Host-Defense Peptides

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Abstract A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide microplate assay was adapted to screen for the ability of 20 host-defense peptides to inactivate herpes simplex virus type 1 and type 2. The procedure required minimal amounts of material, was reproducible, and was confirmed with standard antiviral testing techniques. In screening tests, with the exception of melittin, a highly cytotoxic and hemolytic peptide found in bee venom, the α-helical peptides in our test panel (magainins, cecropins, clavanins, and LL-37) caused little viral inactivation. Several β-sheet peptides (defensins, tachyplesin, and protegrins) inactivated one or both viruses, sometimes with remarkable selectivity. Two peptides were identified as having antiviral activity against both viruses, indolicidin (a tryptophan-rich peptide from bovine neutrophils) and brevinin-1 (a peptide found in frog skin). The antiviral activity of these two peptides was confirmed with standard antiviral assays. Interestingly, the antiviral activity of brevinin-1 was maintained after reduction and carboxamidomethylation, procedures that abolished its otherwise prominent hemolytic and cytotoxic effects.

Introduction

Many peptides with broad-spectrum antimicrobial activity have been isolated from vertebrates and invertebrates. Most are amphipathic molecules that contain between 15 and 40 amino acid residues, and many have well-defined β-sheet or α-helical structures. β-sheet host-defense peptides of mammals, exemplified by defensins and protegrins, manifest potent antimicrobial activity against a wide range of organisms, including *Neisseria gonorrhoeae* [1], *Chlamydia trachomatis* [2, 3], *Escherichia coli* [4], and *Candida albicans* [5]. Defensins also directly inactivate certain enveloped viruses, including vesicular stomatitis and herpes simplex [6, 7]. Tachyplesins and protegrins, small β-sheet peptides found respectively in the blood cells of horseshoe crabs and pigs, have been reported to inactivate HIV-1 [8]. The antiviral properties of α-helical host-defense peptides have been studied less extensively, although both magainin-2 [9] and cecropin-like peptides isolated from the hemolymph of *Heliothis virescens* larvae [10] were reported to lack activity against herpes simplex virus type 1 (HSV-1).

Multiple techniques can be used to evaluate the ability of host-defense peptides to inactivate enveloped viruses such as herpes simplex. These include plaque reduction [11], dye uptake [12, 13], nucleic acid hybridization [14, 15], enzyme-linked immunosorbent assays [16, 17], conventional assays [18, 19], and microtiter virus yield reduction assays [20]. However, such assays typically consume large amounts of antiviral peptide and have limited utility for screening large numbers of synthetically modified peptides. In this report, we used a quantitative microplate assay to screen a panel of 20 host-defense peptides for their ability to inactivate HSV-1 and herpes simplex virus type 2 (HSV-2). This screening assay requires small amounts of peptide and evaluates for cytotoxicity. After initial screening, the best peptide candidates showing antiviral susceptibility were selected and then subjected to standard antiviral

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testing techniques, namely plaque-reduction assays and dose-response assays using the microplate system.

Using sequential screening and confirmatory testing, we were able to identify significant antiviral activity in two of the 20 peptides tested (indolicidin and carboxamidomethylated [CAM]-brevinin). Furthermore, we confirmed that neither of these peptides had significant cytotoxicity and both are thus excellent candidates for topical microbicides.

Materials and Methods

Peptides. The peptides used in this study are described in Table 1. Human defensin HNP-2 [21] and rabbit defensin NP-2 were purified from leukocytes as described previously [22]. LL-37 was prepared on a Perkin-Elmer ABI 431 A synthesizer, using a prederivatized polyethylene glycol polystyrene serine resin (PerSeptive Biosystems, USA), FastMoc chemistry (Perkin Elmer, USA), and single coupling. After reverse-phase high-performance liquid chromatography (RP-HPLC), the peptide appeared homogeneous by capillary zone electrophoresis and had a mass of 4493.16 (expected mass, 4493.3). Synthetic protegrins were prepared by solid-phase synthesis (SynPep, USA), purified after reduction by RP-HPLC, folded, and repurified to apparent homogeneity by further RP-HPLC. Synthetic clavanins (SynPep) and MSI-78, a magainin analogue, were prepared by solid-phase synthesis and purified by RP-HPLC. Brevinin-1 was purchased from Bachem, USA. All other peptides were purchased from Sigma Chemical, USA.

Brevinin-1 was reduced and alkylated by dissolving at 1 mg/ml in 6 M guanidine HCl, 20 mM EDTA, and 0.5 M Tris, pH 8.07, flushing with nitrogen, heating to 52 °C, and adding dithiothreitol (DTT) in 100-fold molar excess. After 15 h, iodoacetamide was added in threefold molar excess (relative to DTT) to alkylate cysteine residues, followed 10 min later by the addition of excess DTT. CAM-brevinin-1 was purified by RP-HPLC. CAM-brevinin showed the expected mass gain of 58 amu/cysteine residue by matrix-assisted laser desorption/ionization mass spectrometry. The synthetic peptides were stored as lyophilized powders or as 1 mg/ml stock solutions in sterile acidified water (0.01% glacial acetic acid) at −20 °C.

Cells and Viruses. Herpes simplex virus 1 M (HSV-1 M), the MacIntyre strain (ATCC VR-539), and herpes simplex virus 2 G (HSV-2 G, ATCC VR-734) were purchased from the American Type Culture Collection, Rockville, MD, USA. ME-180 human cervical carcinoma cells (HTB 33) and Vero African Green Monkey Kidney cells (CCL 81) were also purchased from the

Table 1 Characteristics of peptides used in this study. All of the peptides were synthetic, except for defensins HNP-2 and NP-2, which were purified from human and rabbit neutrophils, respectively

Peptide	No.[a]	Structure	Reference	Original source	Amino acid sequence
Defensin HNP-2	29	β-sheet	21	human neutrophils	CYCRIPACIAGERRYGTCIYQGRLWAFCC
Defensin NP-2	33	β-sheet	22	rabbit neutrophils	VVCACRRALCLPLERRAGFCRIRGRIHPLCCRR
Tachyplesin-1	17	β-sheet	32	*Limulus* hemocytes	KWCFRVCYRGICYRRCR[b]
Protegrin PG-1	18	β-sheet	33	porcine neutrophils	RGGRLCYCRRRFCVCVGR[b]
PG-1, all D	18	β-sheet	34	PG-1 analog	RGGRLCYCRRRFCVCVGR[b]
PG-1 "bullet"	18	β-sheet	3	PG-1 analog	RGGRLCYARRRFAVCVGR[b]
PG-1 "kite"	18	β-sheet	3	PG-1 analog	RGGRLAYCRRRFCVAVGR[b]
PG-1 "snake"	18	α-helical	3	PG-1 analog	RGGRLAYARRRFAVAVGR[b]
Indolicidin	13	mixed	35	bovine neutrophils	ILPWKWPWWPWRR
Brevinin-1	24	mixed	36	frog skin	FLPVLAGIAAKVVPALFCKITKKC
CAM brevinin-1	24	mixed	UD[c]	brevinin-1 analog	FLPVLAGIAAKVVPALF*C*KITKK*C*[d]
Melittin	26	α-helical	37	bee venom	GIGAVLKVLTTGLPALISWIKRKRQQ
Magainin-1	23	α-helical	38	frog skin	GIGKFLHSAGKFGKAFVGEIMKS
MSI-78	22	α-helical	39	magainin-1 analog	GIGKFLKKAKKFGKAFVKILKK
Clavanin A amide	23	α-helical	40	tunicate hemocytes	VFQFLGKIIHHVGNFVHGFSHVF[b]
Clavanin AK acid	23	α-helical	41	clavanin A analog	VFQFLGKIIKKVGNFVKGFSKVF
Clavanin A acid	23	α-helical	41	clavanin A analog	VFQFLGKIIHHVGNFVHGFSHVF
Cecropin P1	31	α-helical	42	porcine intestine	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR
Cecropin A	37	α-helical	43	insect hemolymph	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVG-QATQIAK[b]
LL-37	37	α-helical	44	human neutrophils	LLGDFFRKSKEKIGKEFKRIVQ-RIKDFLRNLVPRTES

[a] Number of amino acid residues in each peptide
[b] Signifies C-terminal amidation
[c] Unpublished data
[d] *C* indicates that the residue was reduced and then carboxamidomethylated
CAM, carboxymethylated

American Type Culture Collection. Vero cell monolayers were infected with HSV-1 M or HSV-2 G, and the infected cells were cultured at 37 °C for 48 h in RPMI medium 1640 (Gibco BRL, USA) supplemented with 2% fetal bovine serum (Bio Whittaker, USA). The infected cells were collected with a cell scraper and lysed by three cycles of freeze-thawing. Cell debris was cleared by centrifugation at $1877 \times g$ for 10 min at 4 °C, and the supernatants, which constituted our virus preparations, were stored at −85 °C until used.

Cytotoxicity Assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation kit (Boehringer-Mannheim, Germany) was used according to the manufacturer's instructions. This assay is based on the quantitation of MTT, a widely accepted procedure for assessing cell viability [23–28]. Briefly, ME-180 cells (2.5×10^4/well) were added to 96-well tissue-culture microtiter plates (Costar, USA) and incubated for 48 h at 37 °C. Serial twofold dilutions of host-defense peptides (10 μl), prepared in acidified water, were added to 90 μl of RPMI 1640 that contained 2% fetal bovine serum. Final peptide concentrations ranged from 100 to 1.56 μg/ml. After 72 h each well received 10 μl of MTT, and 4 h later, 100 μl of 10% sodium dodecyl sulfate in 0.01 M HCl. After keeping the plates overnight at 37 °C, absorbance was measured at 600 nm (tetrazolium peak) and 650 nm (background) with a SpectraMax Microplate Spectrophotometer (Molecular Devices, USA). Optical density $(OD)_{600–650}$ values were calculated with SOFTmax PRO software (Molecular Devices).

Direct Viral Inactivation. Viral titers were checked by infecting ME-180 cell monolayers in 96-well plates with twofold serially diluted viral inocula and incubating the cells for 72 h at 37 °C. A viral dilution that produced 75–80% cell death, based on MTT reduction, was used in the inactivation assays. This corresponded to an inoculum of approximately 6×10^3 pfu/well for HSV-1 M and approximately 1×10^3 pfu/well for HSV-2 G. ME-180 target cells were seeded in 96-well tissue-culture plates at 2.5×10^4 cells/well and incubated for 48 h at 37 °C. For peptide topical screening assays, preinoculation exposure was performed. The virus inoculum (8 μl), in RPMI with 2% fetal bovine serum, was mixed with 2 μl of the 1 mg/ml stock peptide solution and incubated for 2 h at 37 °C. Virus-free controls (peptide only) and peptide-free controls (virus + acidified water only) were incubated in parallel. The peptide-treated viral inocula and controls were diluted with 310 μl RPMI 1640 containing 2% fetal bovine serum. The regular medium was aspirated from the target cells and replaced with 100 μl/well of the peptide-treated viral inocula. This procedure diluted the peptides to 6.25 μg/ml. Appropriate peptide-free or virus-free controls were included. Trays were incubated at 37 °C for 72 h and cytotoxicity was measured with the MTT kit. Since melittin was highly cytotoxic at the above concentration, a less concentrated (0.5 mg/ml) stock of this peptide was used, thereby reducing the final melittin concentration to 3.125 μg/ml. The antiviral compound acyclovir (Sigma, USA) was also tested in the same assay, at 100μM. As expected, since the treatment was performed preinoculation, rather than during intracellular viral replication, its protective effect was marginal: 34.6% on HSV-2 G, 0% on HSV-1 M.

In subsequent confirmatory dose-response experiments conducted on indolicidin and CAM-brevinin, final peptide concentrations ranging from 6.25 to 200 μg/ml were incubated with virus stocks for 2 h, as described above, and added directly to ME-180 cells. Trays were incubated at 37 °C for 72 h and cytotoxicity was measured with the MTT kit. In plaque-reduction assays performed with indolicidin and CAM-brevinin, final peptide concentrations ranging from 6.25 to 100 μg/ml were incubated with virus stocks for 2 h as described above and added directly to ME-180 cells. Plaques were counted 48 h later, after staining the monolayers with crystal violet using standard techniques, for direct visualization of the cytopathic effect [29].

A limited number of experiments in which the preincubation step was eliminated, i.e. cells were treated postinoculation with serial twofold dilutions of acyclovir (100–3.125 μg/ml or 444–13.9 μM) or indolicidin (100–3.125 μg/ml or 52.5–1.6 μM), were also conducted.

Calculations. The calculations used [30] to assess antiviral activity and to correct for background cytotoxicity are shown below. The $OD_{600–650}$ values, provided as examples in square brackets, were obtained in an experiment with brevinin-1.

$a = OD_{600–650}$ of untreated cells [1.666].
$b = OD_{600–650}$ of cells treated with 6.25 μg/ml of peptide [1.499].
$c = OD_{600–650}$ of cells infected with untreated virus [0.375].
$d = OD_{600–650}$ of cells infected with peptide-treated virus [1.422].
$e = b/a$ [calculated value, 0.8897].

For peptides without intrinsic cytotoxicity, percent protection would be calculated conventionally with Equation 1, which assumes that all cytotoxicity results from the virus.

$$\text{Percent protection} = [1 - (a - d)/(a - c)] \times 100$$

Since certain of our peptides were cytotoxic for the ME-180 target cells, even at their low "carryover" concentrations of 6.25 μg/ml (3.125 μg/ml for melittin), cytotoxicity resulted from peptide carryover as well. The *e* provides a correction factor for this peptide-mediated cytotoxicity. For cytotoxic peptides (i.e., those peptides for which b is $< a$), one substitutes b for a in Equation 1 and multiplies c by e. This yields Equation 2, which is shown below. For noncytotoxic peptides, $a = b$, and, consequently, $e = 1.0$, rendering Equations 1 and 2 identical.

$$\text{Percent protection} = [1 - (b - d)/(b - ce)] \times 100$$

Applying Equation 2 to the brevinin data indicates that this peptide afforded 93.4% protection.

Hemolysis Assay. Heparinized human blood was washed once with phosphate-buffered saline (PBS) containing 4 mM EDTA, then three more times with PBS, and resuspended at a 2.5% v/v concentration in PBS. Serial twofold peptide dilutions were prepared in 0.01% acetic acid, and 3 μl of each was added to 57 μl of the suspension in polypropylene microcentrifuge tubes. Final peptide concentrations ranged from 2.5 to 80 μg/ml. The 0% and 100% lysis controls received 3 μl additions of 0.01% acetic acid and Triton X-100, respectively.

The tubes were incubated for 30 min at 37 °C with intermittent mixing. After adding 150 μl of PBS and centrifuging the samples (3 min, $7280 \times g$) an aliquot (150 μl) of each supernatant was transferred into a 96-well plate. Absorbances were measured at 540 nm with a SpectraMax 250 Microplate Spectrophotometer (Molecular Devices).

Results

Inactivation of Herpes Simplex Virus Type 1. As indicated in Table 2, several host-defense peptides displayed considerable activity (79–100% inactivation) against HSV-1 M. These included human defensin HNP-2, rabbit defensin NP-2, bovine indolicidin, and both frog brevinin-1 and its linearized derivative, CAM-brevinin. Porcine protegrin PG-1 was less effective (40.5 ± 9.5% protection), and modified PG-1 molecules ("bullet", "kite", and "snake" variants) that

Table 2 Inactivation of HSV-1 M (MacIntyre strain) and HSV-2 G by host-defense peptides. All peptides (except melittin) were tested at 200 μg/ml. Melittin was tested at 100 μg/ml

Peptide	Molecular weight	μm concentration	Percent protection[a] Mean ± SEM (no. of experiments)	
			HSV-1 M	HSV-2 G
β-sheet				
Defensin HNP-2	3368.5	59.4	79.9 ± 7.0 (7)	53.1 ± 1.1 (2)
Defensin NP-2	3846.0	52.0	91.1 ± 10.0 (7)	−11.4 ± 0.1 (2)
Tachyplesin 1	2263.1	88.4	34.4 ± 4.4 (3)	68.7 ± 3.2 (3)
Protegrin PG-1	2155.0	92.8	40.5 ± 9.5 (7)	68.2 ± 7.0 (3)
Protegrin analogs				
PG-1, all D	2155.0	92.8	82.0 ± 7.8 (4)	75.7 ± 4.6 (4)
PG-1 "bullet"	2093.1	95.6	13.9 ± 2.4 (3)	78.5 ± 5.3 (4)
PG-1 "kite"	2093.1	95.6	−3.4 ± 4.4 (4)	20.4 ± 1.1 (2)
PG-1 "snake"	2031.2	98.5	−8.4 ± 5.8 (3)	2.3 ± 0.9 (2)
Other				
Indolicidin	1906.0	104.9	93.8 ± 5.0 (3)	107.6 ± 1.8 (3)
Brevinin-1	2527.5	79.1	93.7 ± 4.1 (10)	89.8 ± 6.9 (4)
CAM brevinin-1	2527.5	79.1	111.2 ± 16.9 (4)	77.9 ± 5.4 (4)
α-helical				
Melittin	2845.7	35.1	131.4 ± 16.4 (2)	112.4 ± 3.6 (2)
Magainin 1	2408.3	83.1	−2.3 ± 2.2 (3)	−7.3 ± 4.4 (2)
MSI-78	2476.6	80.8	10.9 ± 2.8 (3)	0.8 ± 4.5 (2)
Clavanin A amide	2665.4	75.0	−6.7 ± 5.9 (6)	−25.2 ± 1.0 (2)
Clavanin AK	2629.6	76.1	70.4 ± 7.3 (6)	25.2 ± 8.8 (2)
Clavanin A acid	2665.4	75.0	−3.2 ± 6.9 (6)	−6.7 ± 0.1 (2)
Cecropin P1	3336.9	59.9	−9.5 ± 8.0 (3)	−10.6 ± 6.3 (2)
Cecropin A	4002.4	50.0	7.5 ± 2.6 (2)	−10.1 ± 1.5 (2)
LL-37	4490.6	44.5	27.6 ± 4.4 (2)	46.4 ± 1.2 (2)

[a] The calculated percent protection accounted for any peptide-mediated cytotoxicity, as described in the text

lacked one or both of the intramolecular disulfides found in the parent molecule were inactive. Curiously, the *enantio* PG-1 variant, composed exclusively of D-amino acids, was significantly more active (82.0 ± 7.8%) than normal PG-1 ($P < 0.001$, Student's t test for unpaired samples). Tachyplesin-1, a protegrin-like antimicrobial molecule from horseshoe crab hemocytes, was about as effective as PG-1. Of the α-helical peptides tested, only melittin and clavanin AK showed a substantial ability to inactivate HSV-1 M. The cytotoxic effects of HSV-1 M alone ranged from 75 to 81.6% in these assays.

Dose-response microplate assays were then conducted with the most active peptides, CAM-brevinin and indolicidin, further confirming their antiviral activity at different doses (Figure 1). At 100 μg/ml, CAM-brevinin displayed antiviral activity against HSV-1 M (35.0 ± 2.8% protection; ID50 could not be determined). At this concentration, indolicidin afforded 96.7 ± 5.9% protection against HSV-1 M (ID50, 56 μg/ml) (Figure 1).

To confirm the antiviral activity of these two peptides and gain additional information about the potency of indolicidin and CAM-brevinin, we performed a standard plaque-reduction assay. In standard plaque-reduction assays performed to confirm the antiviral activity of these two peptides, both CAM-brevinin and indolicidin completely eliminated plaque formation by HSV-1 M at 100 μg/ml (Figure 2).

When cells were treated postinoculation with serial twofold dilutions of acyclovir (100–3.125 μg/ml or 444–13.9 μM) in the MTT assay, percent protection ranged from 47 to none against HSV-1 (data not shown). Over the same concentration range, percent protection for indolicidin ranged from 92.8 to none against HSV-1 (data not shown).

Inactivation of Herpes Simplex Virus Type 2. As also indicated in Table 2, indolicidin, brevinin-1, CAM-brevinin, and melittin showed potent activity against HSV-2 G. In contrast to its excellent activity against HSV-1 M (92.1 ± 10% inactivation), rabbit defensin NP-2 showed no activity against HSV-2 G. Protegrin PG-1, *enantio* PG-1, and "bullet" PG-1 showed considerable activity (68–75% inactivation). With the exceptions of melittin (complete inactivation) and human LL-37 (46.4 ± 1.2% inactivation), the other α-helical peptides in the panel displayed little or no activity. The cytotoxic effects of HSV-2 G against ME-180 cells ranged from 75 to 80.1% in these assays.

In confirmatory dose-response experiments, CAM-brevinin displayed strong antiviral activity against HSV-2 G (71.6 ± 1.8% protection; ID50, 75 μg/ml). Indolicidin completely inactivated HSV-2 G at 100 μg/

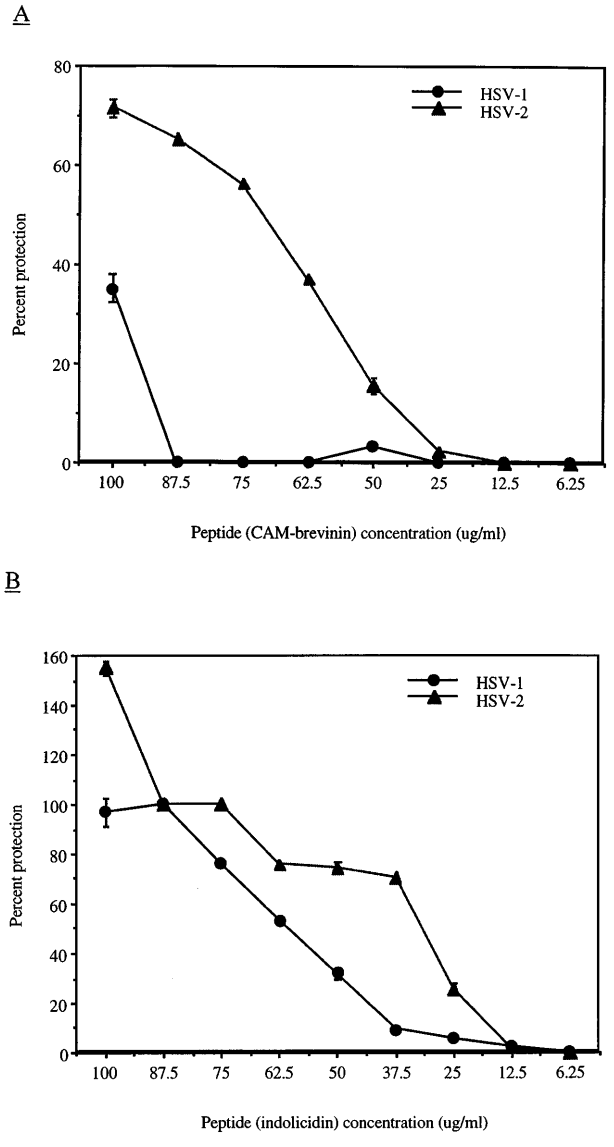

Figure 1 Inactivation of HSV-1 and -2 (dose-response assay). CAM-brevinin (**A**) and indolicidin (**B**) were incubated with the viruses for 2 h and then added to ME-180 cell monolayers. Trays were incubated at 37 °C for 72 h, and cytotoxicity was measured with the MTT kit. Values shown are means ±SEM from two experiments

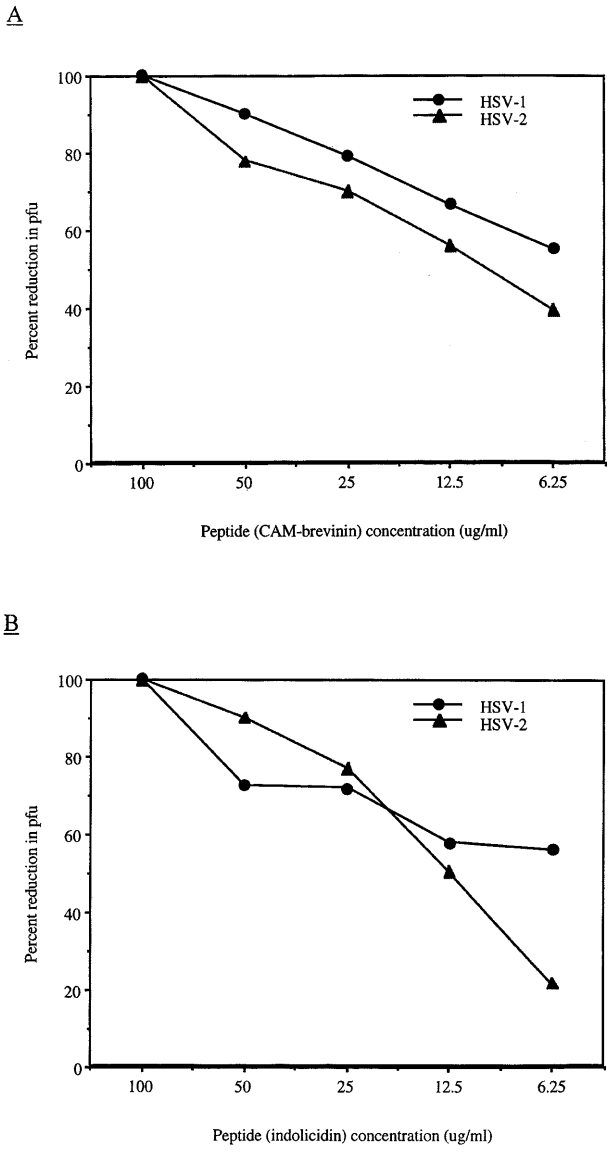

Figure 2 Inactivation of HSV-1 and -2 (plaque reduction assay). CAM-brevinin (**A**) and indolicidin (**B**) were incubated with the viruses for 2 h and then added to ME-180 cell monolayers. Plaques were counted after 48 h of incubation

ml (155.1±2.8% protection; ID50, 37 μg/ml). It also displayed significant antiviral activity against HSV-2 G at 50 μg/ml (74.5±2% protection) (Figure 1). At 100 μg/ml, these two peptides completely inhibited plaque formation by HSV-2 in the plaque reduction assay (Figure 2).

When cells were treated postinoculation with serial twofold dilutions of acyclovir (100–3.125 μg/ml or 444–13.9 μM) in the MTT assay, percent protection ranged from 100 to 56 against HSV-2 (data not shown). Over the same concentration range, percent protection for indolicidin ranged from 100 to none against HSV-2 (data not shown).

Cytotoxic Activity. The cytotoxic effect of peptides (6.25 μg/ml) alone against ME-180 cells in the screening assays ranged from 0 to 36.4%. Peptide concentrations ranging from 3.125 to 100 μg/ml were tested for cytotoxicity against the ME-180 target cells under the culture conditions used in the antiviral assays (Table 3). Several peptides displayed little activity against HSV-1 or HSV-2, even when tested at 100 μg/ml. This group included "kite" and "snake" variants of PG-1, magainin-1 and MSI-78, cecropins A and P1, and clavanin A acid. Many of the peptides with activity against HSV-1 or HSV-2 also caused marked or moderate cytotoxicity to ME-180 cells. Among these were tachyplesin-1, various forms of PG-1, melittin, and brevinin-1.

Table 3 Cytotoxic and hemolytic properties of the peptides. In cytotoxicity experiments ($n = 2$–4), ME-180 cell monolayers were incubated with peptides for 72 h, and cytotoxicity was measured by the MTT assay. In hemolysis experiments, peptides were incubated for 30 min with a 2.5% (v/v) suspension of washed human erythrocytes

Peptide	Percent cytotoxicity		Percent hemolysis	
	Mean ± SEM at 100 μg/ml	EC50 (μg/ml)	80 μg/ml	EC50 (μg/ml)
β-sheet				
HNP-2	15.5 ± 5.1	–	0.9	–
NP-2	48.8 ± 1.5	–	1.9	–
TP-1	99.4 ± 0.5	29.0	66.5	51.9
PG-1	98.7 ± 1.3	42.3	70.3	29.7
Protegrin analogs				
dPG-1	98.0 ± 1.8	39.4	90.0	13.3
PG-1 "bullet"	99.8 ± 0.1	40.8	14.7	–
PG-1 "kite"	37.7 ± 5.4	–	0.0	–
PG-1 "snake"	4.1 ± 8.2	–	0.8	–
α-helical				
Melittin	99.9 ± 0.2	6.4	94.6	7.7
Magainin 1	5.3 ± 4.2	–	0.0	–
MSI-78	36.3 ± 4.7	–	11.4	–
Cecropin P1	18.7 ± 2.6	–	0.3	–
Cecropin A	36.5 ± 1.5	–	0.6	–
Clavanin A amide	60.7 ± 1.6	18.1	3.7	–
Clavanin A acid	9.5 ± 9.6	–	0.0	–
Clavanin AK acid	27.4 ± 2.0	–	30.6	–
Other				
Indolicidin	39.5 ± 5.8	–	38.2	–
Brevinin-1	99.9 ± 0.0	14.4	89.0	13.4
CAM-brevinin	22.4 ± 4.8	–	0.5	–
LL-37	16.6 ± 0.1	–	17.1	–

–, cytotoxicity too low for EC50 to be calculated

Only indolicidin and CAM-brevinin manifested potent viral inactivating activity (Table 2) unaccompanied by significant cytotoxicity for the ME-180 cells (Table 3). In dose-response assays, CAM-brevinin displayed marginal cytotoxicity against ME-180 cells at the higher peptide concentrations (22.4% at 100 μg/ml) (Table 3). At lower peptide concentrations (25 μg/ml), CAM-brevinin was noncytotoxic (data not shown) to the ME-180 cells. Indolicidin exerted a slightly higher (39.5%) cytotoxicity against ME-180 cells at 100 μg/ml (Table 3). With decreasing peptide concentrations, the cytotoxic effect of indolicidin also decreased to marginal levels (9.5% cytotoxicity at 6.25 μg/ml) (data not shown). Both peptides were cytotoxic to host cells at 200 μg/ml (data not shown).

Hemolytic Activity. The peptides varied considerably in their ability to hemolyze human erythrocytes (Table 3). Whereas brevinin-1 was strongly hemolytic, its carboxamidomethylated (and antiviral) derivative, CAM-brevinin, was nonhemolytic. Melittin, tachyplesin-1, protegrin PG-1, and *enantio* PG-1 showed considerable hemolytic activity for human erythrocytes, whereas human and rabbit defensins and most of the α-helical peptides were not hemolytic. Clavanin AK acid and indolicidin were moderately hemolytic, causing 30–40% hemolysis at 80 μg/ml.

Discussion

Host-defense peptides are widely distributed in nature, and appear to play important roles in innate host defenses against bacteria and fungi. Because many of these peptides act by perturbing membranes, we tested their ability to inactivate enveloped viruses, selecting HSV-1 M and HSV-2 G as targets. We used a microplate assay for initial screening of a panel of 20 host-defense peptides [23–28]. Our data analysis allowed us to compensate for any peptide-mediated cytotoxicity in assessing antiviral activity. The resulting quantitative assay was sensitive and reproducible and yielded considerable information while consuming minimal amounts of peptide. The most active peptides were then tested for their antiviral potential in dose-response microplate assays as well as the confirmatory standard method, the plaque-reduction assay.

Hundreds of naturally occurring host-defense peptides have been described, and many additional variants have been created in the laboratory. Although the 20 peptides in our panel represent only a small fraction of this universe, several noteworthy findings have emerged. Two peptides emerged as potential topical microbicides, CAM-brevinin and indolicidin. Our findings with CAM-brevinin and indolicidin also showed that peptide-mediated inactivation of herpes simplex

viruses is not simply a manifestation of indiscriminate damage to target cell membranes. Although both peptides were potently antiviral, they showed little (indolicidin) or no (CAM-brevinin) hemolytic activity, and they caused minimal cytotoxicity in ME-180 cells. Antiviral compounds are considered to have promise when there is a tenfold difference between the ID50 for antiviral potency and the EC50 for cytotoxicity using a range of drug doses. By this criteria, both CAM-brevinin and indolicidin show great potential as antiviral agents against herpes simplex viruses.

Some of the antimicrobial peptides showed remarkable selectivity with respect to their actions on the two viruses in screening experiments. Rabbit defensin NP-2 potently inactivated HSV-1 M but was inactive against HSV-2 G. Clavanin AK showed similar, but less marked, discriminatory behavior. Conversely, the "bullet" variant of PG-1 was considerably more active against HSV-2 G than HSV-1 M ($P < 0.001$, Student's t test for unpaired samples). Perhaps the selective tissue expression characteristic of many antimicrobial peptides contributes to the organotropism of many viruses, including HSV-1 and HSV-2.

These experiments establish that host-defense peptides can impede the ability of herpes viruses HSV-1 M and HSV-2 G to infect host cells. Because some of these host-defense peptides also inactivate HIV-1 [31], *Neisseria gonorrhoeae* [1], and *Chlamydia trachomatis* [2, 3], these peptides hold promise as topical agents to prevent sexually transmitted diseases.

The assay procedure described in this report permits the simultaneous, quantitative evaluation of antiviral activity of both cytotoxic and noncytotoxic peptides. As such, it should be of value in identifying other potentially useful molecules with antiviral properties. Furthermore, the sequential use of screening microplate assays followed by standard confirmatory antiviral assays allows for the conservative use of limited quantities of synthetic peptides.

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