UNIVERSITY OF TARTU

Faculty of Science and Technology

Institute of Technology

Muhammad Nabeel

A Study of Anti-adenoviral activity of antimicrobial peptides

Master's Thesis (30 ECTS)

Curriculum of Bioengineering

Supervisor:

PhD Kai Rausalu

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A Study of Anti-adenoviral activity of antimicrobial peptides

Abstract:

Adenoviruses, non-enveloped DNA viruses, pose a significant health concern. This study explores the antiviral potential of four recombinant proteins with attached cationic antimicrobial peptides (H1mod1R, H2mod2R, HELP and HIn) against the human adenovirus (AdV-Gluc) in vitro. The proteins presented some antiviral activity, but this inhibitory effect was independent of the concentrations of the proteins within the tested range. The results are not aligned with the previous research on the cationic antimicrobial peptides with other viruses. Currently, these proteins are not ideal for use as therapeutic against adenovirus, but the good cytotoxicity profile suggests that further research is needed to optimize the efficacy and to explore their mechanism of action for beneficial future applications.

Keywords:

adenovirus, antimicrobial peptides, antiviral activity

CERCS:

B230 microbiology, bacteriology, virology, mycology

Uurimus antimikroobsete peptiidide adenoviirusvastasest aktiivsusest

Lühikokkuvõte:

Adenoviirused on ümbriseta DNA genoomiga viirused, mis on oluline terviseoht. See uurimus käsitleb antiviraalsid omadusi neljal rekombinantsel valgul, mille struktuur sisaldab katioonseid antimikroobseid peptiide (H1mod1R, H2mod1R, HELP ja HIn). Nende valkude toimet inimese adenoviirusele (AdV-Gluc) uuriti *in vitro*. Uuritud valgud demonstreerisid tagasihoidlikku antiviraalset toimet, kuid see viirusvastane mõju oli testitud kontsentratsioonide piires kontsentratsioonist sõltumatu. Tulemused ei ühti hästi teiste uurimuste tulemustega, mis on tehtud katioonsete antimikroobsete peptiidide ja teiste viirustega. Hetkel pole need valgud valmis kasutuseks adenoviirusvastase ühendina, kuid tsütotoksilisuse puudumine lubab loota, et edasise uurimis- ja arendustööga, mis suurendaks valkude viirusvastast efektiivsust ning aitaks mõista nende viirusvastase aktiivsuse toimemehhanismi, võiks neil valkudel olla potentsiaalne roll tuleviku viirusvastastes rakendustes.

Märksõnad:

adeoviirus, antimikroobsed peptiidid, antiviraalne aktiivsus

CERCS kood:

B320 mikrobioloogia,bakterioloogia, viroloogia, mükoloogia

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ABBREVIATIONS

AdV	Adenovirus
Adv-2	Adenovirus serotype 2
Adv-5	Adenovirus serotype 5
AMP	Antimicrobial peptide
CHDP	Cationic Host Defense Peptides
Cryo-EM	Cryo-Electron Microscopy
dsDNA	double-stranded DNA
DMEM	Dulbecco's modified eagle medium
FBS	Fetal Bovine Serum
GI	Gastrointestinal tract
Glu	Gausia luciferase
HVR	Hypervariable regions
HIV-1	Human immunodeficiency virus-1
In	Indolicidin
Iu	Infectious unit
PBS	Phosphate Buffered Saline
PKC	Protein Kinase C
RLU	Relative Luminescence Units
SARS	Severe acute respiratory syndrome coronavirus 2
TGEV	Transmissible gastroenteritis virus
TAF	Trans Acting Factor
wt	wild type

WST	water soluble tetrazolium

Introduction

Viral infections are a serious threat to human health, and new drug-resistant variants of viruses make the treatment more complicated. This highlights how urgently new antiviral treatments are needed. Cationic antimicrobial peptides (AMPs), have come to light as potential antiviral agents. These naturally occurring peptides have broad-spectrum antibacterial activity and may be useful in the fight against viruses, adenoviruses among them.

Adenoviruses are non-enveloped DNA viruses that can cause a variety of diseases, ranging from serious respiratory and gastrointestinal infections to the common cold. The importance of finding alternate antiviral approaches gains significance by the increasing incidences of adenoviral infections and the limitation of available treatments.

Research has shown that cationic antimicrobial peptides, specifically β- and α- defensins, exhibit antiviral efficacy against a range of viruses. They can act by breaking viral envelopes, limiting viral entrance, or altering host immune responses as part of their complex mechanism of action against viruses.

This thesis explores the antiviral activity of synthetic recombinant protein carriers with attached cationic AMPs, namely H1mod1R, H2mod1R, HELP and HIn, against adenoviruses. In this study the effectiveness of various AMPs is evaluated, and the possibility of using them against viruses discussed.

1. LITERATURE REVIEW

1.1. Biology of Adenovirus

1.1.1. AdV genome structure

Adenoviruses (AdV) have double stranded DNA (dsDNA) genome. Their genome length varies between 26 kbp in case of frog adenovirus and 48 kbp in the fish, the only adenovirus isolated from the fish so far (Liu, 2011).

The most well-characterized human AdVs are serotypes 2 (AdV2) and 5 (AdV5), both belonging to subclass C (there are seven subclasses of AdV, named A to G). The 36 kb AdV-5 genome encodes 39 genes, which are categorized as early or late based on whether their expression occurs prior to or subsequent to DNA replication. The proteins encoded by the four early transcription units (E1a, E1b, E3, and E4) are necessary for immune response modification, host cellular environment modification, and transactivation of other viral areas. Proteins directly involved in viral DNA replication are encoded by E2 (Giberson *et al.,* 2012). The viral DNA does not directly interact with the key capsid proteins, but it nevertheless seems to play a role in the physical stability of the virion; virions containing sub genomic sized DNA (less than 90% of the length of the wild-type (wt) genome) are less stable than those containing full-sized wt AdV DNA. The AdV genome includes a packaging sequence and inverted terminal repeats, which are essential for the replication and encapsidation of the viral DNA, respectively, in addition to encoding the components needed for virion generation (Kennedy, M. A., & Parks, R. J. (2009**).** The scheme of AdV genome is depicted in Figure 1.A. Three major (II, III, and IV) and five minor (IIIa, IVa2, VI, VIII, and IX) polypeptides make up the AdV5 capsid; these proteins don't interact with DNA. Conversely, the viral DNA is linked to three extremely basic proteins, known as core proteins VII, V, and Mu (μ), within the capsid. The protamine-like protein known as protein VII is in charge of condensing and wrapping the viral DNA. The twelve huge spherical nucleoprotein projections, known as adenosomes, that extend into each vertex make up the protein VII-DNA nucleoprotein complex, which is arranged into a central dense core. It is believed that the protein VII-DNA complex is covered in a protein V shell (Kennedy *et al.,* 2009). Protein VII shields the viral DNA from triggering the DNA damage response during the infection phase. Six copies of the viral core protein VII are coiled around 200 bp of DNA per viral nucleosome. (Karen, K. A., & Hearing, P. 2011). Trans-acting factors involved in transcription and replication have limited access to their corresponding sites due to the

chromatin structure formed by the complexation of the viral DNA in infected cells with either histones or basic viral core proteins. Thus, the modification of the viral chromatin occurs prior to the start of transcription and/or replication. This finding implies that the DNA structure is altered to facilitate these processes, and it has been demonstrated that TAF1a and TAF2B activate the replication machinery. In the late stage of replication the pre-protein VII replace the histones to make it feasible for packaging. (Okuwaki *et al.,* 1998).

1.1.2. AdV virion structure and major virion proteins

Adenovirus virions have an icosahedral symmetry. A pseudo T = 25 icosahedral capsid measuring roughly 95 nm from vertex to vertex contains the genome. The icosahedron facets are formed by the primary coat protein, Hexon. The fibers and penton base combine to produce capsomers at the vertices. Since these two proteins are in charge of cell receptor contact, they are important participants in the early stages of infection (Liu, 2011). The scheme of AdV virion is depicted on Figure 1.

Hexon

Hexon, which makes up around 60% of the virion mass overall, is the main structural component of the protein shell of adenovirus. Capsid has 730 hexon monomers in it, arranged into 240 trimers, 12 trimers for each facet. Hexon is a big polypeptide that is present in all known adenovirus strains and is longer than 900 amino acids. The monomer folds into two domains, or eight-stranded β barrels, separated by a little β-sheet (Burnett *et al.,* 1985). Hypervariable regions (HVRs) are found within the hexon towers, which are composed of long loops intercalated between the β-strands. To establish connections between hexons and minor coat proteins IIIa and VIII, the N- and C-termini assume distinct conformations based on where they are located within the capsid (Crawford-Miksza, L., & Schnurr, D. P. 1996). Hexon has a conserved structure with two folded domains and variable loops on the surface. These loops can differ in length and flexibility depending on the adenovirus type, potentially affecting how the virus interacts with the host (Pérez-Illana *et al.,* 2021).

Penton

The void created by the five peripentonal hexons is filled up by pentamers of penton base. The integrin-binding motif known as Arg-Gly-Asp (RGD) sequence found in a hypervariable loop region of the penton base protein, despite the viral penton base exhibiting a high degree of sequence homology. The length of this segment varies between human adenovirus serotype 12 (hAd12) and human adenovirus type 2 (hAd2), ranging from about eight residues

to about seventy. The hypervariable loop of the majority of human adenoviruses contains the integrin-binding motif known as Arg-Gly-Asp (RGD) sequence. Enteric AdV-F40 and AdV-F41 have RGAD and IGDD instead of the RGD motif (Zubieta *et al.,* 2005).

In human adenoviruses, a separate surface-exposed variable loop exhibits divergent sequences and distinct conformations. Human adenoviruses have an extensive (~50 residues) N-terminal arm that extends from the main body of the protein and interacts with two monomers of the inner coat protein IIIa inside the virion (Liu *et al.,* 2010).

Fibers

Adenovirus fibers are made up of three domains: the head, the shaft, and the N-terminal tail. The globular head is in the C-terminus of the protein. The tail secures the fiber to the capsid's penton base, the head binds to receptors on the host cell membrane. Depending on the type of virus, the shaft's length and flexibility can vary (Vassal-Stermann *et al*., 2019). While the shaft is constructed from six to twenty-two repetitions of a repeating design. It allows the knob to emerge from the virion and connect with cellular receptors; its length and rigidity vary between species. With rare exceptions, adenoviruses bind to host cells most frequently through the interaction of the knob domain of the viral fiber protein with a host cell receptor; while internalization requires the penton base.

The fiber protein's central domain folds into a trimeric β-spiral and generates a shaft of varying lengths. Additionally, shaft flexibility and length influence AdV entrance into the cell by promoting virion contact with integrins via the penton base and the main receptor via the fiber base (Wu *et al*., 2003). The means to extract non-icosahedral details (disposition of fiber tail and the trimeric nature of the head) from icosahedral virus capsids is made possible with the developments in Cryo-EM image processing (Stass *et al.,* 2018). Additionally, advanced Cryo-EM investigations demonstrate how the fiber N-terminal peptides wrap around the RGD (Arg-Gly-Asp) loop in the penton base, extending farther than previously seen by X-ray crystallography (Liu *et al.,* 2011**).**

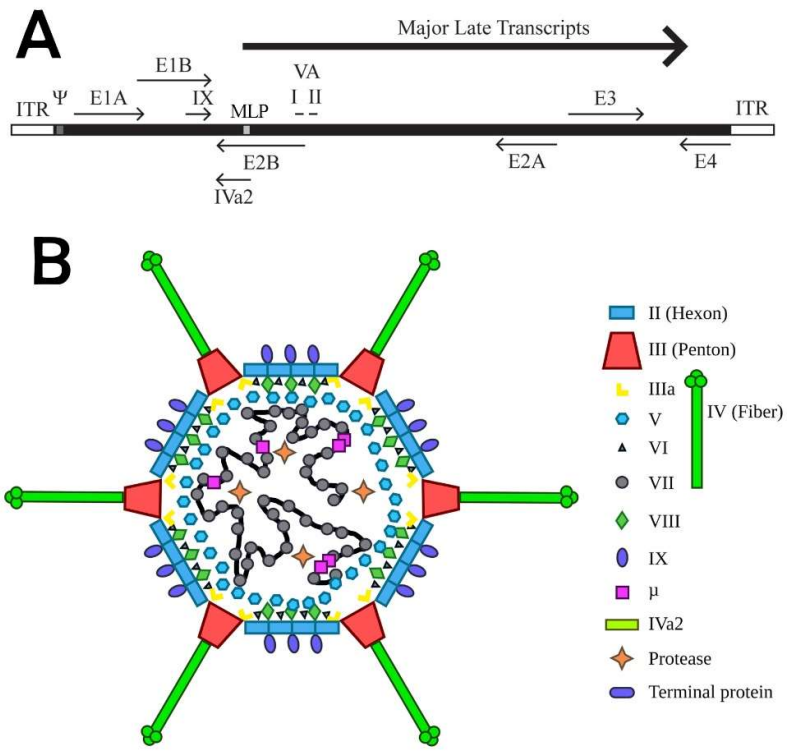


Figure 1. Schematic of the adenovirus genome and virion. **(A)** A simplified map of the AdV5 genome showing the early genes (E1–E4) and the region from which the major late transcript is produced (the extensive spliced L1–L5 transcripts produced from alternative splicing of the major late transcript are not shown). The relative position of pIX, VA RNA I and II and IVa2 are indicated. Also shown are the viral inverted terminal repeats (ITR) located at each end of the genome, the viral packaging element (Ψ) located adjacent to the left ITR, and the position of the major late promoter (MLP). Please note that these features are not drawn to scale. (B) Model of the AdV5 virion (Redesigned by Muhammad Nabeel from (Giberson *et al.,* 2012)).

1.1.3. AdV Disease

AdVs most often infect the upper or lower respiratory tracts, conjunctiva, or gastrointestinal (GI) tract. More than 80% of diagnosed AdV infections occur in children < 4 years old (due to lack of humoral immunity). Immunosuppressed persons are more susceptible. High baseline immunity against AdV confers substantial protection. Crowded settings (like military recruits), children or adults in closed settings might also get infected through AdV (Hawkinson *et al.,* 2013**).** Although there is no seasonal pattern to the development of disease, the most of epidemics occur in the winter or early spring. Illness can result from

outside sources (such pillows, lockers, or weapons), reactivation, or contact with infected individuals (inhaling aerosolized droplets, inoculating the conjunctiva, or spreading the illness orally through feces). Two to fourteen days is the time required for incubation. Most importantly, latent AdV can reactivate in patients with severe immunosuppression; it can remain for years in renal parenchyma, lymphoid tissue, and other organs. Asymptomatic AdV carriage can linger for several weeks or months. Epidemics can spread fast among small groups, such as those in hospitals (Lynch *et al.,* 2016). AdV causes at least 5 to 10% of pediatric and 1 to 7% of adult respiratory tract infections (Lee *et al.,* 2010). The incidence of positive AdV infections varies depending on the age category, with young children ≤ 4 years old accounting for the largest percentage of positive cases (57.2%), followed by adults 20–44 years old (12.5%) (Akello *et al.,* 2020). Adenovirus can be detected through direct or indirect immunofluorescence, conventional or shell vial cultures, PCR, or nasopharyngeal aspirates, swabs, washings, bronchoalveolar lavage, urine, stool, or blood (Lee *et al.,* 2010**).**

1.1.4. Treatment and prevention of AdV infections

No antiviral medication has received approval to treat AdV infection. There aren't enough prospective randomized controlled trials. Cidofovir and ganciclovir are two antiviral medications that have demonstrated *in vitro* efficacy against AdV. The antiviral medications ribavirin, foscarnet, acyclovir, penciclovir, and brivudin showed no anti-adenovirus activity, although ganciclovir showed some activity. Broad-spectrum action against DNA viruses, including adenovirus, is exhibited by intravenous nucleotide analogue DNA polymerase inhibitor cidofovir (Naesens *et al.,* 2005).

AdV infections can be prevented by vaccination. Unfortunetely, there is no vaccine available for civilian use. Vaccines have been used in US military. By 1999, the oral vaccinations against AdV types 4 and 7, that were created for the US military in 1971, had run out. The same live nonattenuated vaccination formulation for AdV4 and AdV7 was successfully reintroduced for military use in the United States in October 2011, following a fresh set of clinical studies and production by a different manufacturer. (Kuschner *et al.,* 2013)**.**

1.1.5. Cationic Antimicrobial Peptides

The antimicrobial peptides (AMPs) are observed in almost all organisms. The first AMP was discovered by Alexander Flaming which was a lysozyme found in vegetables and human tissues and secretions. AMPs are generated as abundant polyprotein precursors, and post-processing delivers active peptides. Almost all the AMPs hold antimicrobial activity, but only

a few of them demonstrate antiviral features. CHDP (Cationic Host Defense Peptides) or AMP plays a diverse role in innate immunity by responding to bacterial and viral pathogens. Therefore, understanding the antiviral and immunomodulatory activities of AMP can play a vital role in developing treatments against viral pathogens in humans.

There are two major families of AMP i.e. cathelicidins and defensins. However, more families exist in mammals, birds, fish, reptiles, and plants. Cathelicidins contain 12-88 amino acids in length and can be characterized by the presence of an N-terminal signal sequence that directs the synthesized protein toward the secretory pathway, a conserved cathelin-like domain that has homology with the porcine protease inhibitor, a variable C-terminal antimicrobial domain, which after the proteolytic cleavage converts into a mature functional peptide. These peptides are produced and stored in macrophages and neutrophils (Gordon YJ, 2005**).** Defensins are often expressed in neutrophils and epithelial cells, and they feature a framework of six disulfide-linked cysteines with a distinctive β-sheet-rich folds.

Human defensins are of two types i.e. α-defensins and β-defensins. Both differ in the length of peptide segments between the six cysteines and the pairing of the cysteines that are connected by disulfide bonds. α-defensins are further classified into six subtypes i.e. HNP1-4, HD 5-6, while the β-defensins are believed to have 32 subtypes (T, 2003).

1.1.6. Effect of AMPs on AdV

There is more information in literature about the effect of α-defensins on AdV (compared to studies of β-defensins and AdVs). The mechanism of cell entry is better defined for the human AdV-C serotypes in cultured epithelial cells. It is initiated by a high-affinity contact between one or more cell surface receptors and the distal knob of the fiber. The viral particle is swallowed by clathrin-mediated endocytosis following the binding to integrin coreceptors and high-affinity attachment receptors (such as CAR and CD46). The viral machinery proteins become active upon the release from the endosomes, and help the virus to enter into the host nucleus (Arnberg N. 2009). In order to stop infection, α-defensin binding to the capsid of serotypes like AdV5 stabilizes the virus capsid and stops the virus from un-coating during cell entry. Studies have shown that the viral capsid, rather than a cellular component, is the important target for neutralization of infection; sensitivity to α-defensin-mediated neutralization is also serotype-dependent. It is also reported that defensins neutralize AdV infection by blocking uncoating and release of the endosomalytic protein VI. HD5 interacts with the exposed surfaces of the three major capsid proteins: hexon, penton base, and fiber of

the AdVs (Smith *et al.,* 2010). There is another evidence that defensins hamper the release of adenovirus particles from endocytic vesicles, resulting in virion accumulation in early endosomes and lysosomes. (Smith, J. G., & Nemerow, G. R. 2008**).** HNP1-4 are expressed mostly by neutrophils, although they are also present in or expressed by natural killer cells, monocyte/macrophages, B cells, some T cells, and immature dendritic cells (DCs)3. On the other hand, Paneth cells, salivary glands, and the mucosa of the genitalia all constitutively express and secrete human α-defensins 5 and 6 (HD5 and HD6) as a pro-peptide (Ouellette A. J. 2006)**.**

2. Aims of the study

The purpose of this study is to investigate the efficacy of AMPs against human AdV5. The studied recombinant proteins included a carrier protein, named HELP, and HELP carrier with attached various AMPs. HIn had indolicidin sequence attached to HELP. H1mod1R and H2mod1R had one or two copies respectively of peptide, which sequence was based on human β-defensin-1.

The aims of the thesis were:

1. To determine whether the studied proteins are cytotoxic to the HEK293T/17 cells.
2. To determine whether the studied proteins have any anti-adenoviral activity.

3. Experimental part

3.1. Materials and Methods

3.1.1. Cell line and virus

The human embryonic kidney cell line HEK 293T/17 (ATCC CRL-11268) was used in this study. Cells were maintained in Dulbecco's modified eagle medium (DMEM; Corning) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, PAN Biotech).

AdV-Gluc virus strain was constructed in University of Tartu by A. Merits group. In human AdV5 genome the E1A/E1B region has been replaced with Gaussia luciferase marker gene between cytomegalovirus promoter and Simian virus 40 terminator.

3.1.2. Recombinant proteins

The studied recombinant proteins (H1mod1R, H2mod1R, HELP and HIn) were kindly provided by A. Bandiera (University of Trieste, Italy).

HELP is a human elastin like polypeptide, Elastin-like polypeptides are biotechnological protein and peptide carriers. This peptide has broad spectrum of applications. Indolicidin (In), a well-studied 13-residue cationic AMP, is often used as a model peptide because of its wide range of biological activity and effectiveness against a number of bacteria. Its fluorescence makes it easier to find because of its high tryptophan content. Figure 2 shows the schematic structure of the unique construct, named HIn,, where a short linker region divides the In domain from HELP. A glutamic acid residue was inserted immediately before the In sequence in order to establish a unique, specific proteolytic cleavage site that would release the AMP, as neither the In nor the HELP sequences contain this amino acid. However, depending on the requirements of the desired application, different specialized proteolytic sites, such as those for factor Xa and TEV protease, as well as linkers, spacers, and other functional sequences, may be introduced in place of amino acids for a given chemical cleavage (Colomina-Alfaro *et al*., 2024).

The structures of H1mod1R and H2mod1R resemble those of HIn. Instead of indolicidin, a 22 aa peptide, which sequence is based on human β-defensin 1, is coupled to the HELP carrier in one or two copies, respectively. The schemes of used recombinant proteins are depicted on figure 2.

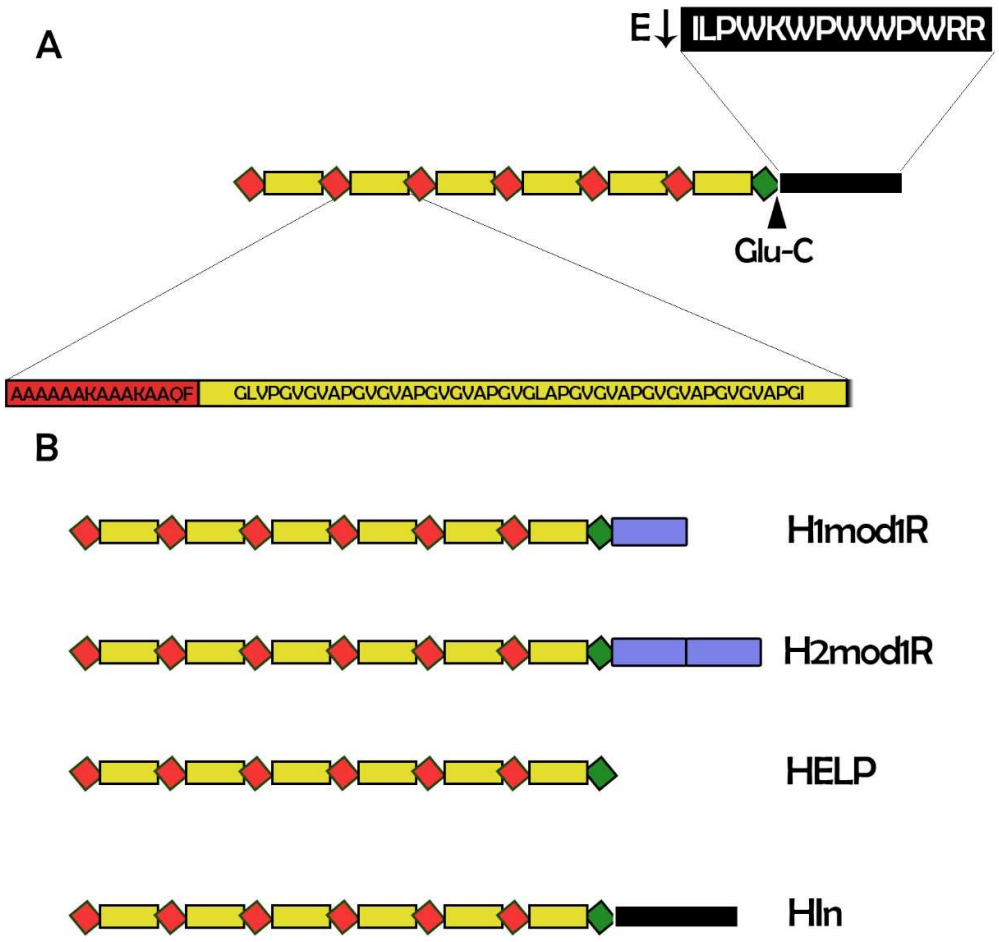


Figure 2. (**A)** Schematic representation of the HIn construct structure. The theoretical molecular mass is 49 kDa. Green, His-tag; hatched, linker; black, In domain; Red, cross-linking domains; yellow, elastin-like hexapeptidic repeats. The image is reproduced from **(**Colomina-Alfaro *et al*., 2024). **(B)** Schemes of used recombinant proteins (Designed by Muhammad Nabeel)

3.1.3. Cytotoxicity assay

I determined the cytotoxicity of all the used peptides (HELP, HIn, H1mod1R, H2mod1R) and $CuSO_4$ (positive control for antiviral effect in further studies) in HEK 293T/17 cells in all the concentrations that were going to be used in antiviral assays. Peptide stocks had a concentration 10 mg /ml. 157 mM $CuSO_4$ solution contains 10 mg Cu /ml. The stock solutions were diluted in 10x series in DMEM (without FBS) to obtain concentrations 1000,

100, 10, 1, 0.1, 0.01 and 0.001 µg/ml. HEK 293T/17 cells were seeded onto 96-well plates on the day before in volume to obtain 70-90 % confluent cells.

Sample dilutions were prepared on separate 96-well, each dilution in triplicate. After the preparation of the samples' dilutions, the old medium was removed from the cells and the samples were added to the wells in a way that each well received 100 µl. The cells were incubated with protein dilutions for 90 minutes. After the incubation, the protein dilutions were removed and DMEM containing 2 % FBS was added to the cells (100 µl/well). The cells were incubated for 48 hours before determining cell viability with cell proliferation reagent WST-1 (Roche). WST-1 was added to the cells (10 µl/well) and incubated for 3 h, then the optical density at 450 nm was measured using Synergy H1 spectrophotometer. Data analysis was done in MS Excel. Triplicate OD450 nm values were averaged, blank OD values (wells without cells, the background) were subtracted from average samples' OD values. The resultant values were compared to controls (which had H_2O added instead of proteins) and cell viability was expressed as percentage of control (viability 100%). If viability was <80%, the compound was considered to be cytotoxic.

3.1.4. Antiviral assay

Protein stocks (10 mg/ml) and 157 mM $CuSO_4$ stock (10 mg Cu/ml) were diluted in H_2O to obtain samples with concentrations 1000, 100, 10, 1 and 0.1 µg/ml after addition of virus. For $CuSO_4$, three additional dilutions were prepared to achieve concentrations 0.01, 0.001 and 0.0001 µg/ml. AdV-Gluc stock with TCID50/ml $5.33*10^8$ was diluted 10,66 times in H_2O to get $5*10^7$ infectious units (iu)/ml. Virus dilution in H_2O was added to protein dilutions in 1:1 ratio (100 µl protein dilution + 100 µl virus in H_2O). Virus was incubated with protein for 1h at room temperature. After incubation, for titration of virus, the samples were diluted 10x in serum free DMEM before infecting the cells.

AdV-Gluc has a linear relationship between virus amount (iu/ml) and Gluc signal measured with luminometer (relative luminescence units, RLU). Therefore, a RLU *versus* iu/ml standard curve can be used to titrate adenovirus. For standard curve, the original virus stock was diluted in DMEM to obtain 10x dilution series with concentrations from 10^7 to 1 iu/ml. The cells were infected with standard curve dilutions in triplicate.
For infection, the old cell culture medium was removed from 100 % confluent HEK293T/17 cells on 96-well plate. The infection mixtures were added to the cells (25 µl/well) in triplicate and incubated for 90 min at 37°C.

Gluc is secreted into cell culture medium and therefore cell culture medium can be used directly to measure Gluc luminescence. 1,5 µl coelenterazine native (TBD Biodiscovery) was added per 1 ml Assay buffer (50mM Tris, pH7.5, 25mM NaCl, 0.5mM EDTA, pH8.0) Cell culture medium (containing Gluc) was added in 1:1 ratio to coelenterazine/assay buffer mix (25 µl + 25 µl) and Promega Glomax 96 microplate luminometer was used to measure Gluc signal.

Data was analyzed using MS Excel. Triplicate RLU values were averaged. Standard curve was created using RLU data from wells with known AdV-Gluc amount. The equation of standard curve was used to calculate the viral titers of samples from RLU signals.

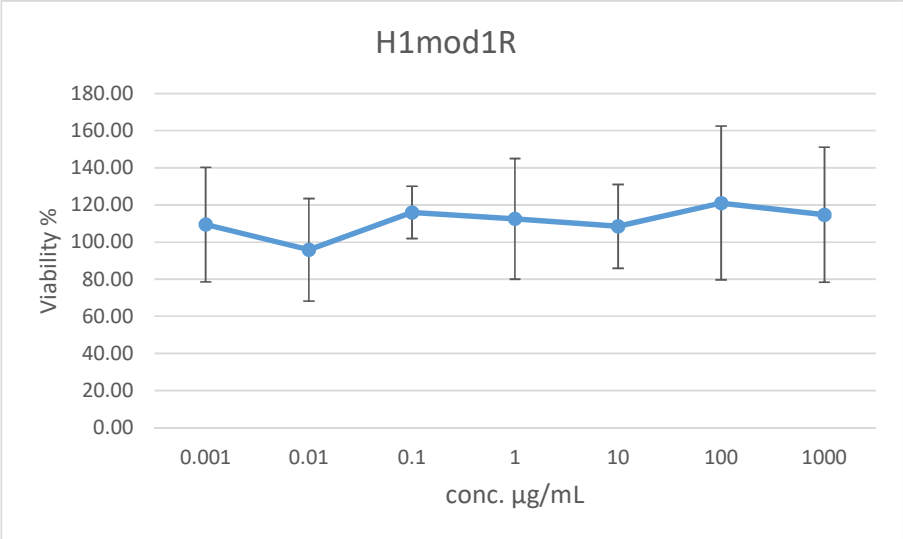
3.2 Results and discussion

3.2.1. Cytotoxicity determination of the studied proteins

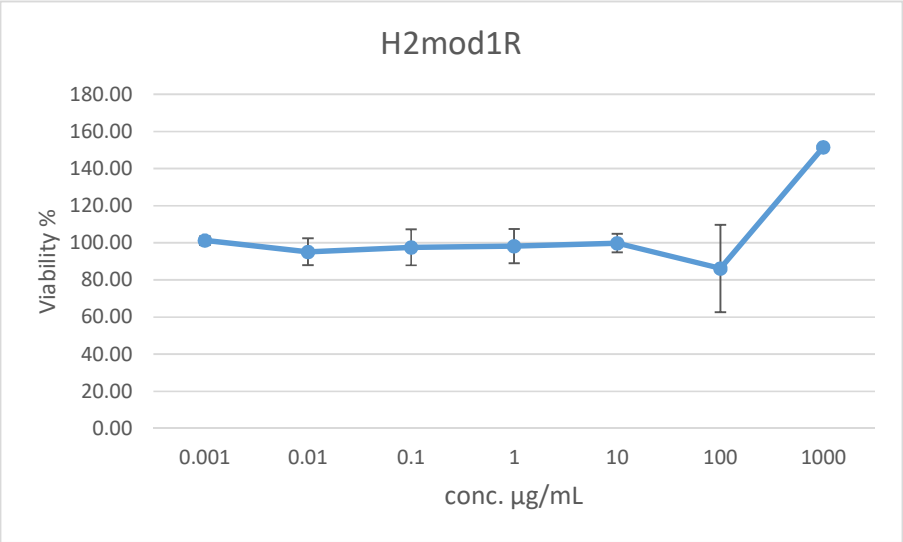
As the purpose of the study was to determine the antiviral activity of the studied recombinant proteins against the adenovirus, it was important to evaluate whether the proteins are toxic to the host cells at the concentrations that were going to be used in virus assay. WST-1 was used as a viability-determining reagent.

The data show that all the studied recombinant proteins H1mod1R, H2mod1R, HELP and HIn are nontoxic to the HEK293T/17 cells (Figure 3.A-D). It can be seen that for HELP, HIn and H1mod1R the cell viability is above 80% of control and almost the same at all the tested protein concentrations. H2mod1R was also not cytotoxic in general. The highest tested concentration (1000 µg/ml) of H2mod1R demonstrated variable cytotoxicity. Sometimes the cell viability dropped to ~50%, in other assays cell viability was ~100%. Altogether, the average viability was ~86%, which is above 80%, that was considered as limit for deciding whether a compound is cytotoxic or not. These results demonstrate, that the proteins would not be harmful to the cells during the virus assay. We have verified that cells would not be dying because the proteins are toxic, resulting in adenovirus not having any cells to infect.

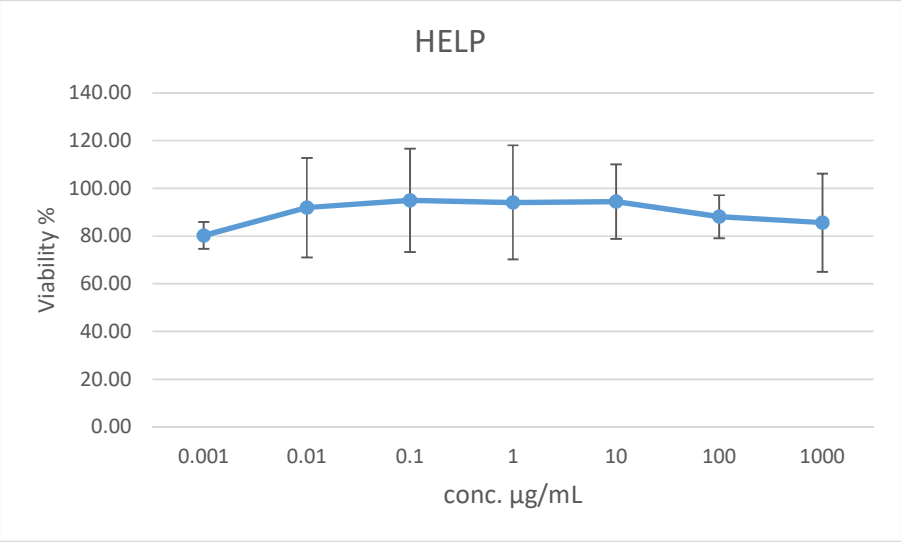
A



B


H2mod1R
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Viability %
0.001
0.01
0.1
1
10
100
1000
conc. µg/mL

C


HELP
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Viability %
0.001
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1
10
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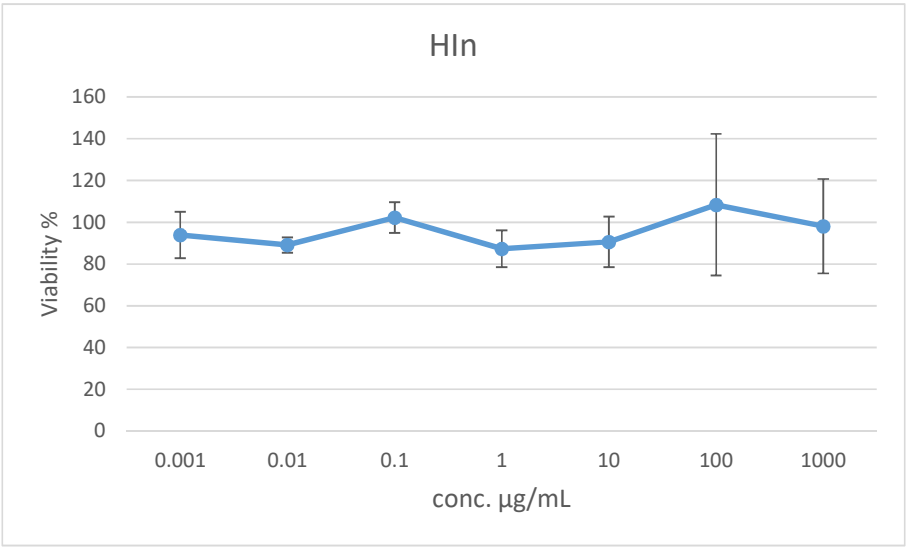
conc. µg/mL

D



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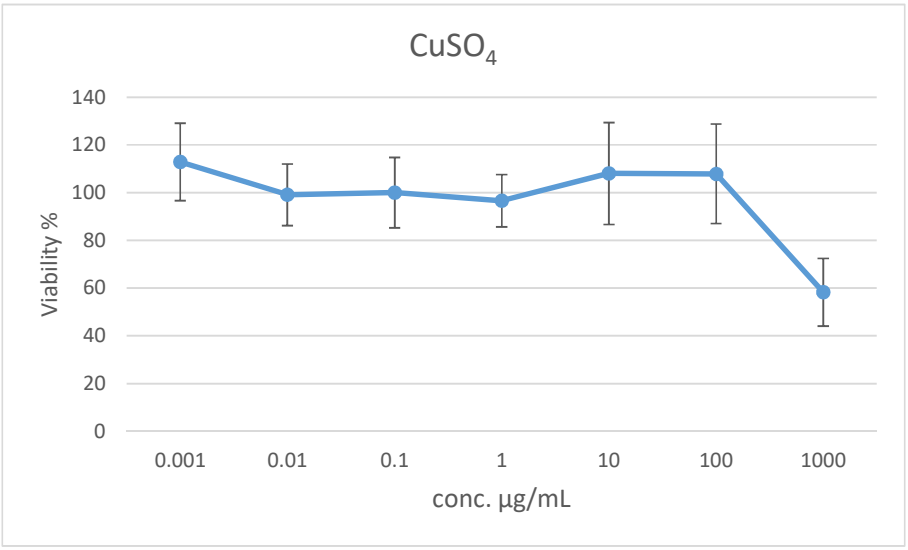


Fig 3. Cytotoxicity data for studied compounds. A) H1mod1R, B) H2mod1R, C) HELP, D) HIn, E) $CuSO_4$.

3.2.2. Anti-adenoviral efficacy of tested recombinant proteins

The data on Figure 4 illustrate the measurements of the amount of the virus in the assay. It represents how much virus has actually infected the cells during the assay. In control samples, incubated with H_2O, without recombinant proteins, the virus amount on average was $2.01*10^7$iu/ml. In the case of H1mod1R the virus amount fluctuates between $5.04*10^6$ and $7.36*10^6$ infectious units (iu)/ml (Fig.4.A) with no clear increasing or decreasing trend as the concentration increases. On the other hand, for H2mod1R the virus presence generally decreases from $6.33*10^6$ iu/ml at 0.1 concentration to $4.67*10^6$ iu/ml at 1µg/ml concentration, then increases steadily to $1.15*10^7$ at 1000µg/ml concentration (Fig 4.B).

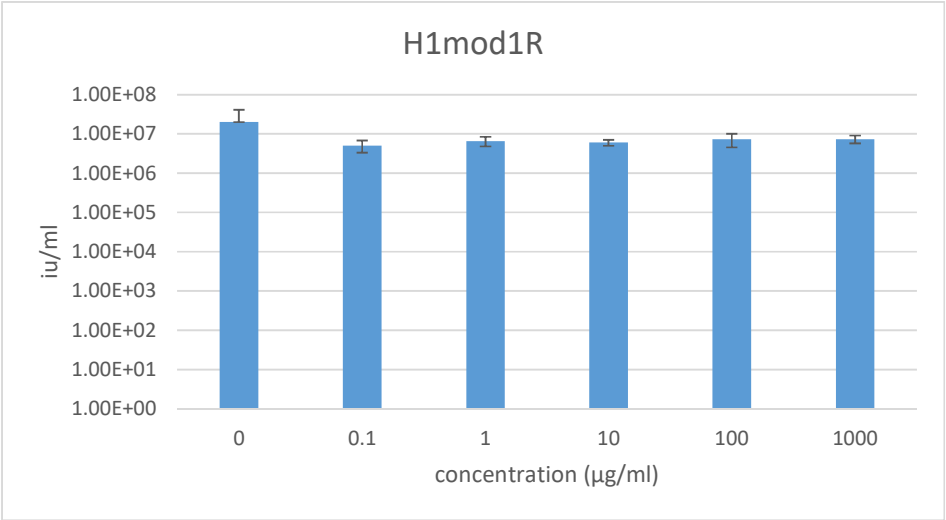
Similar to H1mod1R, for HELP the virus amount varies around a range $6.18*10^6$ to $1.00*10^7$ iu/ml with no clear trend across concentrations (Fig. 4.C). HIn, compared to all other recombinant proteins shows the most distinct trend. The virus presence starts high at $1.57*10^7$ iu/ml at 0.1µg/ml concentration and steadily decreases to $5.43*10^7$ iu/ml at 1000 µg/ml concentration (Fig.4.D).

CuSO4 was included into the assay as antiviral positive control, because $CuSO_4$ has demonstrated antiviral properties in earlier studies against coronaviruses (SARS-CoV-2, TGEV) and influenza A virus (Kubo *et al.,* 2022). As expected, $CuSO_4$ proved to be antiviral against adenovirus (Fig 4.E). Its antiviral effect is dose-dependent, the higher the dose, the higher the antiviral activity and *vice versa*. At the highest tested $CuSO_4$ concentration, 1000 µg/ml, the virus amount decreased to $2.27*10^6$ iu/ml, which is almost 1 log less than control ($2{,}01*10^7$ iu/ml). $CuSO_4$ still decreased the AdV amount at 0,1 µg/ml, which was the lowest concentration tested with the studied recombinant proteins. We wanted to see when the anti-adenoviral effect of CuSO4 disappears, therefore we tested three additional smaller $CuSO_4$ concentrations, 0,01, 0,001 and 0,0001 µg/ml. At the smallest concentration, 0,0001 µg/ml, $CuSO_4$ no longer decreased the amount of AdV.

All the tested recombinant proteins (H1mod1R, H2mod2R, HELP and HIn) had a modest anti-adenoviral activity. Somewhat surprisingly, also HELP demonstrated anti-adenoviral activity. HELP, which is the carrier protein that has no attached AMP sequence was expected to be the negative control without any antiviral activity. There is no earlier research done on the antiviral properties of the studied recombinant proteins, but the antibacterial efficacy of HELP and HIn has been previously studied and 20 µM HELP has been shown to be not harmful to different bacteria (Colomina-Alfaro *et al*., 2024).

In conclusion, H1mod1R and HIn are the least affective against the virus because of which virus presence is the highest against these peptides. H2mod1R is also showing the almost same affect but with the less infection. Only HELP showed inhibition against the virus because of which titer value is lowest as compared to the rest of the AMPs.

A



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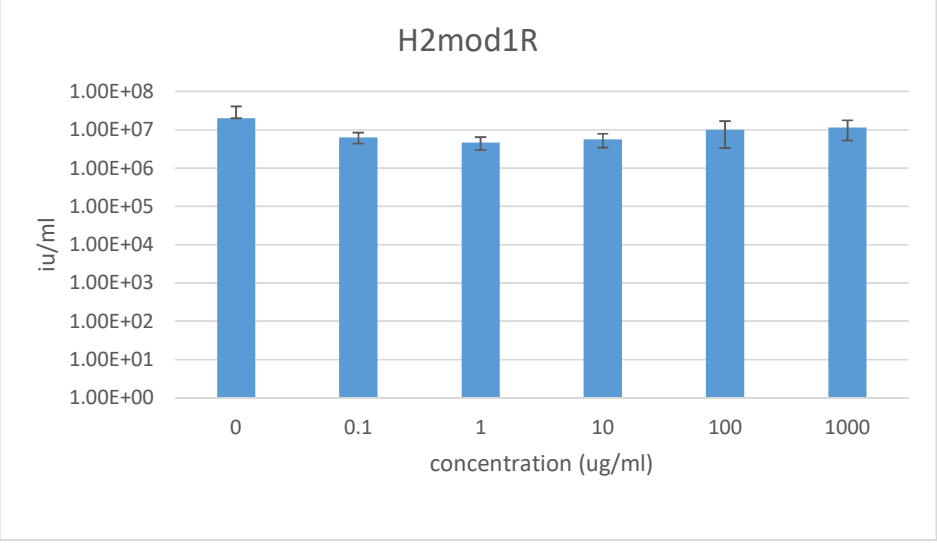

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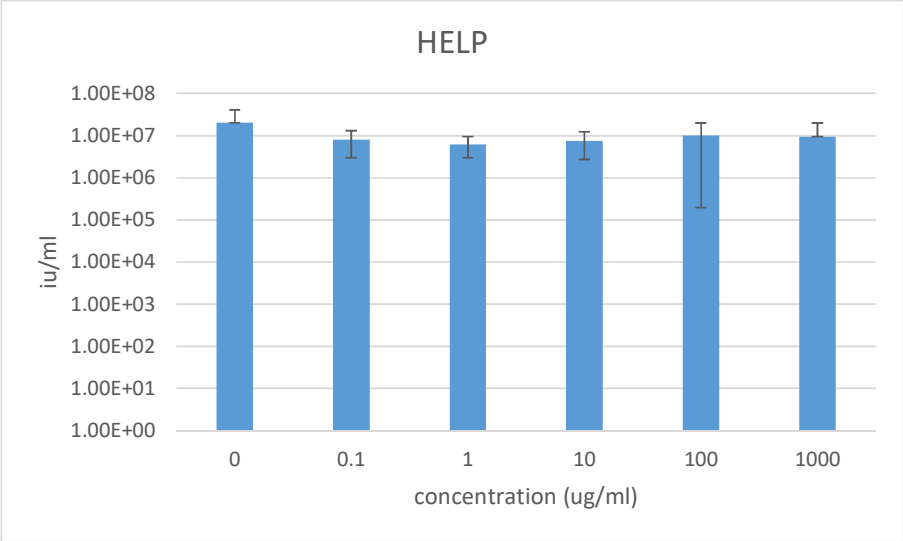
HELP
1.00E+08
1.00E+07
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1.00E+05
1.00E+04
1.00E+03
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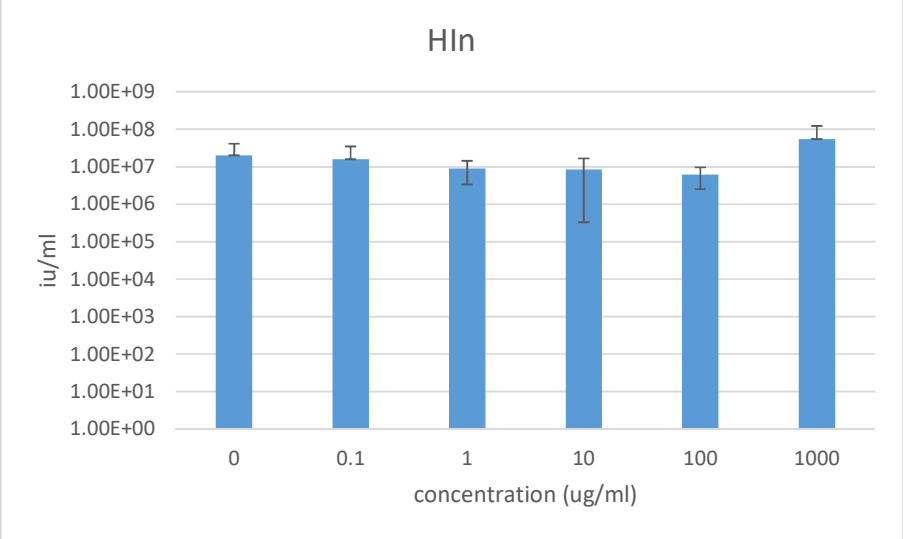
1.00E+00
iu/ml
0
0.1
1
10
100
1000
concentration (ug/ml)

D


HIn
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1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
iu/ml
0
0.1
1
10
100
1000
concentration (ug/ml)

E

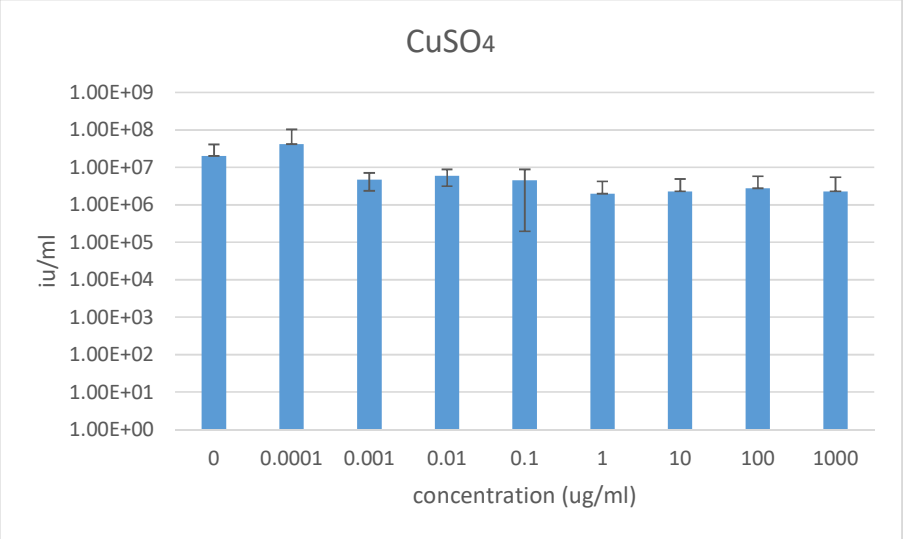


Figure 4. AdV-Gluc amount after treatment with studied compounds.

To better evaluate the antiviral efficacy, we calculated the AdV infectivity percentage of control for the studied compounds. Figure 5 shows the results of calculating the infectivity percentage of control of the studied proteins. Higher infectivity percentage means less inactivation of AdV and *vice versa*.

H1mod1R recombinant protein presents a moderate anti-adenoviral effect, with infectivity ranging from 51% to 64% at different protein concentrations (Fig. 5.A). There is no clear evidence of concentration dependence and increasing or decreasing trend.

H2mod1R protein shows the similar effect on AdV (Fig 5.B), with infectivity decreasing from 53% at 0.1 µg/ml concentration to 38% at 1 µg/ml concentration. From there, the average infectivity percentage gradually increases to 91% at at 1000 µg/ml concentration. However, the variability in data at higher H2mod1R concentrations (100 and 1000 µg/ml) increased, the infectivity percentage varied from 33 % to 179%. Therefore, it seems probable that the anti-adenoviral effect of H2mod1R does not actually disappear at 1000 µg/ml concentration.

HELP, similar to H1mod1R and H2mod1R, exhibited moderate decrease in infectivity, fluctuating between 44% and 65% across concentrations. However, it also showed no evident increasing or decreasing trend with concentration independence. The fact, that HELP decreased AdV-gluc infectivity, was unexpected. We did not anticipate, that the carrier

protein without attached AMP, would have antiviral properties. HELP's structure contains repeating cross-linking domains (Colomina-Alfaro *et al*., 2024). which could facilitate forming clumps of AdV virions and thus hinder the viral infection process.

Recombinant protein HIn had indolicidin amino acid sequence attached to HELP carrier protein. Indolicidin has been shown to have antibacterial (Vergis *et al.,* 2004) and herpes simplex virus (Ron-Doitch *et al*., 2016) Among the studied recombinant proteins, HIn showed the most consistent and antiviral effect up to concentration 100 µg/ml. AdV-Gluc infectivity steadily decreased from 67% at 0.1 µg/ml concentration to a minimum of 41% at 100 µg/ml concentration. However, AdV-Guc infectivity then increased dramatically to 214% at 1000 µg/ml concentration. In two of the three repeat experiments, at this HIn concentration, the infectivity percentage was >200% compared to control, indicating that at this concentration HIn might have, unexpectedly, antiviral properties.

H2mod1R and HIn show an interesting reversal in their inhibitory effect at the highest concentration (1000 µg/ml). H2mod1R appears to lose its inhibitory effect, although it may be due to experimental error, while HIn seems to increase AdV infectivity at high concentration. If the effect is real, then it is an interesing example of non-monotonic relationship between protein concentration and AdV infcetivity. These results also emphasize, that one always has to carry out careful and thorough studies of new potential antiviral compounds to ensure that no unexpected pro-viral properties exist. If a compound is antiviral only in a narrow concentration range and loses its efficacy or even becomes proviral at different concentrations, then it poses a challenge in practical applications. .

In general, all the studied recombinant proteins were quite similar in their anti-adenoviral efficacy. They reduced AdV-Gluc infectivity approximately 50 % across the tested concentration range. There was no clear concentration-dependence in the antiviral effect of studied proteins. The only exception was HIn, which reduced AdV infectivity at most tested concentrations, but unexpectedly seemed to increase infectivity at 1000 µg/ml.

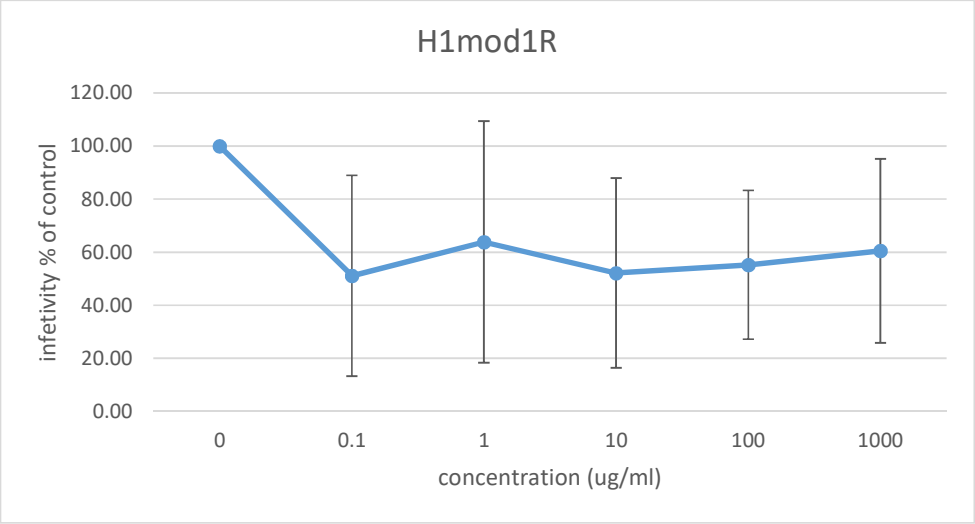
$CuSO_4$ was used as a positive control for antiviral activity in this study. It can be seen from the data that $CuSO_4$ showed an increasing trend of inhibition against the virus. $CuSO_4$ was only compound, that exhibited concentration-dependent inactivation of AdV. 0.0001 µg/ml was the smallest concentration tested and it had no antiviral effect on AdV (average infectivity 129%). As the $CuSO_4$ concentration increased, then AdV infectivity decreased,

from 42% at 0.001 µg/ml to 8.4 % of control while at the highest tested concentration, 1000 µg/ml. $CuSO_4$ was more efficient against AdV-Gluc than any of the tested recombinant proteins. Therefore, it is clear that the design of the studied proteins could be improved to increase their anti-adenoviral efficacy.

As it is clear from the data the studied recombinant proteins proved to be modestly antiviral against the AdV-5 (AdV-Gluc), but the mechanism of action is still not clear. However, previous works shows that defensins binding to the capsid of serotypes such as AdV-5 stabilizes the virus capsid and prevents the virus from uncoating during cell entrance, hence stopping infection. Research has indicated that the viral capsid, as opposed to a cellular component, is the primary target for neutralizing infection (Smith *et al.,* 2010). Additionally, the susceptibility to defensin-mediated neutralization varies depending on the serotype. Defensins are also said to prevent the uncoating and release of endosomalytic protein VI, which neutralizes AdV infection. Hexon, penton base, and fiber are the three main capsid proteins with which the defensins interact on their exposed surfaces (Smith *et al.,* 2008). Some other researches, consistent with the antiviral effect of the defensins, suggest that HNP1 disrupts HIV-1 entry by inhibiting fusion and downregulating CD4 (a critical component of T-cells) receptors. HNP1 can also interfere with PKC (Protein Kinase C) signaling, a pathway crucial for HIV-1 and influenza virus replication (Xu, D., & Lu, W. 2020). Unfortunately, the antiviral assay results were quite variable, which is reflected in large error bars on the graphs. This makes drawing conclusions more complicated. We cannot see any concentration dependent anti-adenoviral activity for studied recombinant proteins. All the differences in infectivity percentages at different concentrations are within the standard deviation range. Therefore, it would be good to repeat the assay in hope to reduce the variability. To test the full potential of the studied proteins repeating experiment with other viruses will help to get a clearer understanding of the anti-viral activity. In bacteria, disruption of the membrane is the main mechanism of action of AMPs, but AdV is a non-enveloped virus, it does not have a membrane. This might be one reason why the studied recombinant proteins demonstrated only modest activity against AdV. These proteins might be more efficient against enveloped viruses. Secondly, different research techniques can be used to investigate the specific mechanism of action for each peptide. In case of AdV, the possible mechanism of action could be bridging the virions. The HELP part of the

proteins contains cross-linking domains (Colomina-Alfaro *et al.,* 2024) which could bind AdV virions into clumps and thus interfere with virus attachment to the cell membrane during infection. This mechanism would also explain, why HELP carrier protein exhibited anti-adenoviral activity without attached AMP sequence (Fig. 3.C and 4.C). The recombinant proteins could also interact with viral capsid proteins or cellular receptors for viral attachment, again thus disrupting the infection process.

A



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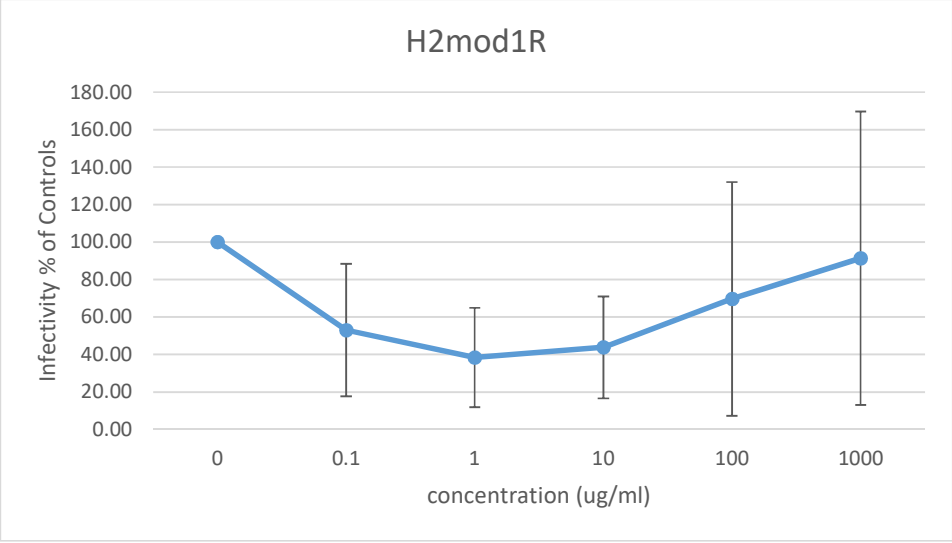

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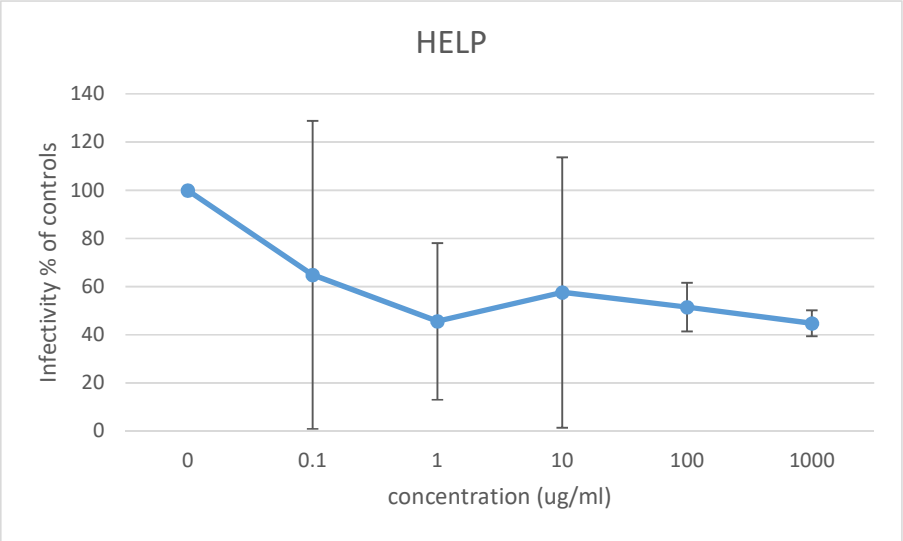
HELP
Infectivity % of controls
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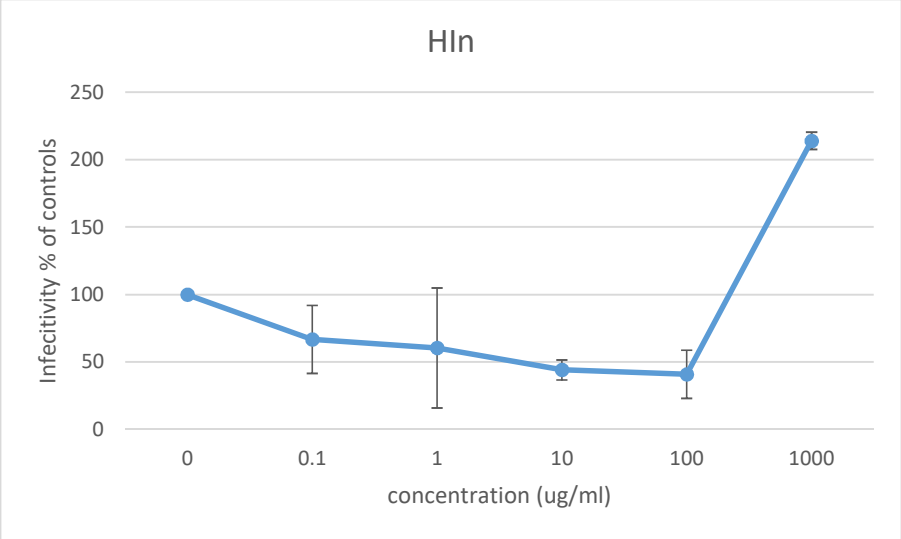
100
80
60
40
20
0
0
0.1
1
10
100
1000
concentration (ug/ml)

D


HIn
Infecitivity % of controls
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200
150
100
50
0
0
0.1
1
10
100
1000
concentration (ug/ml)

E

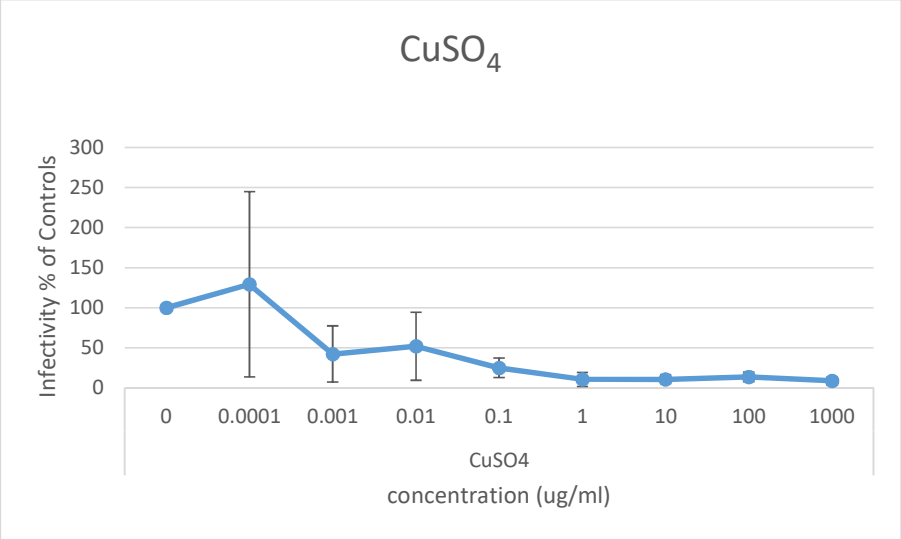

Figure 4. Infectivity percentage of control. A) H1mod1R, B) H2mod1R, C) HELP, D) HIn, E) $CuSO_4$.

Summary

This study suggests that the recombinant proteins H1mod1R, H2mod1R, HELP, and Hin have the potential to inhibit adenovirus infection. All the studied proteins showed a moderate anti-adenoviral activity. The antiviral activity was independent of the protein concentration against Human Adenovirus-5 (AdV-Gluc) *in vitro*. This finding again does align with the previous research based on cationic AMPs against other enveloped and non-enveloped viruses. CuSO4 acted as a positive control and showed antiviral activity with the concentration dependency.

The cytotoxicity data showed that all the studied recombinant proteins H1mod1R, H2mod1R, HELP and HIn are nontoxic to the HEK293T/17 cells. Overall the cell viability was more than the 80% which proved the nontoxic nature of the studied proteins.

The findings offer promising initial evidence to support the use of these recombinant proteins as preventative measures against infections caused by adenovirus-5. To ascertain their effectiveness in vitro and clarify the mechanisms by which they prevent viral infection, more investigation is needed.

Overall, this study emphasizes on the fact that further research with other enveloped and non-enveloped viruses is necessary to test the full potential of cationic AMPs as antiviral agents. Further research might help to develop novel strategies to deal with adenoviral infection and potentially other viruses.

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