Antiviral Activity of Synthetic Peptides Derived from Physiological Proteins

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Keywords

Antiviral activity · Peptides · Antimicrobial peptides · Herpes simplex virus 1 · Adenovirus · Vesicular stomatitis virus · Coxsackievirus B5

Abstract

Background/Aims: New antivirals are needed to supplement or replace currently used drugs. The aim of this study was to evaluate the antiviral activity of synthetic peptides derived from physiological proteins. **Methods:** Vero cell monolayers were infected with herpes simplex virus 1, vesicular stomatitis virus, adenovirus, and coxsackievirus B5 strains in the presence of different concentrations of the selected peptides and viral yield was determined by plaque reduction assays to evaluate the antiviral activity of the peptides. Virucidal activity was evaluated by determining the residual infectivity of viral suspensions treated for 1 h with the peptides at the same concentrations as in the viral yield assays. **Results:** Among the investigated peptides, the killer peptide proved to exert a considerable antiviral activity against herpes simplex virus, attributable to a direct effect on virus particles, while its derivative K10S showed to be effective against the four investigated virus strains only at the

highest concentration tested, yet, the inhibitory effects were only partial. **Conclusion:** Overall, initial evidence is provided on the antiviral activity of several peptides, as well as of their derivatives. Further investigation is warranted to ascertain the mechanism of action in order to develop new potential antiviral drugs.

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Introduction

Drug-resistant infectious agents are emerging and spreading globally, threatening our ability to treat the relative diseases and resulting in prolonged illness, disability, and even fatal outcome. This ever-growing problem also results in enhancement of health care costs due to prolonged hospitalization, need for additional laboratory tests, and use of more expensive drugs [1, 2].

Viruses represent a major cause of disease and mortality worldwide and the emergence of viral drug resistance is of particular concern for public health because of the limited number of antiviral molecules available to date. It is well known that drug misuse produces a selective pressure on viruses during replication, allowing adaptation to

antiviral treatment and emergence of resistant strains [3]. The consequences of newly acquired resistance are particularly serious in chronic infections, as those caused by HIV, HBV and HCV, likely resulting in therapeutic failure. Moreover, the costs of new drugs are extremely high, rendering these therapies unaffordable in developing countries. Therefore, new antivirals are urgently needed to supplement or replace existing drugs [1, 2]. In this context, particular attention has recently been focused on naturally occurring or synthetic peptides endowed with antiviral activity, as potential alternatives to current therapeutic agents [4–7].

Antimicrobial peptides (AMPs) or, more generally, host defense peptides, are commonly considered as immunity primitive mechanisms and have been extensively studied in a wide variety of organisms, from microbes to plants, insects and vertebrates, including humans [8–13]. AMPs share common features regardless of their different origins: they are relatively short (10–50 amino acids), mostly positively charged, and amphipathic; interestingly, they exhibit different properties, based on their structural characteristics. Their range of activity usually includes bacteria, fungi, and protozoa, but also antiviral activity has been shown for several AMPs [14–17].

Biologically active peptides, defined as cryptides or crypteins, may also derive from proteolytic cleavage of physiological proteins, including, among others, hemoglobin, components of the complement cascade, and milk proteins [18, 19]. Cryptides may exhibit a broad spectrum of biological activities, different from those of the precursor proteins.

A peculiar group of cryptides are those derived from either the variable or the constant regions of antibodies (Abs) [20–22]. In particular, the killer peptide (KP), the leading compound of a group of Ab-derived fragments endowed with various biological activities, has been widely investigated. It is a decapeptide, derived from the variable region of an anti-idiotypic Ab representing the functional internal image of a yeast killer toxin, endowed with a wide spectrum of antimicrobial activity [23, 24]. To date, KP has shown an outstanding activity against many different infectious agents, including HIV and influenza A viruses, through different mechanisms of action [25, 26]. Here, we expanded this issue, investigating the activity of KP against viruses with different morphology (enveloped vs. nonenveloped) and genome type (DNA vs. RNA). In addition, we studied other peptides, derived from human serum proteins, including Abs, albumin, fibrinogen, C3c and C4 complement fragments, and peptides encoded by immunoglobulin genes.

Materials and Methods

Cell Line

The Vero cell line was used for all the experiments. Cells were cultured at 37 °C in 5% CO₂ atmosphere in Dulbecco's minimum essential medium containing 10% (growth medium) or 5% (maintenance medium) fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL), ciprofloxacin (100 µg/mL), and L-glutamine (2 mM). Cells were maintained by biweekly passages in fresh medium.

Viruses

Herpes simplex virus 1 (HSV-1), coxsackievirus B5 (CoxV B5), adenovirus (AdV), and vesicular stomatitis virus (VSV) strains used in this study were clinical isolates, identified by monoclonal antibodies and laboratory adapted through serial passages (>50) on Vero cells [27]. Viral suspensions used as inocula were obtained from centrifuged lysates of virus-infected Vero cells, cultured in serum-free medium. All the experiments were carried out in medium without fetal bovine serum in order to avoid interactions between serum components and peptides. Virus batches were titrated on Vero cells, aliquoted and kept frozen at –80 °C.

Peptides

The investigated peptides were synthesized by solid-phase peptide synthesis using a multiple peptide synthesizer (SyroII, Multi-SynTech GmbH), at the CRIBI Biotechnology Center (University of Padua, Italy). Peptide purity was >90% as evaluated by analytical reverse-phase HPLC. The peptides were solubilized in dimethylsulfoxide at a concentration of 20 mg/mL and subsequently diluted in phosphate-buffered saline for experimental use. The origin and the amino acid sequence of the peptides used in the study are reported in Table 1.

Evaluation of Peptide Cytotoxicity by MTT Assay

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the effects of different concentrations of peptides on Vero cell viability. Briefly, cell cultures grown in monolayer in 96-well plates were treated with the peptides, starting from 10, 50, and 100 µg/mL, and, only for KP, up to 800 µg/mL in order to obtain the 90% cytotoxicity concentration (CC₉₀), and incubated for 24 h at 37 °C. Then, the MTT assay was carried out as previously described [28]. Cell viability was expressed as optical density percentage of the treated cultures compared to that of the untreated controls (100% viability). CC₉₀ was calculated by regression analysis, as the peptide concentration capable of reducing the optical density in the MTT assay by 90% in comparison to control.

Evaluation of Peptide Antiviral Activity

In order to preliminarily select peptides endowed with a potential antiviral activity, a plaque reduction assay (PRA) was initially performed [29]. Briefly, Vero cells grown in 96-well culture plates (2 × 10⁴ cells/well) for 24 h at 37 °C were infected with 25 µL of serum-free medium containing the different viruses under study diluted to 10⁴ plaque-forming units (PFUs)/mL with or without (as negative control) each peptide at a concentration of 10 µg/mL. After 1 h of incubation to allow viral adsorption, the inoculum was removed and serum-free medium (200 µL/well) containing 0.6% v/v human Ig (Ig VENA, Kedrion, Lucca, Italy) with or without

Table 1. Amino acid sequence and origin of the investigated peptides

Peptide	Amino acid sequence	Origin
KP	AKVTMTCSAS	peptide derived from the variable region of an anti-idiotypic Ab representing the functional internal image of a yeast killer toxin [24]
K10S	KKVTMTCSAS	KP derivative
N10K	NQVSLTCLVK	fragment of the constant region of human IgG1 [21]
N1A	AQVSLTCLVK	N10K alanine-substituted derivative
L5A	NQVSATCSVK	N10K alanine-substituted derivative
L8S	NQVSLTCSVK	N10K alanine-substituted derivative
K13L	KKLVAASQAALGL	fragment of human albumin [43]
D15R	DSGEGDFLAEGGGVR	fragment of fibrinogen α -chain [43]
D15T	DEAGSEADHEGTHST	fragment of fibrinogen α -chain [43]
G17K	GLEELQFSLGSKINVK	fragment of α -chain of complement C4 [43]
S17K	SEETKENEGFTVTAEGK	fragment of α -chain of complement C3c [20, 43]
G10S	GYCSGGSCYS	peptide encoded by immunoglobulin genes [32]
L12P	LCLRNDQGHPR	peptide encoded by immunoglobulin genes [32]

(negative control) each peptide (10 $\mu\text{g/mL}$) was added to each well. After 24 h of incubation, the cells were fixed with methanol and stained with crystal violet. Plaques were scored visually and PFUs were enumerated. Each assay was performed in triplicate.

The antiviral activity of the selected peptides against the different viral strains was then evaluated by the viral yield reduction assay. Vero cells monolayers were infected for 1 h at 37 °C with virus inocula in the presence of peptides (final concentrations 10, 50, and 100 $\mu\text{g/mL}$) or without peptides (control). After 1 h of adsorption at 37 °C, viral inocula were removed and serum-free medium containing peptides at the same concentration used for the inocula, or medium alone (control), was added. Each assay was performed in triplicate. After 24 h of incubation at 37 °C, the supernatants were harvested, and virus yields were titrated by PRA on Vero cell monolayers [29]. Briefly, Vero cells were seeded in 24-well plates (1.5×10^5 cells/well) and infected with 200 μL of 10-fold serial dilutions of the samples to be titrated. After 1 h of incubation, the inoculum was removed and 200 μL of maintenance medium containing 0.6% v/v human Ig was added to each well. After 48–72 h of incubation, the cells were fixed and stained, then plaques were scored visually, and virus titers were expressed as PFU/mL. Results were expressed as percent viral yield in comparison to control (100%).

Evaluation of Inhibitory Concentration 90 and Selectivity Index

The 90% inhibitory concentration (IC_{90}) was established for the most active peptide by PRA. Vero cell monolayers were infected with virus inocula containing 2-fold peptide dilutions. After incubation, the inocula were removed and serum-free medium, containing 0.6% v/v human Ig and peptide at the same concentrations used for the inocula, was added to each well. After 24 h of incubation, the cells were fixed, stained and PFUs enumerated. IC_{90} was calculated by regression analysis as the peptide concentration capable of reducing the number of PFU by 90% in comparison to control (infected cells in the absence of peptide). The selectivity index (SI) was calculated as the ratio between the CC_{90} and the IC_{90} values.

Evaluation of Peptide Virucidal Activity

To evaluate whether the antiviral activity of the investigated peptides could be due to a direct viral inactivation by interaction with virus particles before adsorption, viral suspensions (10⁴ PFUs/mL) were treated with peptides at concentrations of 10, 50, and 100 $\mu\text{g/mL}$ and incubated for 1 h at 37 °C. The residual infectivity of the samples was then titrated by PRA as described above.

Statistical Analysis

The data reported in figures are the mean values ($\pm$ standard errors) from at least 3 independent experiments. Data were analyzed by one-way ANOVA with the Bonferroni post hoc test. A p value <0.05 was considered as statistically significant.

Results

Peptide Cytotoxicity on Vero Cells

By MTT assay, we preliminarily evaluated the cytotoxic effects (if any) of the peptides on Vero cells. At the lowest concentration(s) tested, a negligible toxicity ($<10\%$) was observed (data not shown), irrespective of the peptide tested, and also at the highest concentration (100 $\mu\text{g/mL}$), 7 peptides (KP, D15R, D15T, G17K, S17K, G10S, L12P) caused only a small reduction of Vero cells viability (2–10%) in comparison with the untreated control. Differently, at the same concentration (100 $\mu\text{g/mL}$), 4 peptides (N10K, L8S, L5A, K10S) reduced cell viability of approximately 20%, while N1A and K13L showed the highest effects (40 and 30% reduction of viability, respectively). On the basis of these data, further experiments were performed at 3 peptide concentrations, namely 10, 50, and 100 $\mu\text{g/mL}$.

Fig. 1. Antiviral activity of the peptides, as evaluated by the viral yield reduction assay. Vero cells were infected with HSV-1 (a), VSV (b), AdV (c), and CoxV B5 (d) and treated with the selected peptides at 3 concentrations (10, 50, and 100 µg/mL). Twenty-four hours following infection, virus yield was determined by PRA. Vi-

ral yield percentages of peptide-treated cells in comparison with mock-treated cells (100% yield) are reported as mean $\pm$ standard errors of 3 independent experiments. $p < 0.05$ for 10 and/or 50 µg/mL vs. 100 µg/mL (a); $p < 0.05$ for 10 and/or 100 vs. 50 µg/mL (b); $p < 0.05$ for 50 and/or 100 vs. 10 µg/mL (c).

Peptide Antiviral Activity

On the basis of preliminary data obtained by PRA, where the 4 viruses were challenged with each peptide at a concentration of 10 µg/mL (data not shown), we selected a limited number of peptides for further analysis against the different viruses by viral yield reduction assays. In particular, KP, L12P, and K10S were tested against HSV-1; KP, N1A, L5A, and K10S against VSV; S17K and K10S against AdV; K13L, D15R, G17K, L12P, and K10S against CoxV B5. Each peptide was tested at three different concentrations. The results, expressed as a percentage of viral yield compared to the controls (medium without peptide), are shown in Figure 1. With respect to HSV-1 (Fig. 1a), none of the selected peptides (KP, L12P, and K10S) showed an appreciable antiviral activity, i.e., a viral yield $< 10\%$ in comparison to controls (100% infectivity), when used at the lowest concentration (10 µg/mL). However, KP caused a high inhibition at 50 and 100 µg/mL, with a viral yield of 0.4 and 0.03%, respectively. L12P and K10S caused a

weaker inhibition, with a viral yield of 16 and 24%, at 50 µg/mL, and 13 and 11% at 100 µg/mL, respectively.

K10S was also tested against VSV, giving an approximately 4% virus yield at 100 µg/mL (Fig. 1b). The other peptides assessed against VSV, namely KP, N1A, and L5A, did not show remarkable antiviral effects, at any concentration.

When the peptides S17K and K10S were tested against AdV (Fig. 1c), K10S gave an appreciable inhibition, with a viral yield of 9% in comparison with the control, at the highest concentration, while no significant antiviral activity was obtained with S17K.

The peptides K13L, D15R, G17K, L12P, and K10S were tested against CoxV B5 (Fig. 1d). None of them exhibited a significant activity at 10 and 50 µg/mL. With the exception of L12P, when used at 100 µg/mL, the other peptides showed a modest inhibiting activity, with a mean viral yield of approximately 13% (K13L), 8% (D15R), 12% (G17K), and 6% (K10S).

Fig. 2. Virucidal activity of the peptides. Viral suspensions of HSV-1 (a), VSV (b), AdV (c), and CoxV B5 (d) were treated with the selected peptides at 3 concentrations (10, 50, and 100 µg/mL) for 1 h at 37°C in serum-free medium, then the residual infectivity was determined by PRA. Infectivity percentages of peptide-

treated viral suspensions in comparison with mock-treated control (100% infectivity) are reported as mean ± standard errors of 3 independent experiments. No significant difference was observed between the different concentrations of the peptides.

Considering the remarkable antiviral activity displayed by KP against HSV-1, the CC₉₀, IC₉₀, and SI were determined. The CC₉₀ of KP, evaluated by the MTT assay, was 2,943 µg/mL, while the IC₉₀ was 58 µg/mL; consequently, the SI value was 50.74.

Peptide Virucidal Activity

In order to evaluate whether the antiviral activity observed for some peptides was related to a direct interaction with the viral particles, each virus was pretreated with any selected peptide at 3 different concentrations for 1 h; then, the residual infectivity was titrated by PRA. The results, expressed as percent infectivity in comparison to controls (virus without peptide), are shown in Figure 2. Only KP showed a direct activity against HSV-1 (Fig. 2a), since the percent infectivity dropped to 13% (at 10 µg/mL) and 3.3% (at 100 µg/mL) compared to the 100% infectivity of the control. No other peptide, even if displaying a considerable antiviral activity as assessed by viral

yield assay (Fig. 1), showed a direct virucidal activity against the other viruses tested (Fig. 2b–d).

Discussion

The lack of therapeutic agents against many important viral infections and the ever-growing problem of drug resistance pose the urgent need for molecules endowed with antiviral activity, possibly also exhibiting a minimal toxicity profile and having limited costs; this would give rise to the development of novel antiviral drugs. In this scenario, increasing interest has arisen for AMPs, a large group of natural molecules present in all micro- and macro-organisms, as an essential part of their own defenses, and for cryptides, known to derive from physiological proteins [4, 5, 9, 10, 30]. The most intriguing peculiarity of these molecules is the wide-spectrum antimicrobial activity, achieved by a direct action against microbial targets

or indirectly, via activation of host immune responses [31].

In the present study, we evaluated the antiviral activity of 13 synthetic peptides against 4 viruses, namely HSV-1, CoxV B5, AdV, and VSV, exhibiting different structural features. As described elsewhere, the amino acidic sequences of the selected peptides are comprised in proteins of various origin, including immunoglobulins, human albumin, fibrinogen α -chain, and complement fragments. Several studies have already demonstrated a wide-spectrum antimicrobial activity by KP and some of the other peptides [21, 23, 32]. Yet, the antiviral activity of such peptides has not been investigated yet, with the exception of KP, which has been shown to inhibit HIV as well as human and avian influenza viruses [25, 26]. The results of the present work expand our knowledge on KP, showing that it drastically reduces HSV-1 viral yield, at both 50 $\mu\text{g/mL}$ (99%) and 100 $\mu\text{g/mL}$ (99.99%) (Fig. 1a); also, the corresponding IC_{90} and SI values are 58 $\mu\text{g/mL}$ and 50.74, respectively.

According to the literature, many different AMPs are able to neutralize enveloped viruses by directly interacting with their lipid membrane, regardless of the nucleic acid nature of the viral genome [14, 33]. On this basis, we investigated the potential virucidal activity of KP against HSV-1 by preincubating a cell-free viral suspension with this peptide before cell infection: the results show a massive virus inactivation (99%) (Fig. 2a), thus strongly suggesting that the antiviral activity of KP is mainly mediated by its direct interaction with the virus.

The neutralization of virus infectivity may be ascribed to the presence of hydrophobic amino acids, allowing KP to interact with the envelope lipid bilayer, thus inducing its destabilization. Alternatively, thanks to its positive charge, KP may interact with envelope lipids and/or glycoproteins [34–36]. It is noteworthy that, in our hands, KP fails to have any significant inhibitory activity against the two nonenveloped viruses, AdV and CoxV B5. With regard to the other enveloped virus tested, VSV, KP has shown a scant viral yield inhibition (78%) (Fig. 1b) and no virucidal activity (Fig. 2b). The different composition and organization of the membranes of HSV-1 and VSV may account for these different results: indeed, while glycoproteins are virus specific, lipids derive from different cell membranes, nuclear or reticular for HSV-1, cytoplasmic for VSV [33, 37, 38].

Previous works have demonstrated an antiviral activity for KP against other enveloped viruses, such as HIV, by internalization of the chemokine receptor type 5 (CCR5) in susceptible cells, and human and avian influ-

enza viruses, by reduction of viral protein synthesis, in particular M1 and HA [25, 26]. In both cases, a direct KP interaction with viral envelope has been excluded. Therefore, the HSV-1 inhibition observed in the present study may likely be ascribed to a different mechanism of action.

We show that K10S exhibits a weak antiviral activity against HSV-1 but, differently from KP, a high inhibition of VSV (Fig. 1a, b). This peptide has the same sequence of KP except for a substitution in the first amino acid (Table 1); thus, we suggest that the differences between KP and K10S are due to such a single amino acid substitution. Accordingly, several studies suggest that the interaction between AMPs and viruses depends not only on the overall peptide charge but also on its sequence specificity [33].

Different peptide-virus interactions may allow different mechanisms of action to occur: similarly to KP, K10S is virucidal against HSV-1 but not against VSV, suggesting that viral neutralization is not responsible for VSV yield reduction. Therefore, other mechanisms are likely involved, impacting one or more phases of virus replication. Furthermore, K10S proves to also mildly inhibit AdV (91% of reduction of viral yield) and CoxV B5 (94%), both of them being without envelope (Fig. 1c, d). As for AdV, a modest virucidal action has also been observed (80% reduction of virus infectivity) (Fig. 2c). Different hypotheses can be advanced to explain such results. First, this peptide may interact with capsid proteins, thus reducing virus binding, uncoating or exit from endocytic vesicles [33, 39, 40]. Another mechanism may be suggested, according to the observations of Gounder et al. [41] on the direct interactions between other AMPs and AdV: they demonstrated that the decrease in virion repulsive charge leads to virus particles aggregation and impairment of virus attachment. The same hypothesis can be drawn for S17K, that displays a virucidal activity against AdV similar to that observed for K10S.

Besides K10S, other 3 peptides (K13L, D15R, and G17K) show a reduction of CoxV B5 yield, of about 90% at 100 $\mu\text{g/mL}$ (Fig. 1d), without any virucidal activity; these findings suggest that, as for K10S against VSV, inhibition of viral replication occurs at stages following virus adsorption.

Overall, our results prove that KP is the most promising peptide among those tested, with a remarkable inhibition of HSV-1. These findings together with those previously observed against HIV and influenza viruses, candidate KP as a broad-spectrum antiviral drug, likely correlated with the multiplicity of viral targets. Since a

single amino acid substitution may change peptide activity, as in K10S, we hypothesize that antiviral properties may be further enhanced by other substitutions in the peptide amino acid sequence. The *in vitro* data here described on the direct antiviral activity do not supply any information on peptide interactions with host immune system, which are also likely to occur; initial evidence has been provided on such a dual role of some AMPs, including KP [12, 42]. Finally, since these peptides have peculiar

mechanisms of action and, in some cases, they exert their activity directly on viral external structures, the risk of selecting new drug-resistant strains should be minimized [13].

Disclosure Statement

The authors declare that they have no competing interest.

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