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An enzymatic assay based on luciferase Ebola virus-like particles for evaluation of virolytic activity of antimicrobial peptides

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ABSTRACT

Antimicrobial peptides are currently considered as promising antiviral compounds. Current assays to evaluate the effectivity of peptides against enveloped viruses based on liposomes or hemolysis are encumbered by the artificial nature of liposomes or distinctive membrane composition of used erythrocytes. We propose a novel assay system based on enzymatic Ebola virus-like particles containing sensitive luciferase reporter. The assay was validated with several cationic and anionic peptides and compared with lentivirus inactivation and hemolytic assays. The assay is sensitive and easy to perform in standard biosafety level laboratory with potential for high-throughput screens. The use of virus-like particles in the assay provides a system as closely related to the native viruses as possible eliminating some issues associated with other more artificial set ups.

We have identified CAM-W (KWKLWKKIEKWGQGIGAVLKWLTTWL) as a peptide with the greatest antiviral activity against infectious lentiviral vectors and filoviral virus-like particles.

1. Introduction

Antimicrobial peptides (AMPs) are found in a majority of living organisms and are involved in a primary defense mechanism against bacteria, fungi, and other potentially hazardous pathogens. AMPs can be found in secretion, invertebrate venoms or in the intracellular space. One example of secreted peptide is bombesin, which is secreted on the skin of the European fire-bellied toad (*Bombina bombina*) [1]. Another peptide secreted by a toad is maximin H5 (*Bombina maxima*) [2]. There is a plenty of other antimicrobial peptides produced by various frog species [3]. Other peptides such as cathelicidins are found among the most of the mammals where they are located in lysosomes of macrophages and polymorphonuclear leukocytes playing a critical role in the innate immune defense. Other peptides are substantial components of insect toxins such as melittin, present in a honey bee (*Apis mellifera*) venom [4], mucroporin from scorpion (*Lychas mucronatus*) venom [5] and/or lycotoxin from the wolf spider (*Lycosa carolinensis*) venom [6].

Shared mechanism of a function of the AMPs is a formation of pores in the cell membrane leading to the loss of membrane potential, ion imbalance, and cell death. Due to membranolytic properties, that are not restricted to bacteria only but are as effective in eukaryotic cells, some of the peptides have been tested as the potential anticancer compounds. Many of them have been shown to have strong anticancer effects either alone or conjugated to a tumor targeting tag [4,7–10]. The ability to specifically interact with cells membranes causing a loss of membrane integrity led to an investigation of virolytic or virus inhibitory properties of AMPs. Li et al. have shown that mucroporin-M1 is active against several enveloped viruses such as measles, SARS-CoV and influenza H5N1 viruses [11]. Hecate, a mellitin analog, was shown to inhibit herpes simplex virus-1 (HSV-1) induced cell fusion and virus spread [12]. The antiviral effect does not necessarily need to be linked to the membrane lysis as it has been shown for melittin and cecropin [13]. Both peptides suppress viral gene expression in micromolar concentrations that are well below toxic concentrations for cells

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suggesting a mechanism unrelated to membranolytic properties of the peptides. These results prompted a search for the new peptides with an improved antiviral and antibacterial activity, cytotoxicity, lower hemolytic activity, *etc.* Several approaches can be used toward these goals. Rational redesigning of existing peptides can greatly improve their desired properties. *In silico* computation led to the construction of hecate, a derivative of melittin, which was designed to alter the surface charge distribution without affecting its amphipathic α-helical character [12].

Alternatively, a series of mutations can be introduced into parental peptide and screened in biological assays. Resulting mutants with the desired properties are selected for further work. This approach was applied to one of the sheep cathelicidins SMAP29 [14–16].

Another viable approach in peptide design utilizes peptide domains swapping between different peptides. Cecropin A – Melittin hybrid was constructed in this way [17] with the most effective hybrid containing 13 N-terminal amino acids derived from Cecropin A connected to first 13 amino acids of melittin [CA(1-13)M(1-13)]. The hybrid peptide was further modified to increase its proteolytic stability and enhance antimicrobial potency [18] by replacing 4 residues with tryptophan reducing hemolytic activity compared to the parental peptide. The fourth approach comprises the design of entirely synthetic peptides. The cationic KALA peptide was synthesized as an agent for DNA oligonucleotides delivery into cells [19]. It is highly charged with a repetitive amino acids motif that destabilizes membranes and binds to DNA with a high affinity. A sequential homolog of KALA is anionic peptide GALA [20]. The peptide has structure homologous to KALA peptide but the positively charged lysine residues were replaced with negatively charged glutamic acids. The peptide was designed to interact with lipid bilayer preferentially at acidic pH of late endosomes.

Despite the fact that many peptides have been described to have virolytic activity, they have never been comprehensively compared to each other. One of the reasons could be the biosafety concerns. Experiments with active viruses, such as Ebola or Marburg can be conducted by very few laboratories in strictly controlled biosafety environment. Even less dangerous viruses such as HIV require special laboratory environment and trained staff. In order to facilitate future research aiming at design and testing of new antiviral peptides, we designed a virolytic assay based on non-toxic and non-infectious virus-like particles (VLPs). VLPs resemble parental viruses by their structure. They usually carry some structural proteins necessary for the virus capsid assembly, virus envelope membrane derived from the host cells and virus envelope protein necessary for recognition of cellular receptors. Hence, we have constructed VLPs imitating the Ebola virus. Expression of structural protein VP40 is actually the only prerequisite for the formation and production of VLPs. Fluorescent VLPs based on filoviruses were described previously. Martines et al. [21] have constructed GFP-VP40 fusion protein for visualization of VLPs in dendritic cells. Fluorescent Marburg virus VLPs containing red fluorescent protein were used for live-cell imaging of Marburg virus-infected cells [22]. However, the aim of this work was to develop a simple system to quantify the release of the viral content or the loss of membrane permeability caused by lytic peptides. For that purpose, we cannot use fluorescent VLPs and that is why we chose an approach where the viral membrane separates an enzyme activity from its substrate. In order to achieve as high sensitivity as possible, we have used luciferase reporter being fused to a structural protein of Ebola virus VP40 and its membrane poorly permeable substrate furimazine.

We have attached NanoLuciferase (NLuc) [23] to the N-terminus of Ebola VP40 protein and we show that cells transfected with a plasmid pNLuc-VP40 and a plasmid coding virus envelope protein produce enzymatic VLPs. The enzyme is encapsulated inside the particle further protected by the lipid envelope making it poorly accessible for the substrate. The NLuc substrate is a newly developed coelenterazine derivative furimazine [23] that has been optimized for NLuc to provide brighter fluorescence and a greater chemical stability. We have designed an assay evaluating membranolytic or pore forming properties of peptides reflecting the activity of NLuc after pre-treatment of VLPs with the peptides. The assay carried out with several different peptides is sensitive, reliable and reflects the results of infectious lentiviral vectors inactivation. It can be used for easy enzymatic screening of new peptides or membrane destabilizing drugs for antiviral therapies. VLPs can be modified with envelope proteins of a broad spectrum of viruses, therefore, cytotoxic peptides selectively targeted to inactivate specific highly pathogenic viruses can be evaluated in the common laboratories without regard for special biosafety conditions.

2. Methods and techniques

2.1. Material and plasmids

Peptides were synthesized on the solid phase with CEM Liberty Blue Peptides Synthesizer (Matthews, NC, USA) from Fmoc protected precursors. The purity of peptides was evaluated using HPLC-UV (ESA Inc., Chelmsford, MA, USA). The final purity was greater than 92%. Molecular weights of the peptides were verified by HPLC-ESI-Q-TOF (Bruker Daltonik GmBH, Bremen, Germany). Peptides were dissolved in distilled water with the exception of anionic peptides Maximin H5 and GALA where a small addition of sodium hydroxide was necessary to dissolve the peptide. The peptides, utilized in this study and their basic characteristics are listed in Table 1.

Plasmid for expression of NLuc-VP40 was constructed by overlapping PCR. NLuc open reading frame (ORF) was amplified from pNanoLuc 1.1.CMV (Promega, Madison, WI, USA) with primers NLfw (atatgctagcgccaccatggtcttcacactcgaagatttc) and NLrv (aacccgcctcatggtggatccgccgctgccgcctccggagcccctaggcgccagaatgcgttcgcacagccg). The reverse primer introduced flexible peptide linker (GSGGGSGG). The Ebola VP40 protein ORF was amplified from plasmid pcDNA3.1-Bla-VP40 (BEI Resources, Manassas, VA, USA) with primers eVP40fw (agcggcggatccaccatgaggcgggttatattgcctactgc) and eVP40rv (tatagaattcttacttctcaatcacagctggaagac). Overlapping PCR was carried out with the mix of primary PCR products and primers NLfw and eVP40rv. Proof reading polymerase Phusion (New England Biolabs, Ipswich, MA, USA) was used in all

Table 1
Peptide sequences, origin and physical characterizations.

Name	Sequence	AA	Mw	pI	Charge	Origin
Hecate	FALALKALKKALKKLKKALKKAL	23	2537.35	10.9	+9	Synthetic, derivate of Melittin
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	26	2847.49	12.2	+5	*Apis mellifera*
Maximin H5	ILGPVLGLVSDTLDDVLGIL	20	2022.41	3.42	−3	*Bombina maxima*
SMAP29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	29	3256.00	12.31	+9	Derivative of ovispirin, *Ovis aries*
CAM-W	KWKLWKKIEKWGQGIGAVLKWLTTWL	26	3197.91	10.3	+5	Synthetic, Cecropin A/Melittin hybrid
GALA	WEAALAEALAEALAEHLAEALAEALEALAA	30	3032.40	3,82	−7	Synthetic
KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	30	3131.86	9.9	+5	Synthetic

PCRs. Resulting PCR product was cleaved with NheI and EcoRI and cloned into pCAG vector.

Plasmids for lentivirus production pMD2.G (Addgene plasmid # 12259), pRSV-Rev (Addgene plasmid # 12253), pMDLg/pRRE (Addgene plasmid # 12251) were a gift from Didier Trono. pCSC-SP-PW-GFP (aka: pBOB-GFP) (Addgene plasmid # 12337) was a gift from Inder Verma.

Plasmid for expression of Marburg (NR-19815) virus envelope protein [24,25] was purchased from BEI Resources. Plasmid coding hybrid envelope protein FuG-C, containing extracellular part of rabies virus, transmembrane, and intracellular part derived from vesicular stomatitis virus (VSV) glycoprotein [26], was a gift from Kazuto Kobayashi.

2.2. Cell culture, transfections, virus-like particles, and lentiviruses production

HEK cells were cultivated in standard cell culture conditions in DMEM/F12 media supplemented with 10% of bovine calf serum and penicillin-streptomycin mix.

Virus-like particles were produced from cells transfected in 25 cm^2 flask with 30 μg of polyethyleneimine (PEI) and 12 μg of pNL-VP40(EBOV), 6 μg of plasmid coding virus envelope protein, (pMD2.G for VSV envelope and pCAGGS-MARV GP for Marburg virus envelope), and 600 ng of pmaxGFP (Lonza, Basel, Switzerland) to permit fluorescent control of transfection efficiency. After 12 h, the transfection mix was removed and replaced with VLPs collection medium (OptiMEM, Gibco, Waltham, MA, USA).

For lentivirus production, we have used the third generation of lentiviral vectors with improved biosafety features. For transfections, HEK cells were seeded into a 24-well plate and transfected at the confluence of 60–70% with the PEI:DNA complex. The co-transfection mix comprised of pCSC-SP-PW-GFP, pRSV-Rev, pMDLg/pRRE and a plasmid pFuG-C coding hybrid envelope protein.

Pseudocoated lentiviruses were produced by a transfection of HEK cells in 25 cm^2 flask with 30 μg of PEI and 2.20 μg pRSV-Rev; 5.00 μg pMDLg/pRRE, 8.25 μg pBOB-GFP, and 3.00 μg pFuG-C. The transfection mix was removed 12 h later and replaced with OptiMEM medium for virus collection. Viruses were collected twice after 24 h each and pooled together. Samples were centrifuged to remove detached cells and cellular debris and cleared supernatant was used for the infection assay.

2.3. Hemolytic assay

Human blood treated with EDTA and equine blood collected in heparinized tube was centrifuged for 5 min at 100*g* and 2 ml of sediment red blood cells (RBCs) were several times washed with PBS and centrifuged until no red staining of the supernatant was visible. The erythrocytes were diluted in 10 ml of phosphate buffered saline (PBS, pH 7.4). Selected peptides were diluted in 400 μl of PBS and 100 μl of washed RBCs was added. Peptide concentrations indicated in the graphs reflect the final concentration of the peptide in the reaction mix (500 μl). The cells were incubated at 37 °C for one hour, centrifuged and then, 100 μl of supernatant was transferred into 96 well-plate. Finally, the absorbance was measured at 540 nm. The percentage of hemolysis was determined by the following equation: %hemolysis = $[(At - Ac)/(A100\% - Ac)] \times 100$, where At is the absorbance of the supernatant from samples incubated with the peptides; Ac is the absorbance of the supernatant from negative control; A100% is the absorbance of the supernatant of the positive control (0.1% Triton X-100), which causes complete lysis of RBCs. The results are expressed as an average of three replicas.

2.4. Inhibition of lentiviruses infectivity

Cleared supernatant (200 μl) of lentiviruses coding EGFP and coated with FuG-C hybrid envelope protein were mixed with individual peptides at final concentration of 1 μM, 2.5 μM and 5 μM and incubated at 37 °C for 1 h. Then 600 μl of DMEM/F12 + 10% FCS was added. HEK cells were seeded in 96-well plate the night before. Cell culture medium was replaced with 200 μl of treated virus mixed with the medium in order to reduce the peptide concentration to non-toxic levels for reporter cells. Estimated virus concentration corresponds to MOI 0.8. The images of GFP-expressing cells were taken 24-h post-infection. The cells were lysed 36 h later with TBS buffer containing 0.5% Tween 20 and GFP fluorescence was measured (λ_{ex} = 485 nm, λ_{em} = 520 nm). GFP fluorescence was related to the amount of proteins measured as absorbance at 280 nm. The results are expressed as an average of three replicas. In order to confirm that final peptide concentrations in virus preparations does not kill the cells we have treated HEK cells with 1.25 μM peptide (corresponding to highest concentration of diluted peptide) for 16 h and then performed staining with 1 μg/ml Hoechst 33258.

2.5. Peptide lysis of VLPs and luciferase assay

Cell culture supernatant from VLPs producing cells was collected, centrifuged to remove cells and transferred into a new tube. 49 μl of cleared supernatant was incubated with 1 μl of 50× concentrated stock solution of the peptide at 37 °C for 20 min. Because commercially available NLuc assay buffer contains detergents we have used a detergent-free buffer (150 mM KCl, 25 mM Tris-Cl pH 7.5, 3 mM β-ME) in order to avoid damaging the viral membrane by a detergent.

Luciferase assay was carried out with 10 μl of analyzed sample and 50 μl of luciferase assay reagent composed of Nano-Glo® Luciferase Assay Substrate (Promega, Madison, WI, USA) and detergent-free buffer in ratio 1:500. As a control, we have used VLPs treated with 0.5% Tween 20. Luciferase activity of samples that were not treated with any peptide was subtracted from activities of the samples treated with peptides. The luciferase activity was related to the activity of sample lysed with 0.5% Tween 20. The results are expressed as an average of three replicas.

2.6. Peptide cytotoxicity evaluation in HEK and HeLa cells

Cells were seeded into 96-well plate overnight. Next day the cell culture medium was replaced with fresh medium containing various concentrations of individual peptides and cells were cultivated for 24 h. The effect of peptides on cells viability was analyzed by an MTT assay. The results are expressed as an average of four replicas.

3. Results

3.1. Hemolytic assay

Hemolytic assay carried out with human and equine RBCs shows that all cationic peptides cause substantial release of hemoglobin from RBCs, though they differ in activity (Fig. 1). Melittin and CAMW are highly hemolytic for both, human and equine RBS. On the other hand anionic peptides, GALA and maximin H5 have virtually no hemolytic activity for any tested erythrocytes. KALA had a moderate hemolytic activity for both types of RBCs. Peptides that differ in the ability to release hemoglobin from human and equine RBSs are hecate and SMAP29. These two peptides moderately lyse human erythrocytes but have virtually no effect on equine RBCs. In general, it seems that horse erythrocytes are less sensitive to peptide-mediated lysis. For example, mellitin in 5 μM concentra-

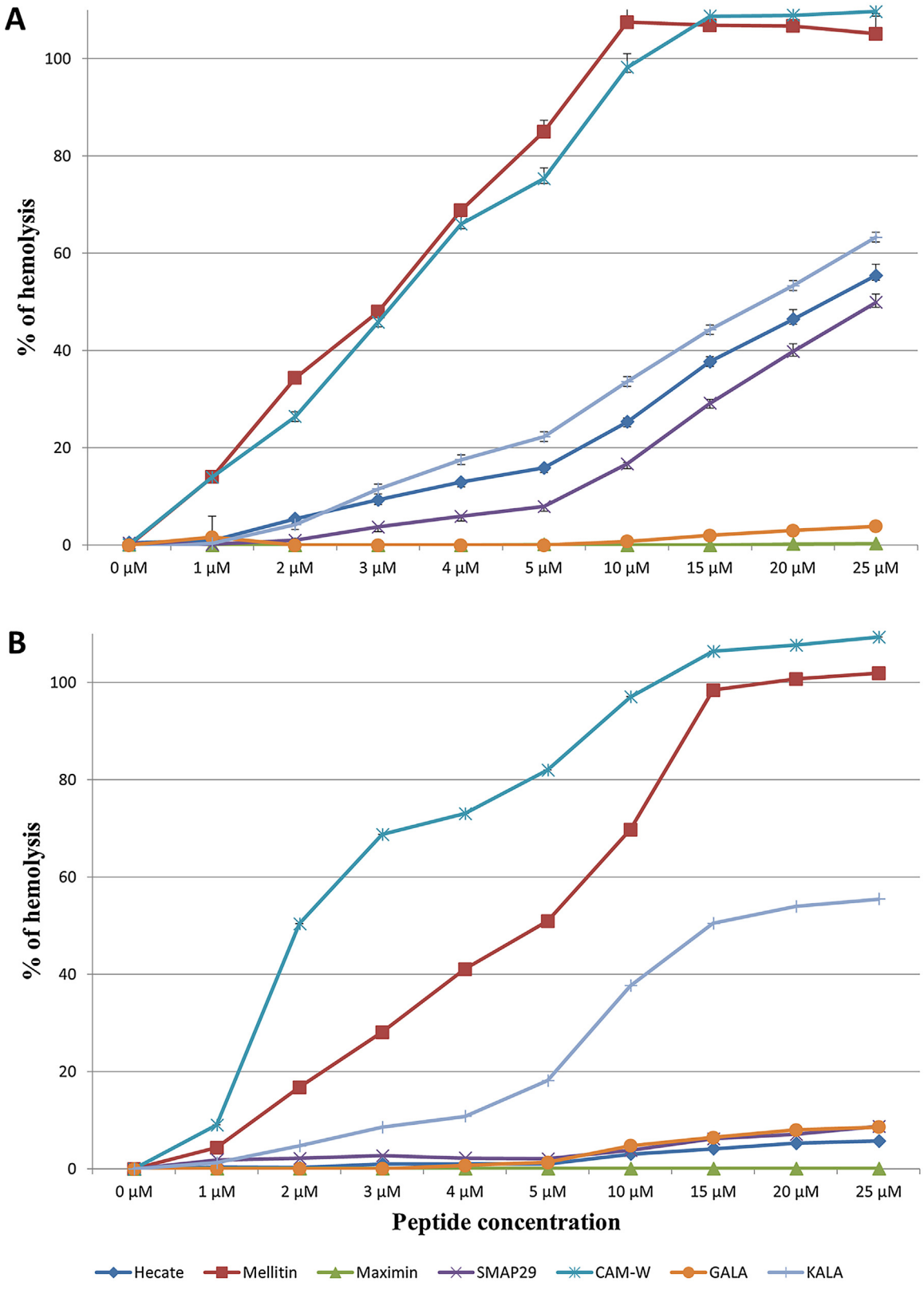

Fig. 1. Hemolytic activities of different peptides show significant differences between cationic and anionic peptides. At a neutral pH, the anionic peptides are mostly inactive. Melittin and CAM-W peptides have the greatest activity in the lowest concentrations while Hecate, SMAP29, and KALA show a moderate activity. The lytic activity was compared to the treatment with 0.1% Triton X100, a detergent commonly used for complete lysis of erythrocytes. (A) Human erythrocytes. (B) Equine erythrocytes.

tion cause the lysis of approximately 80% of human RBCs while only 50% of equine cells are lysed.

3.2. Inhibitory effects of peptides on lentivirus infectivity

In the virus infectivity assay we have used lentiviruses carrying the gene for expression of EGFP reporter protein in the infected cells. The virus particles were produced with a hybrid between rabies and VSV envelope protein leading to the highest yield and infectivity for HEK reporter cells (unpublished observation). Already 24 h after the infection there were apparent differences in the infectivity of viral vectors treated with tested peptides (Fig. 2A). The most profound inhibitory effect was triggered by melittin and CAM-W, where at 5 μM concentration a very few of GFP-expressing cells were found (quantification of GFP fluorescence is shown in Fig. 2B). CAM-W exhibited a strong inhibitory effect even at 1 μM concentration while at the same concentration many viruses remain infectious after melittin treatment. The substantial antiviral effect was noted for other cationic peptides hecate and KALA; though the inhibition became apparent only at 5 μM concentration of the peptides. The least inhibitory peptide was SMAP29. There are surprising differences among anionic peptides. While maximin H5 had only a small effect on virus infectivity, the GALA peptide behaved as a remarkably potent inhibitor despite the fact that the peptide should achieve active conformation in acidic pH. Because we could not exclude a possibility that some peptides might increase the virus infectivity the multiplicity of infection (MOI) was chosen 0.8. Measurements of GFP fluorescence (Fig. 1B) has revealed that maximin H5 and KALA in 1 μM concentration might slightly increase the amount of produced GFP but the increase is not significant. In order to exclude the possibility that the peptides present in the diluted lentivirus samples can inhibit cells growth we have treated HEK cells for 16 h with 1.25 μM peptides, the concentration corresponding to the peptide concentration in diluted virus sample containing the highest 5 μM peptide concentration. Hoechst33258 staining confirmed that the peptides do not interfere with cells growth or the viability (Fig. 2C). The health of cells is further confirmed by the presence of multiple mitotic events apparent on Hoechst33258 stained nuclei. No morphological changes such as cells detachment or rounding were seen in the phase contrast images.

3.3. Enzymatic VLPs lysis with the peptides

VLPs, whose inner core is formed by NLuc fused to Ebola structural protein VP40 and coated with either Marburg virus or

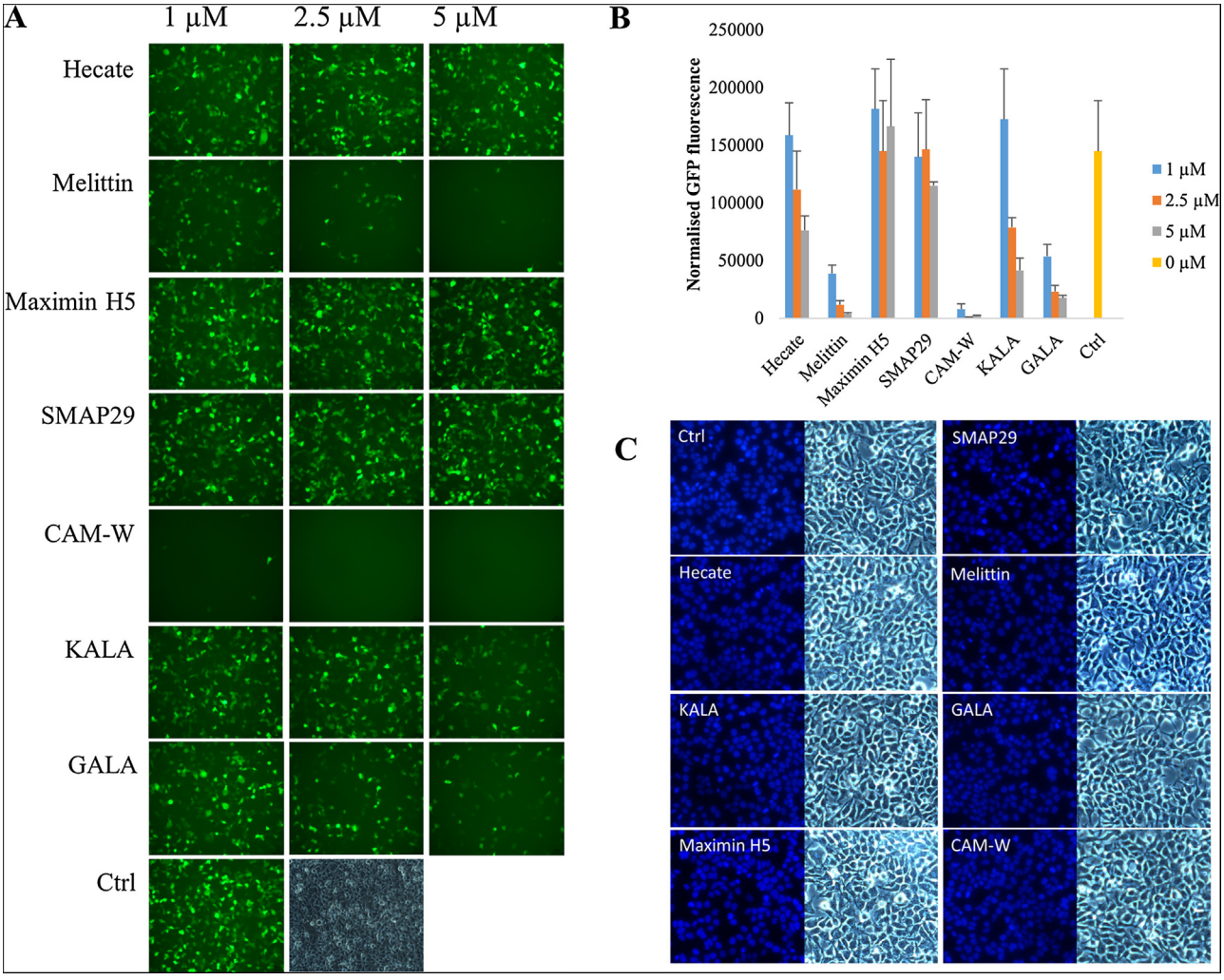

Fig. 2. Peptides inhibit the infectivity of recombinant lentiviruses. The virus supernatants of lentiviruses carrying the gene for expression of EGFP were treated with respective concentrations of peptides before applying to reporter HEK cells. (A) A fluorescent images of HEK cells 24 h after infection with lentiviruses treated with individual peptides. With control sample is included representative phase contrast image of HEK cells 24 h after infection (B) GFP fluorescence of cell lysates 36 h after infection. (C) Hoechst33258 and phase contrast images of HEK cells treated for 16 h with 1.25 μM peptides.

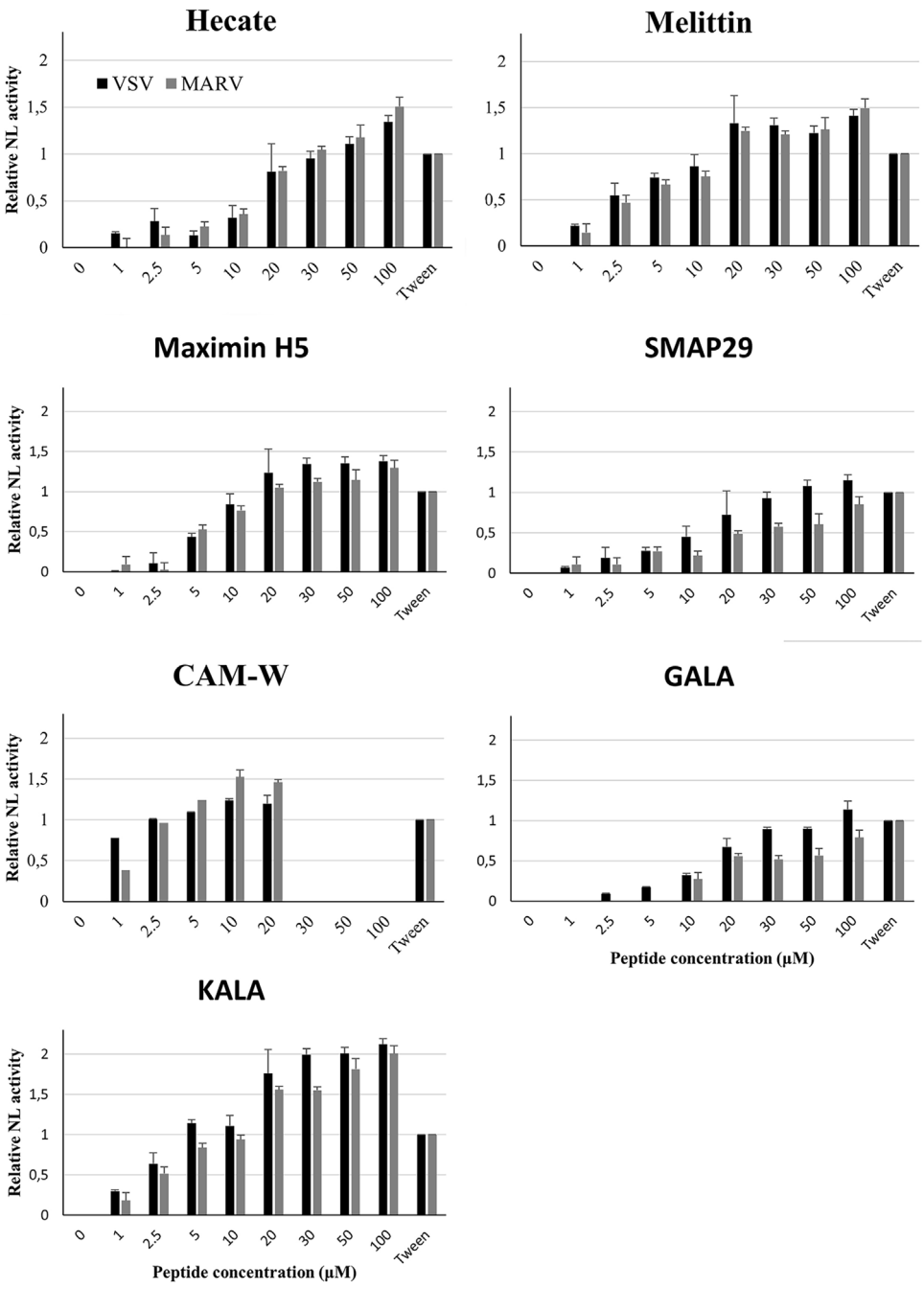

Fig. 3. Results of luciferase assay determining the amount of NanoLuciferase (NLuc) liberated from VLPs coated with VSV or Marburg virus envelope protein and treated for 20 min at 37 °C with different concentrations of individual peptides. As a control for lytic activity was used Tween 20. We found that CAM-W inhibits luciferase activity in concentrations above 20 μM. Corresponding values are absent from the graph.

VSV glycoprotein, were treated with increasing concentrations of individual peptides. All cationic peptides were able to effectively release the enzyme or permit access of the substrate in the concentrations over 1 μM (Fig. 3). The most effective peptide turned out to be CAM-W, a hybrid between cecropin A and melittin that has been further modified by replacing 4 amino acids with tryptophan residues improving its proteolytic stability. The peptide released more than half of enzymatic activity in 1 μM concentration where most of the other peptides had a very small activity. Other cationic peptides were able to release the luciferase activity at the concentrations exceeding 2.5 μM. Noteworthy; we have observed a moderate virolytic activity after treatment with maximin H5 and GALA peptides, which have manifested a negligible hemolytic activity.

We have analyzed two types of VLPs carrying different viral envelopes from distinct viruses. VSV belongs to Rhabdoviridae family while Marburg virus falls into Filoviridae. We have not observed a significant difference in the lytic activity against VLPs differing from each other in the envelope proteins. Maximin H5, SMAP29, GALA, and KALA were at higher concentration able to liberate more NLuc from particles coated with VSV compared to particles with the Marburg virus glycoproteins but the differences were negligible.

During the experiments, we have noticed that 0.5% Tween 20 used for VLPs lysis inhibits NLuc activity. That is why some peptides, mostly at concentrations over 10 μM have apparent activity

exceeding 100% attributed to Tween 20 lysed samples. We have tested other detergents and found that they all inhibit NLuc to a similar extent (data not shown). Despite the inhibitory activity of Tween 20, we decided to use it in order to compare relative activity of individual peptides related to the common lytic compound.

Interestingly, we found that CAM-W for unknown reasons at the concentrations exceeding 20 μM very strongly inhibits the NLuc enzyme activity, which resulted in the total elimination of signal depicted in Fig. 3. The inhibitory effect was confirmed in cell lysates of cells expressing NLuc alone (data not shown).

3.4. Cell viability of cells treated with peptides

In order to compare the results of a virolytic assay conducted on VLPs with cytotoxicity of peptides we performed a viability test on HEK and HeLa cells treated with individual peptides at the same concentrations used previously (Fig. 4). Viability assay with MTT follows the same pattern as the hemolytic assay with the exceptions of the CAM-W peptide. This peptide, while having very high hemolytic activity shows reduced cytotoxicity comparable to hecate, SMAP29, and KALA. Both anionic peptides, maximin H5 and GALA have inconsequential inhibitory activity on the growth of HEK and HeLa cells. Melittin was the most toxic peptide for both cell lines in the assay.

4. Discussion

We have systematically compared several assays commonly used to determine a lytic activity of several antimicrobial peptides and compared it to a newly designed assay based on Ebola-like VLPs containing a sensitive luciferase reporter enzyme. The choice of Ebola virus-like particles was motivated by the ease of VLPs production and increased loading capacity compared to other spherical viruses. Despite the fact, that the VLPs are based on Ebola virus their use is safe and does not require any special biosafety containment because the particles do not contain any genetic information, therefore, they cannot replicate and do not represent any biological hazard and can be used in any laboratory regardless of biosafety level.

The exact mechanism of pore formation by cationic peptides and subsequent membrane lysis and cytotoxicity still remains elusive. The membrane lysis is initiated by the attachment of the peptides to the surface of the membrane. Above a certain peptide/lipid ratio, the peptides invade the cell membrane and form toroidal pores [27]. Ladokhin et al. [28] have shown that pores formed by melittin reach size of 2.5–3 nm allowing passage of compounds up to several kDa. It is assumed that pore formation causes loss of membrane potential and loss of ions gradients incompatible with vital cell functions. However, the proposed mechanism does not explain virolytic properties of peptides because enveloped viruses do not depend on biochemical gradients. Jackman et al. [29] propose that the lysis of the viral membrane is strain induced. The membrane enveloping the virus core is highly curved imposing substantial stress. Formation of pores then induces membrane tearing relieving the curvature induced stress.

In our assays, CAM-W has the comparable hemolytic activity for human RBCs to melittin and higher hemolytic activity for equine RBCs. The data corresponds with the results of Ji et al. [18], where CAM-W at 10 mg/l (equals ~1 μM) has relatively low lytic activity. Unfortunately, as the authors did not analyze the hemolytic activity of the peptide at higher concentrations we cannot directly compare our results. At the concentration of 1 μM CAM-W induces hemolysis of 10% of RBCs, which was the same as melittin (Fig. 1). The complete lysis was achieved at 10 μM concentration for both peptides making CAM-W and melittin the most hemolytic peptides in the assay. In available literature, CAM-W is described as a peptide with the low hemolytic activity, however, the tests were performed with sheep RBCs [17]. Our results show that human RBCs can be lysed quite more efficiently, while equine RBCs were more resistant to the peptides-mediated lysis. Such contradictions are not uncommon. Low concentrations of ovispirin-1 and protegrin PG-1 are hemolytic for human and mouse RBCs, however, they are essentially non-hemolytic for sheep or bovine RBCs [15]. This emphasizes the flaw in the use of hemolytic tests to evaluate the potential lytic activity of peptides against viruses. The results of hemolytic assay correspond to some extend with cytotoxicity assay except for CAM-W peptide. This peptide shows lower cytotoxic activity compared to melittin, which makes it potentially an interesting antiviral compound.

The results of hemolytic assay do not correspond very well with the VLPs lytic assay. Both, anionic peptides, maximin H5 and GALA caused a very small release of hemoglobin from human RBCs but both peptides show a moderate ability to release NLuc from Ebola-like VLPs. The low hemolytic activity of maximin H5 and GALA can be explained by the fact that the assay was performed at a neutral pH where side chains of glutamic and aspartic acids are deprotonated and the peptides cannot form structured α-helix, which is a necessary prerequisite for the pore formation in the target membrane [20]. Interestingly, GALA was able to inhibit infection of cells with the recombinant lentiviruses delivering GFP gene to the infected cells. We assume that the effect is not mediated by direct lysis of the viral membrane but the disruption of the membrane is delayed and probably happens after virus internalization in the endosome and after acidification inside the late endosomes where GALA can adopt α-helical conformation.

Viruses are normally internalized through receptor-mediated endocytosis sequestering the virus particles into endosomes that are acidified along the endocytic pathway. The decline in pH activates the envelope proteins that initiate the process of fusion between the virus membrane and endosomal membrane resulting in a release of the virus capsid into the cell cytoplasm. However, the GALA peptide present in the endosome converts from a random coil to an amphipathic α-helix and forms a channel inside a viral membrane leading to virus inactivation. Alternatively, the peptide can embed itself into the endosome membrane forming an ion pore preventing endosome acidification similar to M2 proton channel of influenza virus [30,31]. The insufficient change in pH of endosome can prevent the viral envelope protein from effective fusion with endosomal membrane and release of the viral cargo into the cytoplasm. The inhibitory activity of maximin H5 is minor compared to GALA. We can only speculate on the reason. One possibility might reflect a total negative charge of the peptides. Maximin H5 carries three aspartic acid residues providing charge −3 while GALA harbors 7 glutamic acid residues reaching net charge −7, which makes it far more negative and, therefore, more sensitive to pH changes. The pH dependent lysis of VLPs with anionic peptides can be probably used in the virolytic assay by adjusting pH to the values common in the late endosomes, though we have not studied the effect of pH in our system.

That is another reason against the use of the hemolytic assay for determination of antiviral properties of newly discovered or designed peptides as the erythrocytes are very sensitive to pH changes and the effectivity of anionic peptides cannot be reliably evaluated in hemolysis assay.

The detectable virolytic activity of maximin H5 and GALA on enzymatic VLPs compared to low hemolytic activity most likely reflects the higher responsiveness of VLPs toward membrane disruptive agents and the high sensitivity of luciferase assay.

Membranolytic properties of peptides are often evaluated with the use of synthetic liposomes. Despite the difficulty with liposome preparation and artificial composition, they are valuable tool due

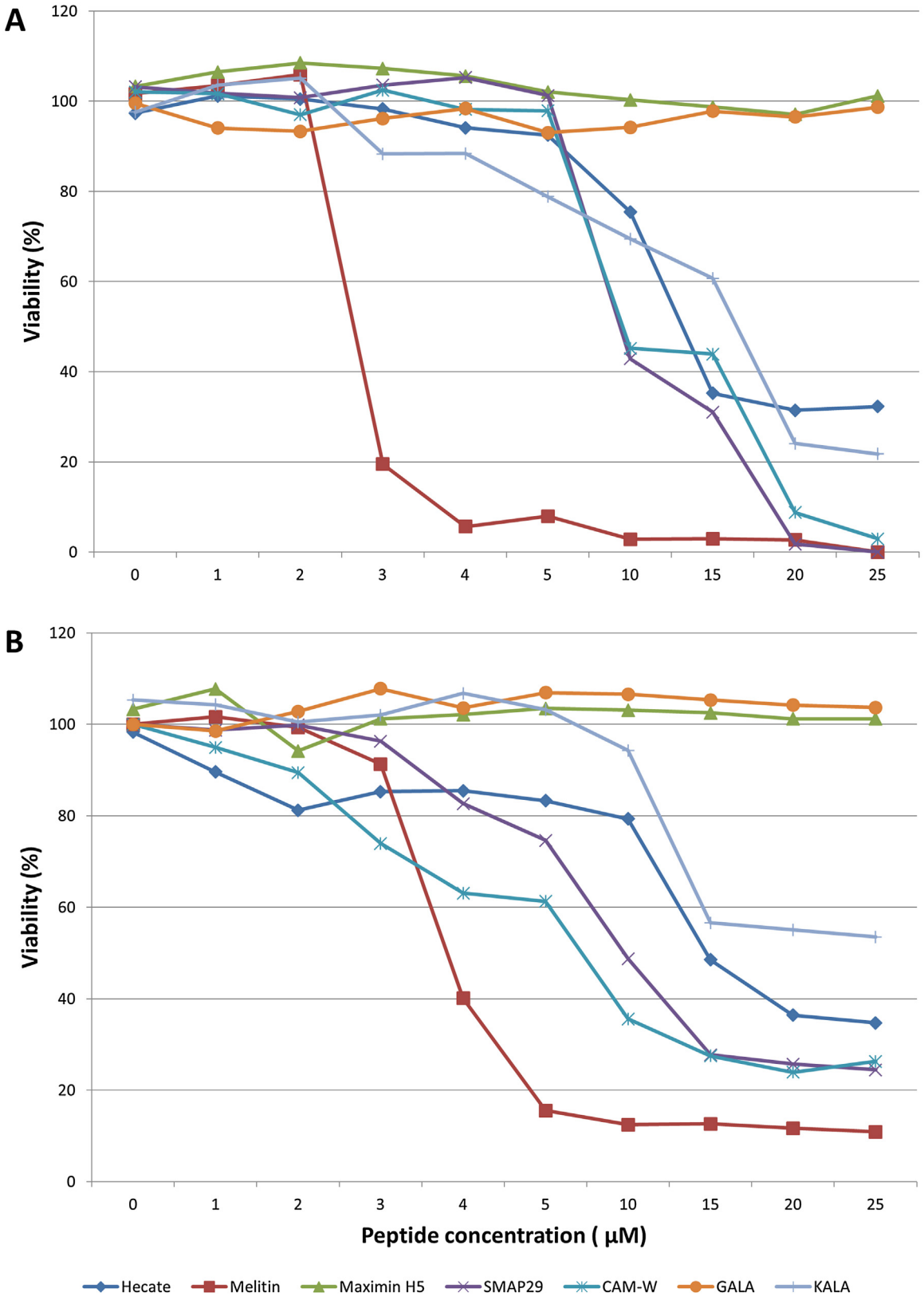

Fig. 4. Cell viability of cells treated with individual peptides. The cells were maintained in the presence of the peptides at indicated concentrations for 24 h and the viability was analyzed by an MTT assay. (A) HEK cells. (B) HeLa cells.

to an easy use and possibility to devise relatively simple biochemical assays. Our enzymatic VLPs provide several advantages over liposomes. The sensitivity of the assay is comparable to the use of liposomes, however, the preparation of particles is not encumbered by the requirements for the equipment necessary for the liposome preparation. The VLPs are inherently stable and do not aggregate as

easily as synthetic liposomes. Due to the fact that they are derived from live cells, the membrane composition is much more closely related to the real viruses. The VLPs can incorporate a specific viral envelope protein further mimicking the studied virus. Besides that, they contain other membrane proteins including glycosylated extracellular proteins identical to infectious viruses. Results of membranolytic assays carried out on naked liposomes can be misleading as many proteins present on the viruses or virus-like particles surfaces can interfere with the peptide. The peptides can be also sequestered by charged peptidoglycans at the virus surface effectively shielding the membrane from the peptide. In that respect, the results derived with the use of enzymatic VLPs are biologically more relevant compared to data acquired with the use of synthetic liposomes.

AMPs are gaining attention as the potential antiviral compounds. Some of them have already found clinical or practical applications. For example, melittin is being incorporated into microbicidal vaginal gels preventing the HIV transmission [32]. Another peptide with a low vaginal epithelial toxicity and anti-HIV activity considered as a potential component of virucidal gels is derivative of caerin 1 [33]. Our results indicate that the use of CAM-W should be considered in similar clinical applications due to its reduced cytotoxicity compared to melittin. Another interesting candidate is GALA peptide that has negligible hemolytic and cytotoxic activity even up to concentrations of 25 μM, but can effectively block the virus entry in concentrations above 5 μM.

5. Conclusions

The results demonstrate that the newly designed assay to evaluate virolytic properties is very sensitive and better reflects the potential of peptides to interfere with a viral membrane compared to a standard hemolytic assay. The greatest advantage is a relatively low cost of VLPs preparation and potential for high-throughput screenings. The assay can be accomplished in a short time compared to tests carried out on the reporter cell lines. The use of VLPs provides the flexibility in respect to a surface of particles adding versatility for the assays targeting specific viral epitopes. The assay also does not use infectious virus particles allowing the use in standard biosafety level laboratories. This assay avoids certain drawbacks of hemolytic assays, mainly false positive results due to different membrane composition or species-specific sensitivity to a given peptide.

Among the peptides analyzed in this work, the modified peptide CAM-W is the most active peptide against viruses or VLPs surpassing its progenitor melittin or melittin derivative hecate. CAM-W also has reduced cytotoxicity compared to melittin. Surprisingly, we found that GALA peptide possesses the virus entry inhibitory activity that is probably related to the adoption of active α-helical conformation within endosomes.

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