Antiviral effect of natural compounds on Human alphaherpesvirus 1

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DIPLOMA THESIS

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TABLE OF CONTENTS

1. INTRODUCTION

1.1 Herpesviruses

Herpesviruses are widely spread in nature, most of all animal species are hosts to at least one herpesvirus, moreover some herpesviruses infect more than one species. Until today around 200 herpesvirus species have been identified but the number is probably much greater (Liu, 2014). The similarity of herpesviruses and DNA bacteriophages has been noticed, and it can be partially explained by the convergent evolution but not completely. In combination with many genetic elements belonging to the oldest branches of evolutionary trees this supports the theory that ancestry of herpesviruses could be placed at the early stages of evolution of parasitism. Based on everything discovered it is assumed that herpes viruses and tailed bacteriophages descend from a common ancestor despite differences in their morphology and hosts (Baker et al., 2005).

1.1.1. Herpesviridae family

Morphologically, herpesviruses are highly divergent with a range of hosts from bivalves to humans. They have originally been classified in a single family, but the extensive sequence of DNA led to a new taxonomic order – *Herpesvirales*. This order is further divided into three virus families: *Herpesviridae* – the herpesviruses of mammals, birds and reptiles, *Alloherpesviridae* – the herpesviruses of fish and amphibians and *Malacoherpesviridae* – the herpesvirus of bivalves.

In human population nine herpesviruses have been identified: *Human alphaherpesvirus 1* (HHV-1) also known as *Herpes simplex virus 1* (HSV-1), *Human alphaherpesvirus 2* (HHV-2) also known as *Herpes simplex virus 2* (HSV-2), *Human alphaherpesvirus 3* (HHV-3) also known as Varicella zooster virus (VZV), *Human gammaherpesvirus 4 (HHV-4)* also known as *Epstein-Barr virus* (EBV), *Human betaherpesvirus 5* (HHV-5) also known as *Human cytomegalovirus* (HCMV), *Human herpesviruses 6A, 6B,* and *7* (HHV-6A, HHV-6B, HHV-7), and Kaposi's sarcoma–associated herpesvirus (also known as HHV-8) (Davison, 2010; Raab-Traub, 2012). At the end of the 1970s, *Herpesviridae* was subdivided into three subfamilies (Montague and Hutchison, 2000). The *Alphaherpesvirinae* subfamily includes lytic viruses with relatively short life cycle that infect different cell types, replicate rapidly, and establish

latency mainly in sensory ganglia. In this subfamily there are HHV-1 and HHV2 (from genus *Simplexvirus*) and HHV-3 (from genus *Varicellovirus*). HHV-3 provokes chickenpox in children and herpes zoster in adults. Second subfamily is *Betaherpesvirinae* and it includes viruses with long life cycle and slow progression of infection of a limited number of hosts. Infected cells thrive and increase in size (cytomegaly). Latent infection localizes in secretory glands, lymphoreticular cells, kidneys, and other tissues. Herpesviruses from this subfamily that have human hosts are HCMV and HHV-6. The third subfamily, *Gammaherpesvirinae,* includes HHV-8 and EBV (Kukhanova et al., 2014).

1.1.2. Structure of herpesviruses

Herpesviruses have been defined based on structure of virion. A typical herpesvirion has complex and characteristic structures consisting of both symmetrical and non-symmetrical components. The spherical virion is comprised of the core, capsid, tegument and an envelope. Electron-dense core which consists of a large linear double stranded DNA (dsDNA) ranging from 125 to 295 kbp in size and from 32 to 75% in G+C content (Furlong et al., 1972). The genome is wrapped as a toroid or spool in liquid crystalline state. A small fraction of DNA may be circular (Whitley et al., 1998).

Early studies on purified HHV-1 virions suggested that they contain more than 30 distinct proteins that were designated as virion polypeptides (VP) and given serial numbers (Davison et al., 2009). HHV proteins have also been named based on serial numbering of the virion proteins on a gel (e.g., VP1/2), on the open reading frame (ORF) encoding them (e.g., UL8), or as infected cell proteins (ICPs; e.g., ICP5. Of the approximately 30 known and 10 suspected virion proteins, at least 11 are on the surface of the virion (accessible to antibody) and at least 10 are glycosylated (Liu, 2014).

Icosahedral capsid is 125nm in diameter and is built from 161 capsomers, with a whole running down their long axis and one capsomeric structure which has a role of packaging and releasing of the viral genome. Complex of core and capsid forms the nucleocapsid (Grünewald et al., 2003).

Tegument is an amorphous layer of proteins that surround the capsid and an envelope containing viral glycoprotein spikes on its surface (Whitley et al., 1998). It is largely unstructured, except for the fibrils and some apparent icosahedral structure around the pentons

and it is comprised of at least 18 viral proteins, some of which are involved in the transport of the capsid to the cell nucleus and other organelles (UL36, UL37, ICP0) (Radtke et al., 2010), penetration of viral DNA into the nucleus (VP1-2, UL36) (Jovasevic et al., 2008), activation transcription of early viral genes (VP16, encoded by the UL48 gene) (Ace et al., 1989), suppression of cellular protein synthesis and mRNA degradation (VHS, UL41) (Barzilai et al., 2006).

The envelope consists of a lipid bilayer with as many as 13 distinct viral glycoproteins embedded in it. The virion envelope glycoproteins are gB (VP7 and VP8.5, encoded by the UL27 gene), gC (VP8, UL44), gD (VP17 and VP18, US6), gE (VP12.3 and VP12.6, US8), gG (US4), gH (UL22), gI (US7), gK (UL53), gL (UL1), and gM (UL11). The presence of gJ (US5) and gN (UL49.5) in virions has not been demonstrated. Virion envelopes also contain at least two (UL20 and US9) and possibly more (UL24, UL43, and UL34) nonglycosylated intrinsic membrane proteins (Chowdhury et al., 2013).

The DNA of HHV-1 and HHV-2 consists of two covalently bounded components, L (long) and S (short), with unique sequences - UL (unique long) or US (unique short) - flanked by large, inverted repeats. The two components can invert relative to one another, creating four different types of DNA molecule that differ only in their orientation of DNA sequences.2 With understanding of its' structure, the virus was genetically engineered and used in vaccine development and gene therapy (Boehmer and Nimonkar, 2003).

1.1.3. Life cycle of herpesviruses

Biological cycle of herpesviruses can be divided into three major stages which are initiation of infection, lytic replication and latency. These stages are related to the biological resolution in the infected cell to follow either lithic or latent pathway, as well as by the ability of reactivation of latently infected cells into the lytic state. Lytic replication can then be divided into the following major stages: entry into the host cell, expression of viral genes, replication, virion assembly, and egress of the new generation of viral particles. In permissive cell lines, this cycle takes about 18-20 h (Kukhanova et al., 2014). To replicate, enveloped viruses must be able to fuse with the membrane of a living cell and deliver their genetic material into its cytoplasm. The process of membrane fusion is initiated by binding of a virus to an appropriate receptor on the cell surface and is mediated by a virus-encoded membrane fusion protein (fusogen).

Herpesviruses are viruses that establish lifelong latent infections in their natural hosts (Davison et al., 2009).To date, two HHV-1 entry pathways have been proposed. The main mechanism assumes the fusion of the viral envelope with the plasma membrane and further transport of the viral capsid to the nucleus. The essential stage of this process is interaction of surface glycoproteins of the virus with specific cell surface receptors. The additional pathway by which the virus enters the cell is endocytosis of the enveloped virion followed by fusion of the envelope with intracellular vesicles (Arii et al., 2009). Attachment of the virion to the cell surface is mediated by viral glycoproteins C (gC) and B (gB), which interact with cell surface glycosaminoglycans, in particular heparan sulfate (Peteranderl et al., 2016). The interaction between four glycoproteins; gD, gB, and the heterodimer gH/gL, is required for viral entry into the host cell by fusion of the viral outer envelope with the plasma membrane (Avitabile et al., 2009). Glycoprotein gD can bind to the receptors of three types: nectin-1 and nectin- 2, herpes virus entry mediator (HVEM), and 3-O-sulfated heparan sulfate (3-O-S-HS). The last is produced by 3-O-sulfotransferases 2-7 (3-OST) making them attractive therapeutic targets for the development of antiherpetic drugs (Arii et al., 2009). In addition to binding of gD to cellular receptors, it triggers membrane fusion by interaction with the gB and gH/gL complex. When gD is not bound to the ligand, the C-terminal domain is blocked (Gianni et al., 2009). An interesting additional function of gD is suppression of apoptosis in the HHV-infected cells (Zhou et al., 2000). Interaction between gB and paired immunoglobulin-like type 2 receptor α (PILRα) is necessary for viral entry into the cell (Satoh et al., 2008). To initiate infection, HHV attaches to at least three different classes of cell-surface receptor and fuses its envelope with the plasma membrane. The capsid, without its envelope, is transported to the nuclear pore, through which it releases viral DNA into the nucleus.

The synthesis of viral mRNA is carried out by RNA polymerase II of the host cell with viral factors at all stages of infection. Viral proteins regulate sequential transcriptional cascades (α, β, and γ genes) and several post-translational modifications (Fig.1.). Transcription of very early α genes requires the VP16 tegument protein (Mackem and Roizman, 1982). The main function of proteins encoded by α genes is the activation of β transcript expression. Proteins and enzymes encoded by β genes are involved in viral genome replication (e.g., HHV DNA polymerase, UL30), regulating nucleotide metabolism (e.g., thymidine kinase UL23), suppression of expression of early α genes, and activation of late γ genes. The regulation of the expression of β genes and γ genes is more diverse; therefore, the onset time, duration, and expression levels of these genes, unlike α, do not coincide. DNA polymerase is the main protein

of HHV-1 replication; its level reaches its maximum only four hours after infection (Yager et al., 1990). The HHV-1 DNA polymerase belongs to the B-family of polymerases, which also includes human DNA polymerases α, δ, and it has been used as a target for antivirals. HHV-1 genes are transcribed by cellular RNA polymerase II (RNA Pol II). While four viral immediate early proteins (ICP4, ICP0, ICP27, and ICP22) function in some capacity in viral transcription, the mechanism by which ICP22 functions remains unclear.

After primary infection, the virus either implements a lytic infection in epithelial cells or enters the nerve cell's axon and, using retrograde transport, enters the neuron nucleus. There, the viral DNA is retained in a circular form without significant expression of lytic genes. Still, the latency-associated LAT transcript is expressed and is spliced to form several RNA products. It was shown that both transcriptionally active and silent regions of viral DNA in a latent state are associated with nucleosome-like structures similar to the chromatin of eukaryotic cells (Deshmane and Fraser, 1989).

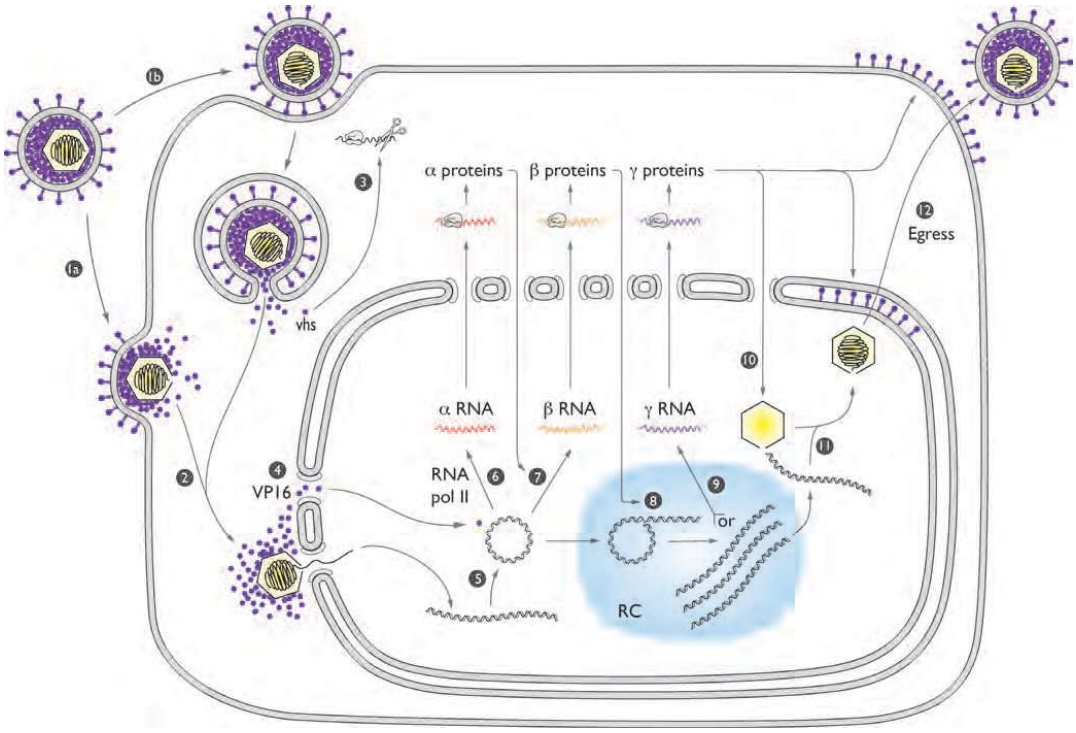


Figure.1: The virus binds to the cell plasma membrane and the virion envelope fuses with the plasma membrane 1a) or the virus enters by endocytosis (1b), releasing the capsid and tegument proteins into the cytoplasm. 2: The capsid is transported to the nuclear pore, where the viral DNA is released into the nucleus. 3: The *vhs* protein causes degradation of host messenger RNAs (mRNAs). 4: VP16 localizes into the nucleus. 5: The viral DNA circularizes. 6: It is then transcribed by host RNA polymerase II to give first the a mRNAs. a gene transcription is stimulated by the VP16 tegument protein. Five of the six immediate-early proteins act to regulate viral gene expression in the nucleus. 7: a proteins transactivate b gene transcription. 8: The b proteins are involved in replicating the viral DNA molecule. 9: Viral DNA synthesis stimulates g gene expression. 10: The g proteins are involved in assembling the capsid in the nucleus and modifying the membranes for virion formation. 11: DNA is encapsidated in the capsid. 12: The filled capsid buds through the inner membrane to form an enveloped virion, and the virion exits from the cell by mechanisms described in the text. (Liu, 2014)

The latency of HHV has been recognized biologically since the beginning of the century. Following entry and infection of nerve endings, both HHV-1 and HHV-2 are transported by retrograde movement to the nuclei of sensory ganglia (Stevens and Cook, 1971). The available evidence indicates that the virus multiplies in a small number of sensory neurons. In the majority of the infected neurons, the viral genome remains in an episomal state for the entire life of the individual. Reactivations occur following a variety of local or systemic stimuli such as physical or emotional stress, fever, exposure to ultraviolet light, tissue damage and immunosuppression (Whitley et al., 1998). The data describing LAT functions are rather contradictory; however, presumably, the main one is the formation of miRNA and siRNA, which suppress the expression of ICP0 and other lytic genes. Latency, reactivation, and recurrent herpes infection have been studied in animal models of a rabbit and a mouse (Digard et al., 1993).

1.1.4. Pathogenesis of HHV-1

The transmission of *Human alphaherpesvirus 1* (HHV-1) infection is dependent upon intimate, personal contact of a susceptible seronegative individual with someone excreting HHV-1. Virus must come in contact with mucosal surfaces or abraded skin for infection to be initiated. With viral replication at the site of primary infection, either an intact virion or, more simply, the capsid is transported retrograde by neurons to the dorsal root ganglia where, after another round of viral replication, latency is established. The more severe the primary infection, as reflected by the size, number, and extent of lesions, the more likely it is that recurrences will ensue. Although replication sometimes leads to disease and, infrequently, results in life-threatening infection (e.g., encephalitis), the host-virus interaction leading to latency predominates. After latency is established, a proper stimulus causes reactivation; virus becomes evident at mucocutaneous sites, appearing as skin vesicles or mucosal ulcers. Mucocutaneous manifestations of HHV-1 infection include gingivostomatitis, herpes genitalis, herpetic keratitis, and dermal whitlows. Neonatal HHV-1 infection and HHV-1 encephalitis also occur.

1.1.5. Therapy for HHV-1 infection

The first effective application of antiviral therapy for a herpesvirus infection was the local application of the nucleoside analog, 5-iodo-2-deoxyuridine, for the topical treatment of HHV keratitis (Kaufman et al., 1962). Topical treatment permits high local concentrations of antiviral at the site of infection without the risk of systemic toxicity. Other anti-HHV drugs are now used for this application. The first effective antiviral for systemic use against HHV was vidarabine, a nucleoside analog converted by cellular enzymes to an inhibitor of HHV DNA polymerase. It was subsequently shown that this intravenously administered drug was effective against neonatal herpes and VZV infection in immunocompromised patients (Whitley et al., 1980).

Acyclovir (ACV) carried the beginning of the second generation of antivirals for herpesviruses and set the standard for the development of antiviral drugs. It became the standard treatment for serious systemic infections such as herpes encephalitis, neonatal herpes, and varicella zoster in immunocompromised hosts (Balfour et al., 1983). In addition, it was shown to be effective for the prophylaxis of HHV and VZV in transplant patients (Saral et al., 1981). As an orally available agent with negligible toxicity, it has become widely used for the treatment of primary and recurrent HHV-1 and HHV-2, the prophylaxis of recurrent HHV, and the treatment of uncomplicated VZV (Douglas et al., 1984; Dunkle et al., 1991; Huang et al., 1999). The earlier in the presentation of symptoms, the more effective the treatment, and prophylaxis is more dependable than treatment. The appearance of resistant isolates has been remarkably rare in immunocompetent patients and occurs in a minority of immunocompromised patients. ACV-resistant HHV and VZV did become a significant challenge in patients with AIDS preceding the availability of combination antiretroviral therapy (Liu, 2014). The expanded number of resistant infections reflected the number of patients with AIDS, the prevalence of recurrent HHV and VZV infection in these patients, and the duration and magnitude of these opportunistic infections, because of the severely reduced antiviral immunity in these hosts. In many, but not all of these patients, cidofovir or foscarnet showed activity against resistant infections, but resistance to these DNA polymerase inhibitors has developed as well. Two limitations of oral ACV have been its limited oral bioavailability (15%) and short half-life (Safrin et al., 1991). These limitations require administration of large pills as often as every 4 hours. Valacyclovir and famciclovir overcome these limitations, and would have completely displaced the use of ACV except for the expiration of the ACV patent resulting in the

availability of low-cost generic drug. Both valacyclovir and famciclovir can be used for applications of oral ACV. In addition, because of the more prolonged maintenance of higher levels of antiviral activity, they are also used for the treatment of serious but not immediately life-threatening infections previously treated with intravenous ACV, for example, herpes zoster in the immunocompromised host (Gershon et al., 2015).

1.2. Influenza viruses

Influenza viruses were probably responsible for the disease described by Hippocrates in 412 bc, and thus they have been with us for a long, long time. Influenza remains a major cause of morbidity and mortality worldwide, and large segments of the human population are affected every year. In addition, many animal species can be infected by influenza viruses, and some of these viruses may give rise to pandemic strains in humans, as in the case of the 2009 H1N1 pandemic. Most threatening is the possibility of another pandemic similar to that experienced in 1918, which is estimated to have caused on the order of 50 million deaths worldwide (Liu, 2014). In the family of *Orthomyxoviridae* there are seven genera: *Alphainfluenzavirus* which contains Influenza A virus, *Betainfluenzavirus* in which is Influenza B virus, *Deltainfluenzavirus, Gammainfluenzavirus, Isavirus, Quaranjavirus,* and *Thogotovirus.* (www.talk.ictvonline.org)

Influenza viruses that infect human population can be classified into 3 types: influenza type A, B and C. The difference between them is mainly in their NP and M proteins. In 2011, novel influenza type D has been identified which primarily infects animals such as cattle and pigs, although specific antibodies have been found also in human serum samples from cattle-exposed workers (Ducatez et al., 2015; Trombetta et al., 2020).

The most common causative agents of seasonal flu epidemics in humans are Influenza A and B. Influenza B virus (IBV), which generally circulates later in the season, is responsible for 15–30% of total influenza infections but it doesn't cause pandemics. In contrast, strains of influenza A virus (IAV) are often responsible for seasonal influenza epidemics and pandemic outbreaks due to frequent genetic mutations and inter-subtype reassortment (Mosnier et al., 2015). The IAV virion is covered by a lipid-protein envelope containing the transmembrane proteins hemagglutinin (HA), neuraminidase (NA), and M2. The genome of IAV consists of single-stranded, negative-sense RNA that is split into eight segments encoding a total of 11

viral proteins: HA, NA, M1, M2, NP, non-structural protein 1 (NS1), non-structural protein 2 (NS2), polymerase acid (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase basic protein 1-F2 (PB1-F2).

The matrix protein (M1) lies just beneath the envelope, and the core of the virus particle is made up of the RNP (ribonucleoprotein) complex, consisting of the viral RNA segments, the polymerase proteins (PB1, PB2, PA and NP). The M1 protein interacts with vRNPs (Bui et al., 2000). HA, NA and M2 are transmembrane proteins that are located in the lipid bilayer of the IAV envelope which give it characteristic morphology with spikes. HA recognizes the sialic acid (SIA) receptors expressed on the surface of host cells in the respiratory tract and it is responsible for viral attachment and entry.

M2 is a proton-selective ion channel which is activated by a decrease in pH that occurs after virion endocytosis and endosomal acidification. It mediates the acidification of the viral core via the introduction of protons and results in the release of vRNP into the host cell's cytoplasm. NA is essential for the spread of newly synthesized viruses from host cells. It cleaves the SIA residues of glycans which allows viral release and to prevents aggregation of individual virions. At present, 18 subtypes of HA and 11 subtypes of NA have been documented (Cohen et al., 2013). While there are many different HA and NA subtypes for inluenza A viruses found in circulating influenza viruses, only three HA (H1, H2, and H3) and two NA (N1 and N2) subtypes have caused human epidemics, as defined by sustained, widespread, person-to-person transmission. Antigenic drift and antigenic shift are primary mechanisms causing the antigenic variation of the influenza virus and they occur in both HA and NA. The accumulation of random mutations (antigenic drift) in HA and NA, and new combinations of sequences from two or more flu strains (antigenic shift) can generate novel viruses that are different from pre-existing subtypes which can make them capable of bypassing pre-existing adaptive immunity and consequently cause influenza pandemics (Nelson and Holmes, 2007).

Different influenza virus strains are named according to their genus (type), the species from which the virus was isolated (omitted if human), location of the isolate, the number of the isolate, the year of isolation, and, in the case of the influenza A viruses, the hemagglutinin (H) and neuraminidase (N) subtypes. For example, the 220th isolate of an H5N1 subtype virus isolated from chickens in Hong Kong in 1997 is designated influenza A/chicken/Hong Kong/220/97(H5N1) virus (www.cdc.gov).

1.2.1. Life cycle of influenza viruses

To achieve successful infection, the influenza virus must first pass through the respiratory mucus layer that forms a primary physical barrier. Mucus in the respiratory tract contains sialylated glycoproteins. As mentioned before, influenza virus cleaves sialylated mucins using NA, which disables the inhibitory functions of the mucus, consequently allowing penetration into the mucus layer. Next, virions bind to the SIA-containing receptor using HA and enter the host cell via receptor-mediated endocytosis. However, also SIA-independent influenza infection has been reported (Brockwell-Staats et al., 2009; Gill et al., 2010). C-type lectin receptors are thought to be alternative receptors that allow entry of the influenza virus. The macrophage's galactose-type lectin and mannose receptors play important roles in influenza infection (Hancock et al., 2009). DC-SIGN (DC209) and L-SIGN (CD209L) have been identified as influenza attachment receptors (Garten et al., 2009; Sym et al., 2009) which indicates that C-type lectin receptors can act as specific receptors for IAV infection. M2 proton channels mediate endosomal acidification, which leads to the fusion of viral and endosomal membranes and the release of vRNPs into the cytosol (Harper et al., 2009; Jain et al., 2009; Munster et al., 2009). Released vRNPs are then translocated into the host nucleus via nuclear pore complexes, where they transcribe and replicate viral RNA (vRNA). RNA-dependent RNA polymerase (RdRP) is responsible for both transcription and replication of the viral RNA genome. The influenza RdRP protein consists of three subunits: PB1, PB2, and PA. Specifically, a "cap-snatching" mechanism is required for the transcription of viral mRNA. PB2 recognizes the 5′ cap of host pre-mRNA, and PA cleaves host mRNA to generate 5′-capped RNA fragments. They are then used as primers to initiate viral mRNA transcription (Bender and Small, 1992; Gill et al., 2010). PB1 carries out viral mRNA synthesis using these short fragments (Fesq et al., 1994). In contrast, vRNA replication, which occurs through a complementary RNA intermediate, is primer-independent (Julkunen et al., 2001). NS1 plays an important role in the inhibition of host antiviral immune responses. Previous studies have shown that NS1 suppresses the expression of host mRNAs that enable interferon (IFN)-induced antiviral phenotypes (Perrone et al., 2008; To et al., 2010). Further, NS1 restricts nucleocytoplasmic export of host mRNA by targeting nuclear RNA export factor 1–nuclear transport factor 2-related export protein 1, inhibits caspase-1 activation as well as the production of interleukin-1β (IL-1β), and disturbs the RIG-I signalling pathway (Cheung et al., 2002; Lee et al., 2008). NS2, also known as the nuclear export protein, transports newly synthesized RNPs out of the nucleus after amplification (Le et al., 2005). Further, it has been

suggested that NS2 is important for efficient influenza virion formation and budding due to its interactions with the F1Fo-ATPase. The IAV virulence protein PB1-F2 contributes to inflammatory responses and flu-induced pathogenesis through activation of the NLRP3-inflammasome (Itoh et al., 2009).

1.2.2. Therapy if influenza virus infection

Therapeutics targeting various stages of the influenza virus' replication cycle have been developed. There are three classes of FDA-approved anti-influenza drugs: M2 inhibitors (Amantadine, Rimantadine), NA inhibitors (Oseltamivir, Peramivir, Zanamivir), and a cap-snatching inhibitor (Baloxavir marboxil), M2 inhibitors block the release of vRNPs into the cytosol. However, most circulating viruses are now resistant to the existing M2 blockers, and they are no longer recommended for treatment or prophylaxis of influenza. NA inhibitors prevent virions from budding from host cells, and the cap-dependent endonuclease inhibitor targets influenza polymerase to prevent viral replication (Fig.2.). While these drugs are currently effective, constant changes in the viral genome cause drug resistance and reduce therapeutic efficacy.

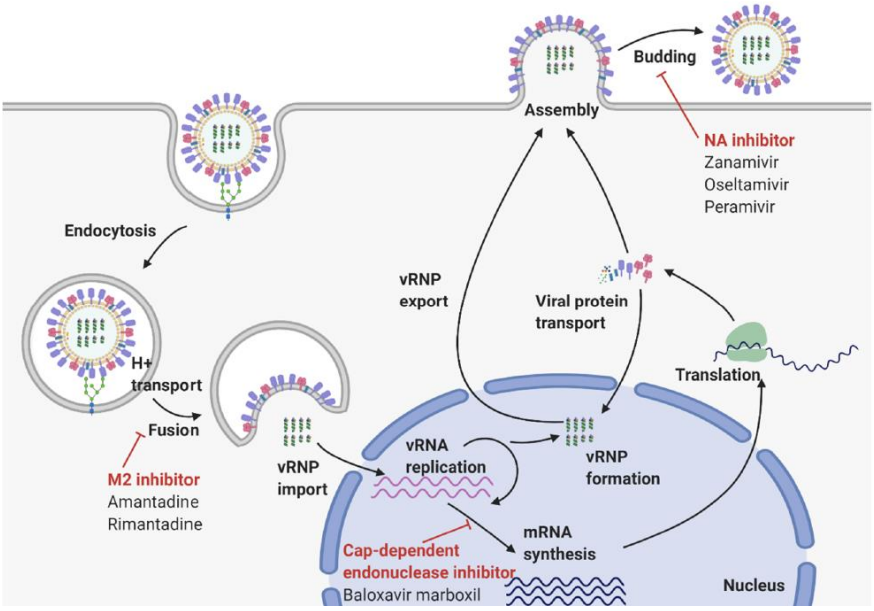


Figure 2. Influenza A replication cycle with inhibitors. The virus recognizes specific receptors expressed on the host cell surface using HA and enters cells via endocytosis. After fusion of the viral envelope and endosomal membrane, vRNPs are released into the cytoplasm and translocate into the nucleus to initiate replication. RdRP is responsible for both viral mRNA transcription and vRNA replication. Newly synthetised vRNPs are exported to the cytoplasm, and assembly of progeny virions occurs near the plasma membrane. NA facilitates budding of new virions from host cells. Anti-influenza drugs inhibit various steps of the influenza virus replication cycle. From (Jung and Lee, 2020).

1.3. Polyomaviruses

1.3.1. *Polyomaviridae* family

Polyomaviridae is a family of small, non-enveloped viruses with dsDNA genomes of approximately 5,000 base pairs. It belongs to the realm *Monodnaviria*, in the kingdom *Shotokuvirae*, phylum *Cossaviricota*, class *Papovaviricetes*, order *Sepolyvirales*. Phylogenetic relationships among polyomaviruses, based on the amino acid sequence of the viral protein large tumor antigen, have resulted in the delineation of six genera: *Alphapolyomavirus*, *Betapolyomavirus*, *Gammapolyomavirus*, *Deltapolyomavirus*, *Epsilonpolyomavirus* and *Zetapolyomavirus*. The members of these genera can infect mammals and birds, also their genomes have recently been detected in fish. Each family member has a restricted host range. Some members are known human and veterinary pathogens causing symptomatic infection or cancer in their natural host. Clinical manifestations are observed primarily in immunocompromised patients (https://talk.ictvonline.org).

First polyomavirus has been isolated and identified as a Maurine K virus in 1952 (Gross, 1953). Since then, 117 species have been identified, 14 of which in human population. First two human polyomaviruses discovered were JC polyomavirus (JCPyV) and BK polyomavirus (BKPyV) from immunocompromised patients and named after their initials. JCPyV was isolated by the transfer of brain tissue from a patient with the demyelinating disease progressive multifocal leukoencephalopathy (PML) into cultures of human foetal brain tissue. BKPyV was isolated from the urine of a renal transplant patient after inoculation into African green monkey kidney cells (Gardner et al., 1971; Padgett et al., 1971). Later species were renamed - name consists of a combination of the Latin host species name plus “polyomavirus”, followed by a consecutive number – BK polyomavirus became Human polyoma virus 1 and JC polyomavirus became Human polyoma virus 2, but they are still known by the earlier name. Both viruses belong to the *Betapolyomavirus* genus (www.talk.ictvonline.org).

For many years, polyomaviruses were studied mainly as model systems for understanding basic eukaryotic cell processes including DNA replication, RNA transcription, splicing and processing, and oncogenic transformation. The cloning and sequencing of the SV40 genome ushered in the era of recombinant DNA research. The SV40 genome is possibly the most intensively studied DNA molecule per base pair. Several genetic elements from the SV40

genome are used in most of molecular biology laboratories in the world today (Poulin and DeCaprio, 2006).

Interest in the polyomaviruses as human pathogens lagged behind these more basic biological studies because for many years the incidence of polyomavirus-associated diseases was rare and not well recognized basic biological studies, However, the onset of the human immunodeficiency virus type 1 (HIV-1)/acquired immunodeficiency syndrome (AIDS) epidemic led to a dramatic rise in the incidence of PML, as mentioned before a JCPyV-induced disease. In addition, recent advances in immunosuppressive regimens for bone marrow and solid organ transplant recipients and biological therapies for autoimmune diseases led to increases in JCPyV-, BKPyV-, and TSPyV-associated diseases. More recently, new findings about an apparent transforming activity of the MCPyV and its contribution to Merkel cell carcinoma has generated widespread interest in the polyomaviruses. Studies have led to the emerging view that while many polyomavirus features are shared, there are many fundamental differences that distinguish each virus (Liu, 2014).

1.3.2. JCPyV structure

Polyomavirus is nonenveloped virus ranging from 45 to 50nm that contains a circular dsDNA genome wrapped with cellular histones H2A, H2B, H3, and H4 (Liu, 2014).The nucleosome of this minichromosome exhibits the same nucleosome structure as cellular chromatin, the only difference is absence of histone H1 that becomes associated with the viral genome only when in the infected cell. The polyomavirus capsid contains 360 molecules of VP1 arranged in 72 pentamers or capsomeres each containing 5 molecules of VP1 and 1 molecule of VP2 or VP3. Out of 3 types of proteins, only VP1 molecule is exposed on the surface of the capsid. The particles have a T=7 icosahedral symmetry. The icosahedral capsid has both five- and sixfold axes of symmetry, with 12 pentamers surrounded by 5 other pentamers and 60 pentamers surrounded by 6 pentamers. Capsomeres with fivefold symmetry are unusual but supported by the high-resolution structure of SV40 (Liddington et al., 1991; Stehle et al., 1996). The C-terminus of each VP1 molecule extends out of the pentamer and contacts the neighbouring capsomere. This structure is flexible and thereby provides the means to form an icosahedron. Capsomere contacts are stabilized by the presence of calcium ions, and mutations in residues that bind calcium result in premature disassembly (Li et al., 2003). Treatment of virus with

EGTA under reducing conditions results in the dissociation of the capsid into VP1 pentamers. In addition to VP1, VP2, and VP3, APyV expresses VP4 (agnoprotein 1a) that interacts with the C-terminus of VP1 and may be incorporated into viral capsids (Shen et al., 2011). The capsid contains posttranslational modifications including disulphide bridges that form between the pentameric capsomeres. In addition, VP2 undergoes myristoylation at its N-terminus. A recent report found a large number of posttranslational modifications on the BKPyV VP1 protein, although the role of these modifications during infection is not known (Fang et al., 2010). Polyomaviruses are relatively resistant to heat and formalin inactivation. Furthermore, since polyomaviruses are nonenveloped, they are also resistant to lipid solvents.

1.3.3. JCPyV Genome

The prototype JCV genome is 5130, although individual variants differ in length, due to alterations in their noncoding regions. The genome encodes 6 major viral proteins (large T and small t antigens, VP1, VP2, VP3, and agnoprotein) as well as several splice variants of T antigen. Early- and late-transcribing sides of the genome are physically separated by a noncoding control region (NCCR), often called the hypervariable regulatory region (HVRR) or regulatory region (RR), and are transcribed in opposite directions from opposite strands of DNA. The early side of the genome, which is transcribed before DNA replication begins, is composed of large T antigen and small t antigen genes, as well as the splice variants T′135, T′136, and T′165. The late side of the genome is transcribed concomitant with DNA replication and encodes the three viral structural proteins; VP1, VP2, and VP3, as well as the accessory agnoprotein (Ferenczy et al., 2012).

1.3.4. Life cycle of JCPyV:

In the life cycle of JCPyV there are two levels of restriction: extracellular on the cell surface according to the presence or absence of virus (co)receptors and intracellular which is at any of the following steps in viral life cycle. As a classical viral infection in the host cell, it is initiated by interactions between viral capsid and cell surface receptors. In 2010 researchers identified a linear sialylated pentasaccharide, with the sequence NeuNAc-α2,6-Gal-β1,4-GlcNAc-β1,3-Gal-β1,4-Glc, also called lactoseries tetrasaccharide c (LSTc), present on host glycoproteins and glycolipids as a specific JCPyV recognition motif. The crystal structure of the JCPyV

capsid protein VP1 was solved alone and in complex with LSTc. It discloses extensive interactions with the terminal sialic acid of the LSTc motif and specific recognition of an extended conformation of LSTc (Fig.3.) Furthermore, mutations in the JCPyV oligosaccharide binding sites abolish cell attachment, viral spread and infectivity, which further confirms the importance of this interaction (Neu et al., 2010; Tsai and Inoue, 2010). JCPyV uses also 5HT2A serotonin receptor for viral entry perhaps as a cell type–specific receptor since it is is expressed on glial cells, the major target cell for this virus. Antibodies to the 5HT2A receptor and receptor antagonists block JCPyV infection, while transfection of H5T2A-negative HeLa cells with H5T2A receptor makes them susceptible to infection (Elphick et al., 2004).

After binding to these viral-specific receptors, viral particles use clathrin-dependent endocytosis mechanism to be transported into the cytoplasm. Once the virus enters into the host cell, it travels to the endoplasmic reticulum after which VP1 protein facilitates entry into the host cell nucleus, it has been shown that JCPyV particles do not disassemble before they reach the nuclear pores. Replication of the viral genome, viral protein synthesis, assembly of the viral particles and their release from the cell use the host cellular machinery. JCPyV life cycle is quite a slow process, even in susceptible cells with Tag is already present DNA replication is detectable after some days. Furthermore, it has been demonstrated that cell-specific factors regulate viral transcription, while the viral DNA replication is most likely regulated by species-specific factors, which can be one or more components of the DNA polymerase. These species-specific factors allow JCPyV replication only in primates. The final viral products are released via host cell lysis (Ferenczy et al., 2012).

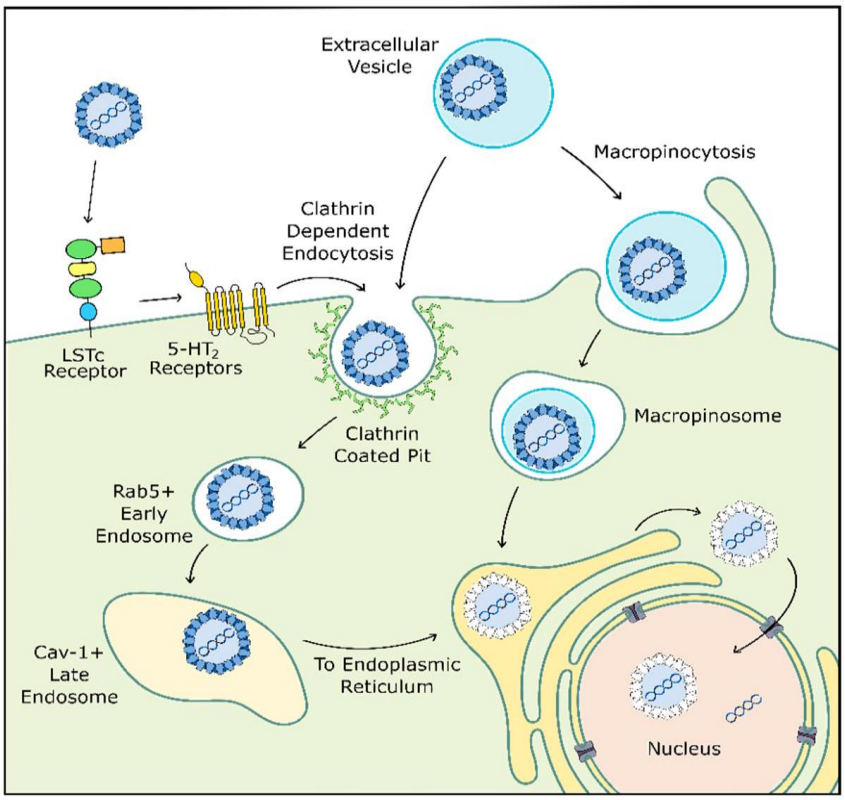


Figure 3. JCPyV cellular entry may occur through multiple pathways. JCPyV initially binds to LSTc receptors before transient interaction with 5-HT2 receptors which facilitate internalization through clathrin-mediated endocytosis. JCPyV then switches from a Rab5+ early endosome to a caveolin-1+ late endosome before entering the endoplasmic reticulum (ER) where the virus is uncoated. JCPyV exits the ER into the cytosol before entering the nucleus for replication. Alternatively, JCPyV may bind to the cell within an extracellular vesicle. Here, the vesicle is internalized through micropinocytosis or clathrin-mediated endocytosis before tracking to the ER. (Atkinson and Atwood, 2020)

1.3.5. Pathogenesis

JCPyV infection is widespread in the general population with more than 80% being seropositive by adulthood (Tyler, 2009). Nevertheless, the mode of transmission is not yet well defined although the presence of JCPyV DNA in B cells and stromal cells of the tonsils supports the proposal that tonsils may serve as an initial site of viral infection (Monaco et al., 1998). Virus might enter the upper respiratory tract by close interpersonal contact or via fomites and presumably spread by the hematogenous route from the primary site of infection to secondary sites such as kidneys, bone marrow, lymphoid tissues, and brain to establish focal areas of infection or persistence (Chapagain and Nerurkar, 2010). Potential alternative modes of transmission include urino-oral, transplancental, and transmission by blood transfusion, semen, and organ transplantation (Jiang et al., 2009). Archetype virus is also isolated from

sewage samples from different geographical areas suggesting a possible transmission by contaminated food, water and fomites (White and Khalili, 2011).

Upon the suppression of $CD4^+$ and $CD8^+$ T-cell mobilization, as occurs with HIV infection or during immune-modulating therapy (such as natalizumab, efalizumab, and rituximab), the JCPyV enters the brain, either within B cells or as cell-free virus, where it infects and kills oligodendrocytes, leading to demyelination. In particular the lack of surveillance, normally imposed by the immune system, could enhance the transcriptional activity of both NFAT4 and NF-κB, that are under proinflammatory cytokine control and can also increase JC early genes (in particular TAg) transcription in response to TNF-α stimulation (Wollebo et al., 2011). The nuclear factor of activated T cells (NFAT) is another transcription factor family under proinflammatory cytokine control. In particular, NFAT is the primary target of Ca^{2+}-calmodulin-dependent serine phosphatase calcineurin, a crucial component of the calcium-signaling pathway that can stimulate the production of inflammatory cytokines in response to extracellular stimuli (Ho et al., 1996). B cells may play an important role in the pathogenesis of PML, in addition to be a potential site of viral latency. Since it has been suggested that the viral genome rearranges during DNA replication; an attractive model is that these events occur in B cells, since these cells possess the Rag1 and Rag2 enzymes for immunoglobulin gene rearrangements. Additionally, evidence that changes in transcription factors can affect viral transcription could be found in the observation that natalizumab treatment upregulates factors involved in B cell differentiation, including Spi-B (Lindberg et al., 2008). Spi-B binding sites in the promoter/enhancer of JCPyV variants are located directly adjacent to TATA boxes, that are essential for transcription of early and late viral genes. Spi-B is a transcription factor that can cooperate with pRB and TATA-binding protein (TBP) to alter expression of proteins involved in B cell maturation (Schmidlin et al., 2008). TBP binds TATA box elements in promoters and it is a subunit of the basal transcription complex TFIID, which increases RNA polymerase II activity. Recruitment of the TFIID complex to JC viral promoters by Spi-B and TBP is an attractive model for the activation of JCV gene expression.

JCPyV is a neurotropic virus; nevertheless, it is still incompletely understood how the virus infects the central nervous system (CNS). There are two possibilities: JCPyV infects the CNS in case of alteration of the immune response; alternatively, the virus infects the CNS and persists there for many years in immunocompetent individual. When alteration of the immune system occurs, viral infection emerges. Within the brain, JCPyV can infect both oligodendrocytes and astrocytes; occasionally JCPyV infects the cerebellar granule cells (Del

Valle et al., 2008). Immune control of JC virus is based mainly on cellular immune response. Cytotoxic $CD8^+$ T lymphocytes (CTLs) recognize the epitopes of viral proteins presented on the class I HLA molecules preventing further spread of the virus. CTLs are usually detected in the blood of PML survivors, in PML lesions where they aggregate around infected cells and rarely in patients with PML, who have a fatal outcome within 1 year from disease onset. Specific $CD4^+$ T cells have been detected in the blood of patients who have survived PML, and the number of these cells correlates with JC virus clearance from the cerebrospinal fluid (CSF) (Lima et al., 2007).

The humoral immune response against the JC virus has been extensively studied. The first test to estimate seroprevalence was the haemagglutination inhibition assay based on the ability of JCPyV antibodies in the serum to prevent agglutination of human type O erythrocytes (Padgett and Walker, 1973). Nowadays the use of different technologies in the tested populations (i.e., quantitative enzyme immunoassay on recombinant VP1 protein) revealed a great variability of viral seroprevalence in adults and children. It might be explained on the basis that primary infection is not associated with a recognizable clinical event. Eleven JCPyV genotypes have been found to be serologically distinct and there is no clearly defined JC virus seronegative population (Kean et al., 2009). Regarding HIV positive patients, although intrathecal JC virus antibody becomes detectable with JC viral clearance after treatment with highly active antiretroviral therapy (HAART), neither the presence of intrathecal nor serum specific antibodies prevent the onset of PML (Weber et al., 2001).

1.3.6. Therapy for JCPyV infection

There is currently no specific antiviral drug against JCPyV but use of antiretroviral drugs in HIV patients and early detection and monitoring have played a significant role in reducing PML mortality (Ferenczy et al., 2012; Garvey et al., 2011). Numerous drugs have been tested for eficacy against JCPyV, but none have been consistently successful thus far (Pavlovic et al., 2015). One candidate called Retro-2.1 (a retrograde transport inhibitor) was able to reduce in vivo murine polyomavirus infection in mice and has in the past inhibited JCPyV in vitro, but this method has yet to be translated to human treatments (Maru et al., 2017; Nelson et al., 2013). Instead, what is currently considered the best option for slowing the effects of PML is restoration of the immune system, although as stated above this increases the risk of IRIS.

1.4. Antiviral peptides

First antiviral drugs were synthetised during 1960s and early 1970s but the battle with viral infections is still ongoing today. Recent research highlights antiviral activity of proteinaceous compounds and it is also being demonstrated that some antimicrobial proteins (AMPs) show activity against broad spectrum of viruses (Pereira et al., 2019). They are called antiviral peptides (AVPs). Many of discovered AVPs have a shared virucidal mechanism of action, they act directly on inhibiting the virion, but there are also other examples including the suppression of viral gene expression. Research is focusing on identifying structures and mechanism of action of these molecules so it could be possible to use them as antiviral drugs or to synthetise an analogue with better properties including lower toxicity and higher antimicrobial activity. Natural AVPs can derive from plants, arthropods, amphibians, marine organisms but also mammals. Commonly shared characteristics of natural AVPs are cationic, amphipathic and positive net charges could be a potential reason behind their antimicrobial activity (Bulet et al., 2004).

Wang at al. (2017) made the first computational study using large-scale AMPs to examine the relationships between antimicrobial activities and two major physiochemical properties of AMPs: amphipathicity and net charge. The results showed that among all kinds of antimicrobial activities, amphipathicity and net charge best differentiated between AMPs with and without anti-gram-negative bacterial activities. In terms of amphipathicity and charge, all the AMPs whose activities were significantly associated with amphipathicity and net charge were alike except those with anti-gram-positive bacterial activities (Wang et al., 2017). Furthermore, the higher the amphipathic value, the greater the proportion of AMPs possessing both antibacterial and antifungal activities. In this research neither amphipathicity nor net charge were found to significantly associate with antiviral or antiparasitic peptides in this study. Any AMPs involved with viruses or parasites showed no distinctions, although those with concurrent antibacterial, antifungal, and antiviral activities which did pass the initial screen failed the Bonferroni correction. It was stated that additional data might help to affirm whether amphipathicity or charge significantly associates with the coexistence of antibacterial, antifungal, and antiviral activities of AMPs (Badani et al., 2014).

Benefit of natural AVPs could be possibility of specificity, effectiveness, low toxicity and peptidase biodegradability which would limit accumulation in tissues. Some limitations of

natural AVPs could present short half-life, immunogenic potential, high cost of production and low oral absorption (Galdiero et al., 2013).

Frog skin is considered as an abundant source of AMPs, but still not many of them have been described in literature (Marcocci et al., 2018b; Shartouny and Jacob, 2019). The main function of molecules contained in frog skin secretion is inhibition of microbial growth on their skin surface which is more than necessary considering many of the species live in humid environments susceptible to fast growth of microbial organisms. Besides antimicrobial peptides, other proteins related to mammalian hormones and neurotransmitters can also be found in frog skin secretions. Mixture of different peptides serve as a good defence mechanism from the predators (Mangoni and Casciaro, 2020).

1.4.1. Bombinin family

Frogs of the Bombinae family have been used in research as models to study barrier against microbial organisms. (Miele et al., 1998, 2001). Three main species that are commonly researched are *Bombina bombina*, *Bombina variegata* and *Bombina orientalis*. In Bombinae skin secretion have been found several bombinins and bombinin like peptides which have not been detected in other amphibian genra. Skin secretions frog *Bombina variegata* contain a family of hydrophobic peptides, called bombinins H, which probably play a role in the defence against microbes (Mangoni et al., 2000). Letter H indicates their haemolytic properties. These peptides are rich in glycine (25%) (Simmaco et al., 1991). Because of that it was thought that the formation of an amphipathic alpha-helix is unlikely but later results from circular dichroism spectra showed that both bombinin H2 and H4 can adopt alpha-helical structure. Furthermore, probability of different conformations is increased by the presence of several Gly residues (Mangoni et al., 2000, 2006). Also, here is increasing evidence that bombinin H can, dependent on the environment, adopt different conformations. Moreover, some of these peptides contain a D-amino acid; the bombinins H2 and H4 differ from each other in that they contain either L-Ile or D-allo-Ile at position 2. Bombinin H2 (IIGPVLGLVGSALGGLLKKI-amide) and bombinin H4 (I-(D-allo-I)GPVLGLVGSALGGLLKKI-amide) (Zangger et al., 2008). Several binding sites for transcription factors (IL-6, NF-kB, and a GATA motif) have been identified. Remarkably, after processing of the precursor, bombinin H is in part further modified by a recently characterized enzyme that catalyses the inversion of the stereochemistry of the second

amino terminal residue from L-Ile to D-allo- Ile (Mignogna et al., 1993; Jilek et al., 2005). Research on structure of bombinin peptides from 2008. showed results of the TALOS 4-angle restraints which were confirmed by the experimental scalar coupling constants indicating a large α-helical content. In this research the Ribbon diagram of the closest-to-mean structure of bombinin H2, with bundles of the 20 lowest energy side-chain structures is shown. Residues facing the interior of the micelle are drawn downwards. The ribbon diagram was prepared with the program MOLMOL (Fig.4) (Koradi et al., 1996; Zangger et al., 2008).

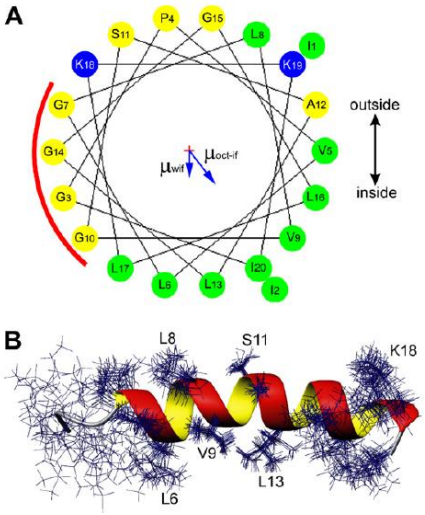


Figure 4. A) Helical wheel representation of bombinin H2. Large hydrophobic residues are green, polar and small hydrophobic residues are yellow and cationic residues are colored in blue. The glycine ridge is indicated by a red arc. respectively. B) Ribbon diagram of the closest-to-mean structure of bombinin H2, with bundles of the 20 lowest energy side-chain structures. Residues facing the interior of the micelle are drawn downwards. (Zangger et al., 2008)

A glycine ridge is exposed at one side of the helix and may provide a helix–helix interaction site. In this respect, the structure of bombinin H resembles the influenza hemagglutinin fusion peptide (Han and Tamm, 2000) and the helical conformer of Alzheimer peptide Ab (Shao et al., 1999). Neither structure nor orientation of bombinin H are affected by the chiral inversion. Environmental conditions can trigger self-aggregation of bombinin H in solution due to hydrophobic interaction. Under these conditions the stereochemistry of the randomly ordered N-terminal segment modulates the preference to fold into a particular conformation (Zangger et al., 2008).

4.1.2. Temporin family

A family of amphibian AMPs, originally isolated from the European red frog Rana temporaria, is called temporins. These peptides represent the shortest AMPs found in nature to date with a typical length of 10 to 13 amino acids and a conserved sequence (Mangoni, 2006; Simmaco et al., 1996). They are amidated at the C terminus and are characterized by a weak cationic charge (net charge ranging from +2 to +3) owing to the presence of only one or two positively charged amino acids, such as lysine or arginine, in their sequence. Temporins, like other AMPs, exert antimicrobial activity against bacteria and fungi (Grassi et al., 2017; Merlino et al., 2017), while their antiviral activity has been reported only against a few enveloped or nonenveloped viruses of ectothermic animals (Chinchar et al., 2004). Furthermore, no studies regarding the activity of temporins against human viruses have been reported to date. Here, we demonstrate for the first time the in vitro antiviral activity of temporin B (TB) against HHV-1. When added to HHV-1-infected cells, TB significantly reduced the virus titer, but, more importantly, the greatest inhibition was obtained by preincubation of HHV-1 with the peptide for 1 h at 37°C, thus demonstrating its virucidal activity (Marcocci et al., 2018).

2. RESEARCH OBJECTIVES

No matter the how much science evolved in last 100 years, viruses still remain one of the main causes of human diseases. Discovery and development of new vaccines is usually challenging and time consuming (Mahmoud, 2016). For these reasons, usually the most utilized alternative available for viral control is treatment with antiviral drugs (Enquist, 2009; Lou et al., 2014). Mechanisms of action for antiviral drugs that are most commonly used are virus-targeting antivirals and host-targeting antivirals (Lou et al., 2014). Virus-targeting antivirals act inhibitory on important transcription and replication enzymes, such as proteases and polymerases (Kiser and Flexner, 2013; McDonald and Kuritzkes, 1997), or the directly inactivate viral structural proteins. In contrast, host-targeting antivirals focus on the inhibition cyclophilins, known to be important cellular factors that are hijacked by some viruses during the replication cycle (Lou et al., 2014); the use of immunomodulators such as interferons (Lin and Young, 2014; El Raziky et al., 2013); and gamma globulins (Buttinelli et al., 2003).

First-generation antiviral molecules were described in the 1960s and early 1970s, they weren't specific which led to serious side effects on humans. For example, vidarabine, an adenosine analogue used as a replication inhibitor, can affect not only viral DNA polymerase, but also the eukaryotic analogue (Thompson and Whitley, 2011). With time and more knowledge new, better molecules were discovered and synthetised such as acyclovir, the first nucleoside analogue, and antiviral drug to be considered successful for the treatment of human herpes virus (HHV) 1 and 2, and varicella zoster virus (VZV) infections. Because of its specificity which is achieved by the need for a phosphorylation step mediated by a viral protein - this molecule causes much lower toxicity for the host when compared with previously used treatments (Elion, 1982; Thompson and Whitley, 2011). Unfortunately, the low efficacy of antiviral treatments is still evidenced by the ever-increasing reports of viral resistance (Musiime et al., 2013; Le Page et al., 2013), concomitant viral infections (Deming and McNicholl, 2011), and the emergence and re-emergence of viral epidemics in relatively short periods of time, as observed for H1N1, Ebola and zika virus (ZIKV) only in the first 5 years of the last (Hui et al., 2017; Marston et al., 2017; Suijkerbuijk et al., 2018). Therefore, the demand for production of new antiviral drugs is higher than ever, with increased preference for molecules capable of presenting broad-spectrum activity (Zhu et al., 2015). The search for these new molecules involves different approaches such as bioinformatics-assisted predictions based on molecule interaction with important viral structures or enzymes (Wang et al., 2010)

and the isolation of new compounds obtained from natural sources (Cantatore et al., 2013; Rothan et al., 2014). Using such techniques, many new molecules have been described so far, and, most recently, the description of antimicrobial peptides has been gaining attention (Chen et al., 2017; Hakim et al., 2013; Rothan et al., 2012).

Focus of this thesis was on bombinin H2 and bombinin H4 which are interesting antimicrobial peptides from frog skin. To test a possible antiviral effect of those molecule, different types of viruses were chosen to understand the mechanism of peptides better, including HHV-1 which is an enveloped DNA virus, H1N1 influenza virus which is an enveloped RNA virus and JC Polyomavirus which is non-enveloped dsDNA virus. These viruses have many differences, but the most important similarity is the human host and a big problem they cause in our healthcare system. Both HHV-1 and JCPyV cause major problems in immunocompromised patients which puts already vulnerable peoples lives in an even greater danger and further burdens already burdened healthcare system. On the other side, Influenza viruses cause major pandemics every few decades, there may have been at least 14 influenza pandemics in last 500 years (Taubenberger and Morens, 2010).

Specific aims:

- Plaque assay to test antiviral effect of bombinin H2 and H4 on HHV-1
- Western blot employed to investigate and confirm antiviral effect of bombinin H2 and H4 on enveloped viruses HHV-1 and IVA H1N1
- In-Cell Western blot analysis to further nvestigate antiviral effect of bombinin H2 and H4 on enveloped viruses HHV-1 and IVA H1N1
- Q-PCR employed to test antiviral effect on non-enveloped virus JCPyV

3. METHODS AND MATERIALS

3.1. Cell lines

3.1.1. Vero cells

African green monkey kidney (Vero) cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine, 50 U per ml penicillin, and 50 mg/ml streptomycin. In all experiments with Vero cells, reagents were purchased by Sigma–Aldrich, St. Louis, MO, United States. The monolayers of cells were maintained in 75 cm2 tissue culture flasks at 37°C in humidified air containing 5% CO2.

3.1.2. A549

Adenocarcinomic human alveolar basal epithelial cells (A549, ATCC catalogue no. CCL-185) were used to determine the antiviral activity of bombinin on influenza virus. Cell lines were maintained in high glucose Dulbecco's Modified Eagle's Medium with sodium pyruvate and L-glutamine (DMEM; Euroclone, Milan, Italy) supplemented with 10% Fetal Bovine Serum (FBS; Euroclone, Milan, Italy) and 1% Penicillin/Streptomycin (Pen/Strep, Euroclone, Milan, Italy).

3.1.3. SVGp12

SVGp12 cells were obtained from the ATTC (ATCC® CRL-8621™). The SVGp12 cell line was established by transfecting cultured human fetal glial cells from brain material dissected from 8- to 12-week-old embryos with DNA from an ori - mutant of SV40. Eagle's Minimum Essential Medium (EMEM) supplemented with 100 U of penicillin, 100 µl of streptomycin per ml, (Sigma-Aldrich, Milano, Italy) and fetal bovine serum (FBS) (10%) was used as maintenance medium for the cell line.

3.2. In vitro cytotoxicity (MTT) assay

MTT assay was performed following the manufacturer's instructions (Cell growth determination kit MTT based, Sigma-Aldrich, Milan, Italy). The MTT system is a simple, accurate, reproducible means of measuring the activity of living cells via mitochondrial

dehydrogenase activity. The key component is 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl tetrazolium bromide or MTT. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in colour. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance. Cells were plated in 96-well plates at a density of $2.0x10^4$ cells / well in 100 µl of complete RPMI medium (for Vero cells) or DMEM medium (for A549 cells) containing 10% v/v of FBS. Confluent monolayers were treated with different concentrations of bombinin H2 or H4 in complete medium free of phenol red. Each sample was tested in duplicate. The treated cells were incubated for 24h at 37 °C with the subsequent addition of MTT Solution in an amount equal to 10% of the culture volume. The microplate was incubated at 37 C for another 2 - 3 hours under the same experimental conditions. After the incubation period of 2-3 h the resulting MTT formazan crystals were dissolved by adding 10 microliters of MTT solvent to each well which was then resuspended. The optical density (OD) of wells was measured at 570 using 96-well plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

3.3. Infection

3.3.1. HHV-1 infection of VERO cells

For in vitro HHV-1 infection, confluent monolayers of Vero cells were incubated with HHV-1 at 1 m.o.i. (multiplicity of infection) for 1 h at 37 C in an atmosphere of 5% CO2, to allow virus adsorption to the host cells (adsorption phase). Then, the HHV-1-containing medium was then removed and after 2 washes in PBS, the medium was replaced with fresh medium supplemented with 2% FBS, and the plates were maintained for 24 h at 37 C in an atmosphere of 5% CO2 (post-infection phase). After the post-infection phase supernatant was directly collected and frozen. The cells were detached with Trypsin, after centrifugated with the addition of FBS and frozen dry at -80 C, later to be used for WB studies.

3.3.2 Influenza A virus (H1N1) infection of A549 cells

Allantoic cavities of 11-day-old embryonated chicken eggs were used to growth influenza virus A/Puerto Rico/8/34 H1N1 (PR8 virus). Viral suspension was inoculated in the allantoic cavity and incubated for 48 h at 37 C, then infected eggs were maintained overnight at 4 C. Subsequently, the allantoic fluid was collected and clarified by centrifugation (2500x g for 30 min). The recovered virus was used for the infection of A549 cells. Cells were plated and infected with H1N1 at a dilution of virus at 1:100. Cells were then incubated for 1 h at 37 C in an atmosphere of 5% CO2, to allow virus adsorption to the host cells (adsorption phase). Then, the virus-containing medium was then removed and after 2 washes in PBS, the medium was replaced with fresh medium supplemented with 2% FBS, and the plates were maintained for 24 h at 37 C in an atmosphere of 5% CO2 (post-infection phase). After the post-infection phase supernatant was directly collected and frozen, so it could be used later for Odyssey assays. The cells were detached with Trypsin, after centrifugated with the addition of FBS and frozen dry at -80 C, later to be used for wb analysis.

3.3.3. JC Polyomavirus infection of SVGp12 cells

SVGp12 cells were plated with a density of 2×10^4 and grown for 24 h in complete growth medium to reach 50-70% confluence on the day of infection. The cells were then infected with supernatants (SPNTs) recovered from previous infection experiment containing virions corresponding to 10^4 copies/mL.

3.4. Standard Plaque assay

The antiviral activity of peptides was evaluated by standard plaque assay. Confluent monolayers of Vero cells were incubated with 3 different 10-fold dilutions of the earlier mentioned supernatants for 1 h at 37 °C. After the incubation supernatants were aspirated, cells washed two times with PBS, and incubated with 2% carboxymethyl-cellulose (CMC) in 2% FBS-RPMI for 3 days at 37°C in 5% CO_2 to allow plaque formation. After 3 days, the cells were fixed with ice-cold 100% methanol for 20 min at -20°C, stained with 0.5% crystal violet in 10% ethanol for 10 min., washed with water and visualized for plaque detection. The data

were analysed as the means of quadruplicates ± SD for each dilution. All the reagents were purchased from Sigma–Aldrich (Milan, Italy).

3.5. Western Blot Analysis

Infected or mock-infected cell pellets were washed twice with PBS and resuspended in cold lysis buffer (RIPA buffer:50mM Tris-HCl, 150 mM NaCl, 10M EDTA, 1mM phenylmethylsulfonylfluoride, 1% Triton X-100, 0.1%SDS, 5% deoxycholicacidsodiumsalt and complete Protease and Phosphatase Inhibitor cocktails (Sigma-Aldrich, Milan, Italy) pH 7.4), incubated for 30 min on ice. After incubation cellular suspensions were centrifuged (10 000 g for 30 min at 4 °C) and the amount of the extracted proteins contained in the sup was determined by Micro bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Equivalent amounts of proteins were resolved in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels with 10% acrylamide and blotted onto nitrocellulose membranes for Western blot analysis (WB). Membranes were coloured with Ponceau Red (Sigma-Aldrich, Milan, Italy) and, after a washing with 1% Tween-20-TBS (T-TBS), blocked with 10% non-fat dry milk in T-TBS for 1 h at room temperature, and incubated with primary antibodies diluted in 5% not-fat milk in T-TBS overnight at 4 °C or during 1 hour at room temperature. The following primary antibodies were used: anti- HHV 1/2 1:1000 (#4956-0104, Bio-Rad, Milan, Italy, Milan, Italy); anti- HHV-1/2 gB (10B7) 1:500 (sc-56987, Santa Cruz Biotechnology, Heidelberg, Germany); anti- HHV-1 ICP0 1:1000 (sc-53070, Santa Cruz Biotechnology, Heidelberg, Germany); anti-tubulin (T6074 Sigma-Aldrich, Milan, Italy), After 3x10 min washes with T-TBS, the membranes were incubated with secondary horseradish peroxidase-conjugated antibodies, Jackson ImmunoResearch Laboratories, followed by 3x10 min washes in T-TBS. The chemiluminescence reaction was obtain using Clarity or Clarity Max Western ECL Substrate (Bio-Rad, Milan, Italy) and detected by Chemidoc Imaging System (Bio-Rad, Milan, Italy). Densitometry was performed to quantify the signal intensity using Quantity One software (Bio-Rad, Milan, Italy).

3.5. In-cell Western blot (ICW)

Vero cells were seeded in 96 well plate and grown to 90% confluency for 24 h. Then, cells were directly infected with serial dilutions of virus (HHV-1 or IAV), or with supernatants from infected cells. After 1 h of incubation at 37°C, supernatants were aspirated, cells washed two times with PBS, and then incubated with 2% FBS-RPMI for 24 h at 37C_ in 5% CO_2. Cells were washed with PBS twice and fixed with 50 microl of 4% paraformaldehyde in PBS for 15 min at room temperature (rt), and then were permeabilized in 0.1% triton X-100 PBS for 5 min at rt. Following the incubation with Odyssey Blocking Buffer for 1 h at rt, cells were incubated:

a) For HHV-1 with anti-glycoprotein B (gB) or anti-ICP4 antibodies (sc-56987 and sc-S9808N, Santa Cruz, Heidelberg, Germany, respectively, 1:1000 dilution in Odyssey Blocking buffer)

b) For IVA with Anti-influenza A antibody (AB1074, Chemicon, Milano, Italy) or antibody against Hemagglutinin (sc-52025, Santa Cruz, Heidelberg, Germany)

for 1 h and then washed three times with PBS containing 0.1% Tween-20. Afterward, labeled secondary antibody IRDye 800 CW Goat Anti Mouse (926-32210 LI-COR Biosciences, 1:1000 dilution in Odyssey Blocking buffer) and CellTag 700 Stain (926-41090, LI-COR Biosciences, 1:500) were added to each well, and after 1 h, cells were washed four times with PBS containing 0.1% Tween-20. Finally, the plate was scanned on the Odyssey Infrared Imager, and the integrated intensity value of each well read by LI-COR Image Studio Software developed for Odyssey analysis.

3.6. Extraction of viral DNA from SVGp12 cells and from supernatants and Quantitative PCR (Q-PCR)

The total DNA was extracted from 1×10^6 SVGp12 cells using a QIAmp® DNA Mini Kit (QIAGEN S.p.A., Milan, Italy), following the instructions provided by the manufacturer. Once extracted, the DNA was stored at -20 °C until use. SPNTs from SVGp12 cells was initially subjected to six cycles of freezing and thawing and then centrifuged at 2000 rpm for 10 minutes, and the resulting supernatant was used directly in molecular biology assays.

Extracted DNA was analyzed using Q-PCR for the detection and quantification of the JCPyV genome using a 7300 Real-Time PCR System (Applied Biosystems, USA). Each sample was analyzed in triplicate, and the viral loads were given as the mean of at least three positive reactions. Standard precautions designed to prevent contamination were followed, and a negative control was included in each run. Viral DNA was quantified using a standard curve consisting of serial dilutions of a plasmid containing the entire JCPyV genome with a known titer (range, 105 gEq/ml–102 gEq/ml). The amount of cellular DNA was quantified simultaneously using a SYBR GREEN PCR for the housekeeping β-globin gene and used to normalize the JCPyV DNA. The data were expressed as genome equivalents (gEq) of viral DNA per cell based on DNA content (gEq/cell) for the SVGp12 cells and as genome equivalents (gEq) of viral DNA per milliliter (gEq/ml) for the supernatants.

3.7. Statistical analysis

The results of the MTT test were shown as mean value (n=2). The 50% cytotoxic concentration (CC_{50}) for H2 and H4 was calculated by regression analysis of the dose-response curves.

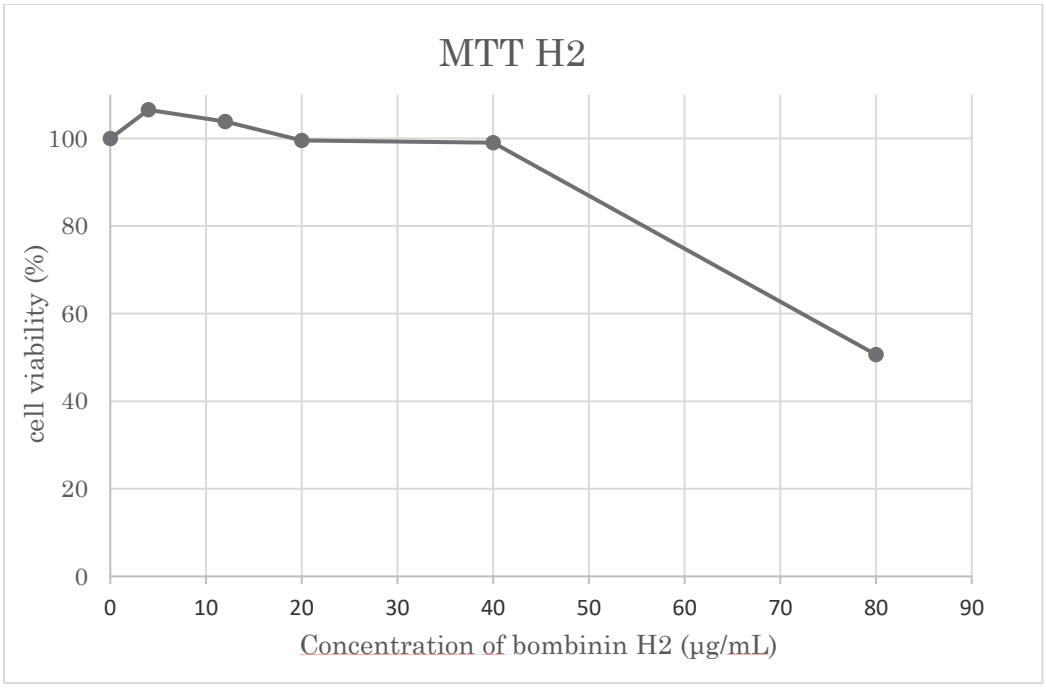
Results of plaque assay in the experiment of testing virucidal effect of bombinin H2 and H4 on HHV-1 were statistically analysed and compared to positive control (infection with virus without treatment) by one-way analysis of variance (ANOVA) followed by a multiple comparison procedure (Tukey's test), using the statistical software Graphpad Prism v. 6.0. Data is presented in graphs as mean ± SD (standard deviation). The level of $p<0.05$ was considered statistically significant.

Results of other plaque assays and of ICW are presented as a mean value (n=2).

4. RESULTS

4.1. Cytotoxicity

To test a potential antiviral effect of bombinin H2 and H4 peptides, preliminary studies were made to assess their possible toxicity in virus-permissive cells. For this purpose, MTT assays were performed. Cytotoxicity of bombinins H2 and H4 was evaluated according to cell viability upon 24 h treatment of Vero and A549 cells with different concentrations of both peptides, ranging from 1,5 to 80 μg/mL. Results are shown on figures 5 and 6. The 50% cytotoxic concentration (CC_{50}) of bombinin H2 on Vero cells is 93,795 μg/mL and of bombinin H4 is 106,485 μg/mL; on A549 cells CC_{50} of H2 is 70,199 μg/mL and of H4 is 434,520 μg/mL, but the values in the case of H4 seem inconsistent. As shown in Fig.6., Bombinin H2 shows a higher cytotoxicity on A549 cells. A big drop in cell viability can be noticed after cellular treatment with concentration >20 μg/mL. In the case of bombinin H4 the values are not consistently dropping or staying similar to control, but CC_{50} calculation shows that it is less cytotoxic to A549 cells. Based on obtained results, the next experiments were performed using H2 and H4 at 10 μg/mL and 20 μg/mL.



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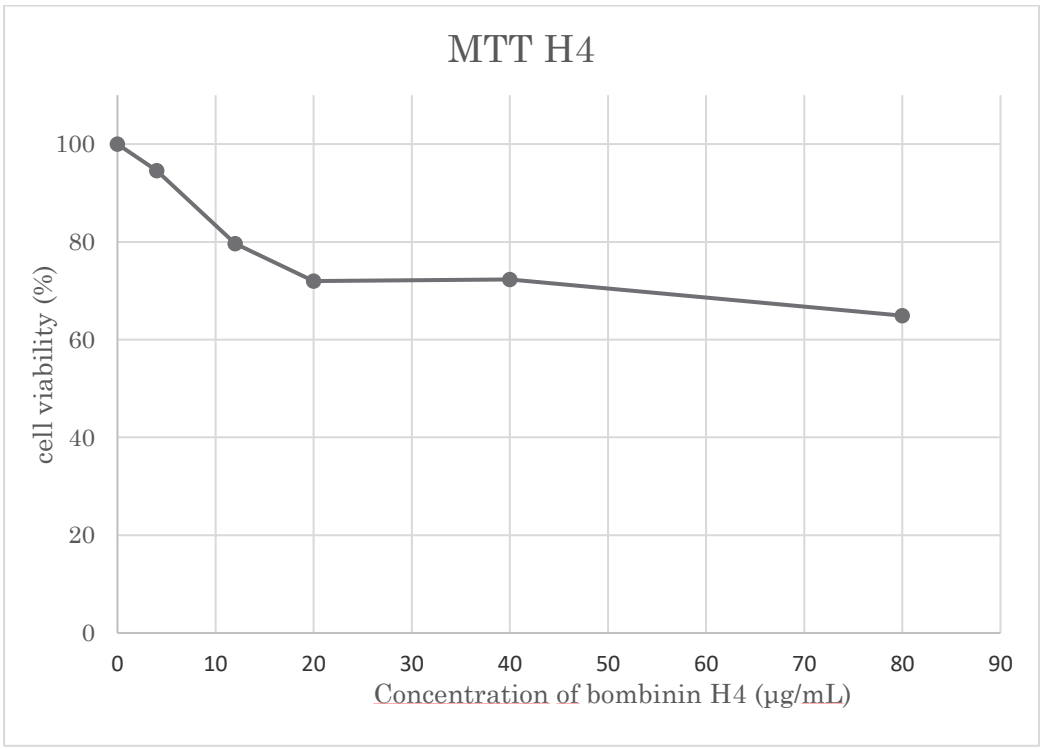

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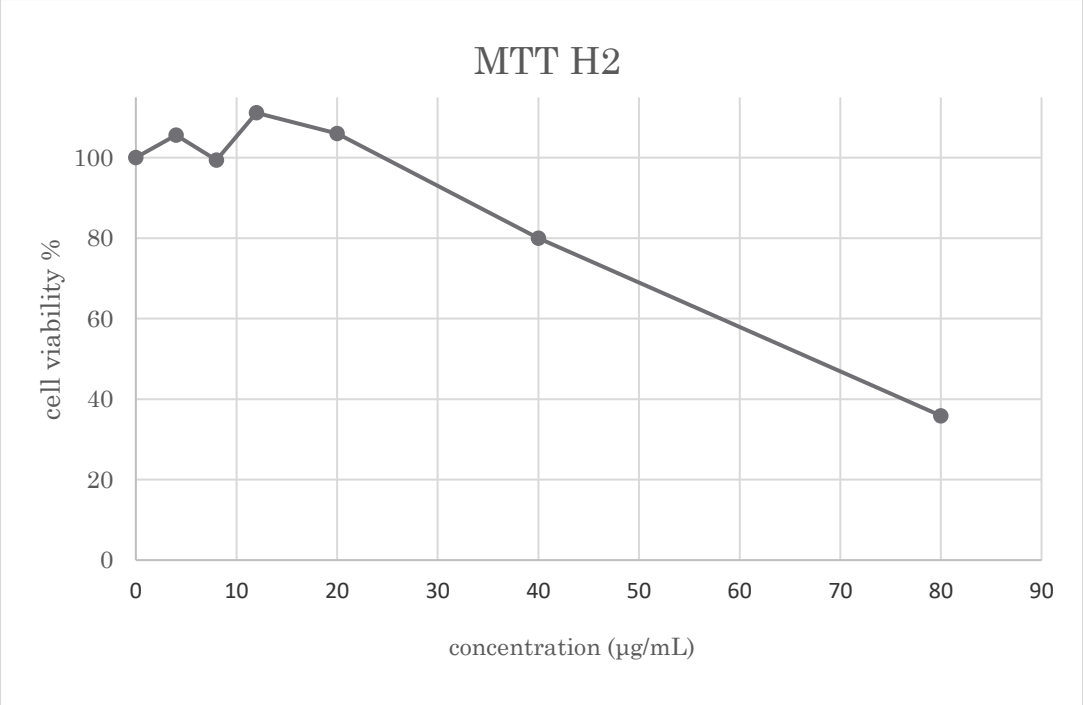
Figure 5. Citotoxicity of a) bombinin H2 and b) bombinin H4 on Vero cells



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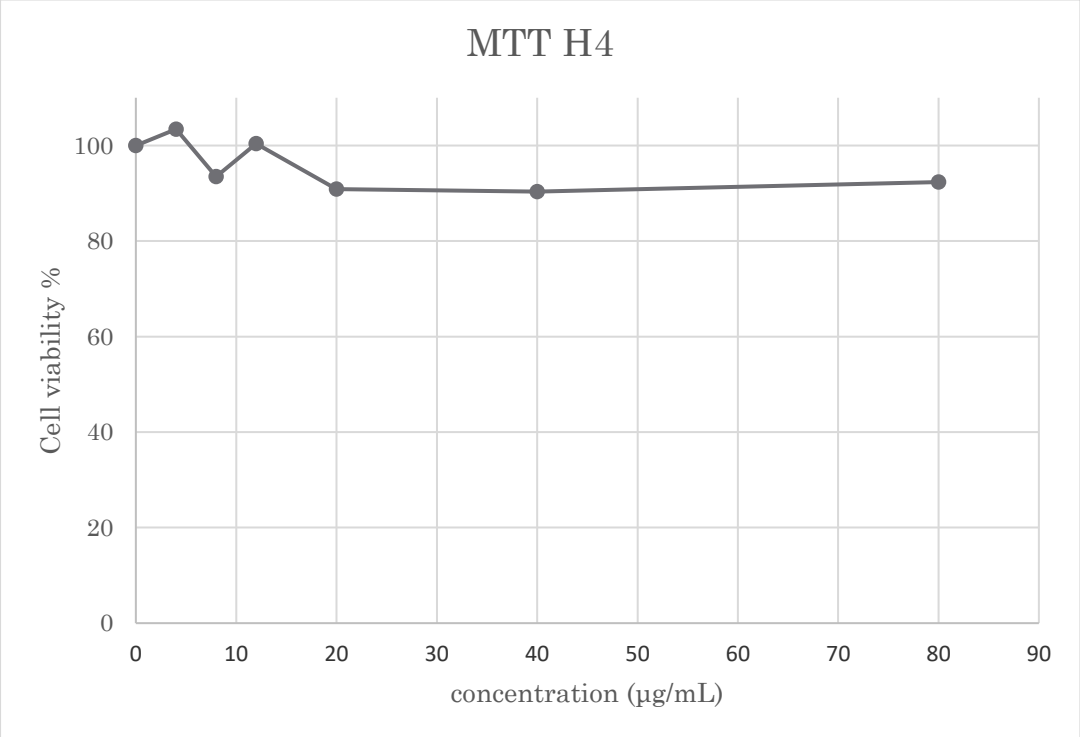


b)

Figure 6. Citotoxicity of a) bombinin H2 and b) bombinin H4 on A549 cells

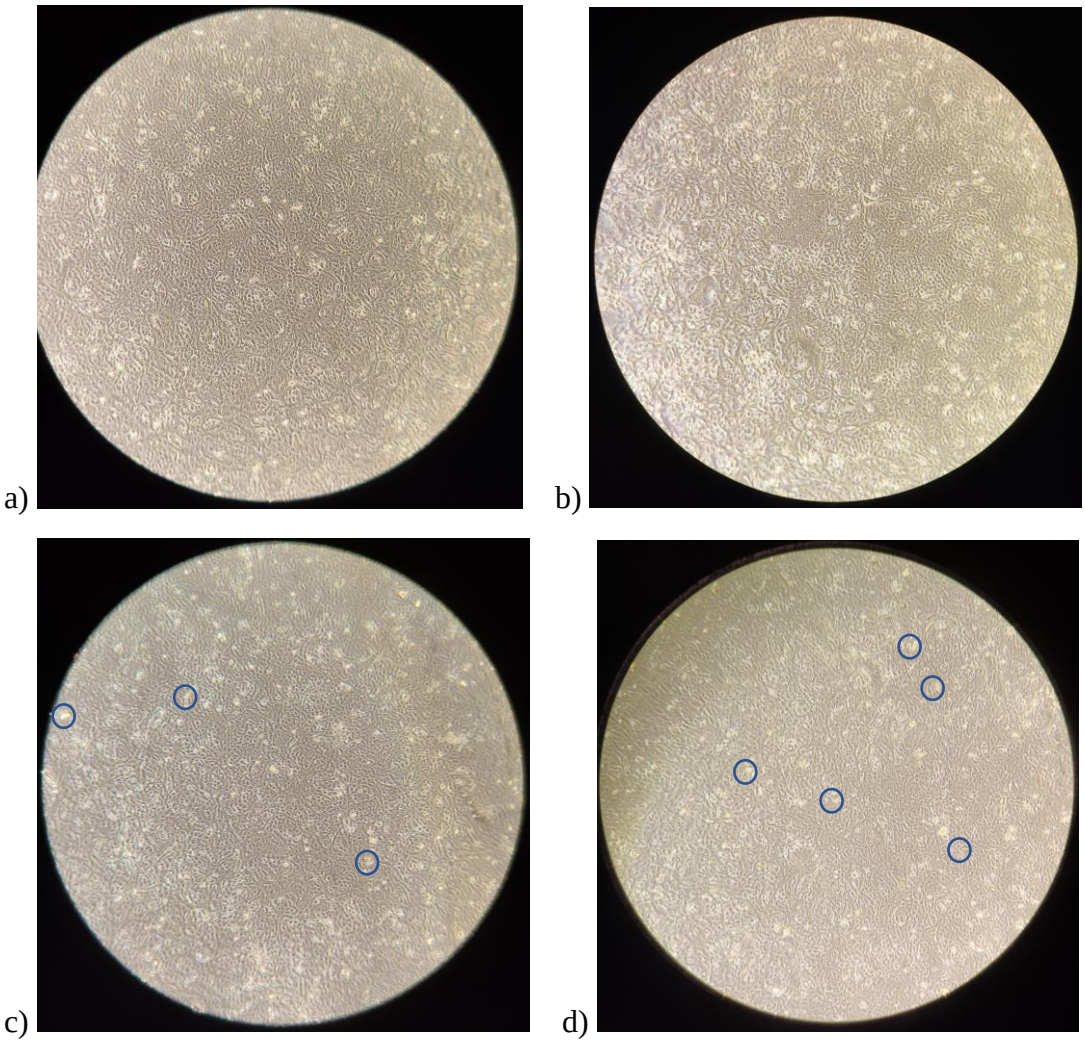


Figure 7. Pictures of experiment testing virucidal effect of bombinin H4 on HHV-1, tested in Vero cells, after 24h infection. a) control b) infection c) bombinin H4 10 μg/mL and d) bombinin H4 20 μg/mL. Yellow formation marked with blue circles.

4.2. Effect of peptides on HHV-1

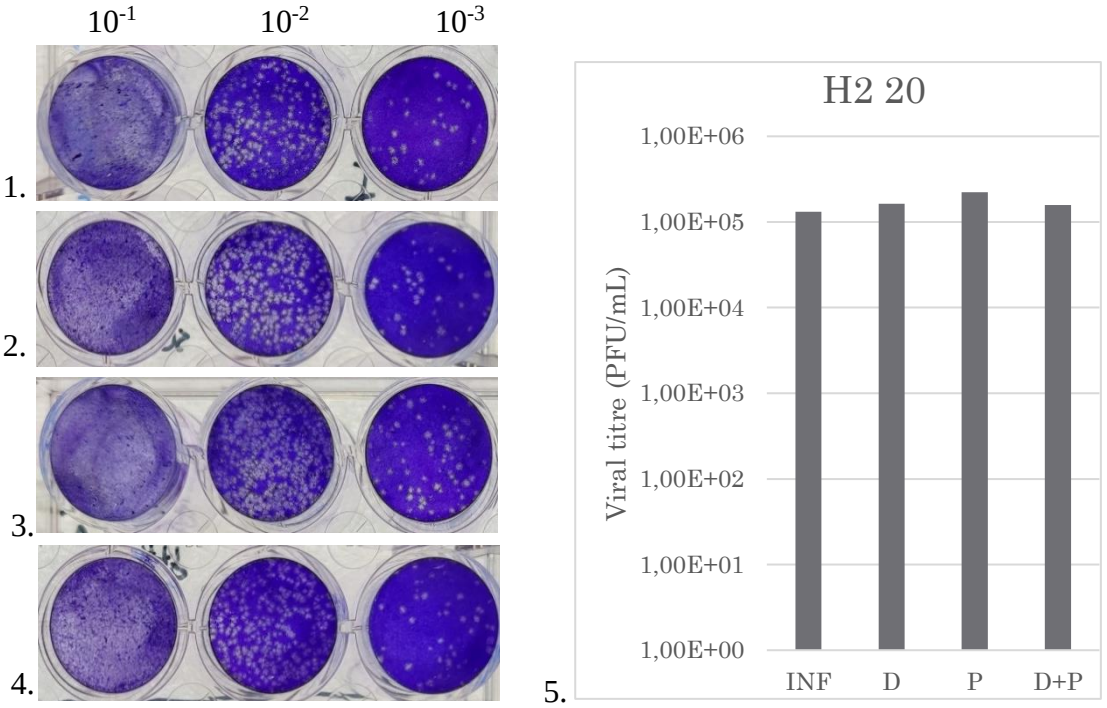
To test the potential antiviral effect of H2 and H4 against HHV-1, time of addittion assays were performed. To this aim, cells were infected and treated with H2 or H4 (10 and 20 µg/ml) at different times of infection: before (PRE), during (D) or after (P) the virus adsorption to the host cells or during and after (D+P, double dose) the adsorption phase and for the following 24 h.

In particular, to test is the substance working when it is added to the cells before the infection occurs, acting on some cellular receptors, the peptide was added to the cellular monolayers for 1-2 h in the fresh media containing 10% FBS. After which the medium was removed, and cells were washed and infected. Furthermore, to test do the peptides have an effect during the viral absorption phase, they were added to the cells at the same time as the virus and left for 1h, after which the cells were washed and the medium was replaced. In the case of testing effect in a post-adsorption phase, substance was added immediately after the adsorption phase, for 24 h. As a comparison the untreated virus was used as well as the uninfected cells.

At the end of each experimentation, supernatants were used to perform standard plaque assays. After which the plaques were counted and compared between infected and treated samples (Fig.8., 10., 12.). Cellular monolayers were pelleted, lysed and extracted proteins were analysed by Western blot (Fig.11., 13.).

As shown in Fig.8 and 9., the administration of H2 and H4 during- and/or post- adsorption phase doesn't reduce HHV-1 infection.

Moreover, to verify the potentially direct effect of bombinins on virions (a virucidal action), virus was preincubated with each peptide for 1 h at 37°C, and then the mixtures were used to infect the cells. After 24 h, supernatants were collected and viral titre was tested by standard plaque assay (Fig.13.). The cell monolayer was harvested and subjected to Western blot analysis of viral proteins. Surprisingly, the plaques count showed that bombinins reduce by 2 log the HHV-1 titre compared to the untreated infected cells. This strong reduction corresponds to 99% inhibition of HHV-1 replication, suggesting that bombinins act directly on viral particles. These data were also confirmed by the reduction of viral proteins in bombinin-treated infected samples compared to untreated HHV-1 samples.



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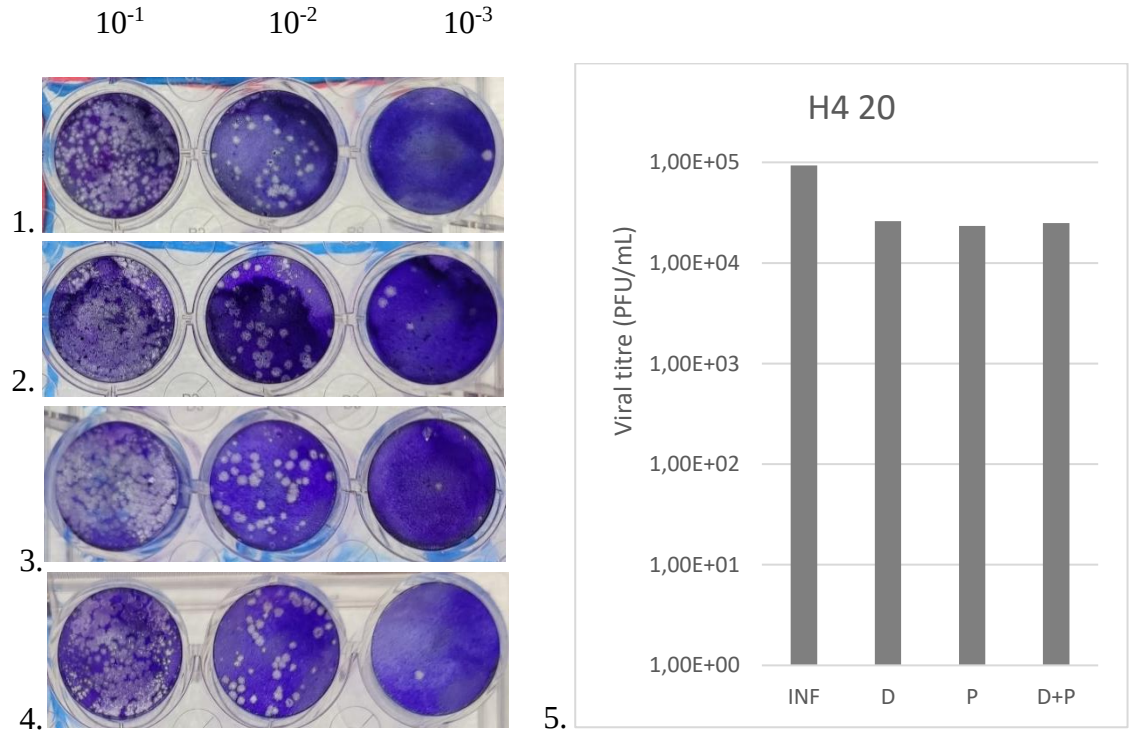
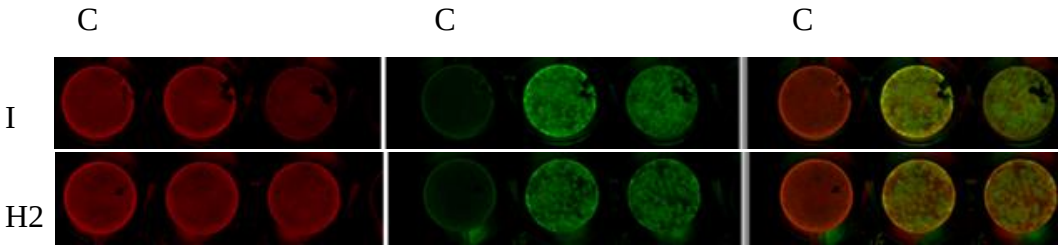


b)

Figure 8. Plaque assay on Vero cells with HHV-1. a) treatment is done with bombinin H2 and b) treatment is done with bombinin H4. In both cases: 1. Infected with HHV1 without treatment (INF), 2. treated with bombinin during adsorption phase (D), 3. treated with bombinin H4 post adsorption phase (P), 4. treated during adsorption phase and and post-adsorption phase (D+P), 5. results of plaque assay showed with a graph, y-axis is logaritmic.



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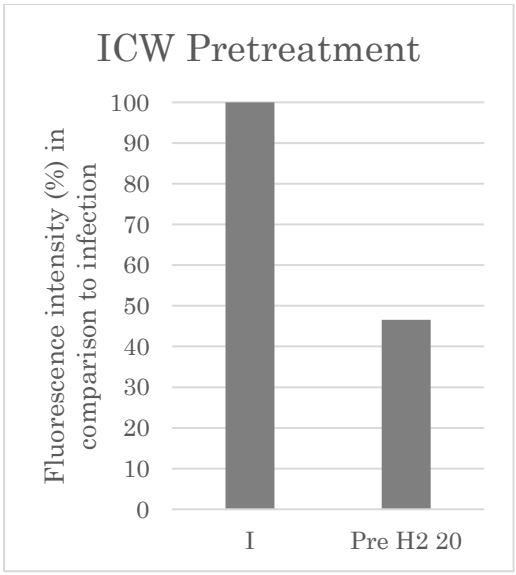
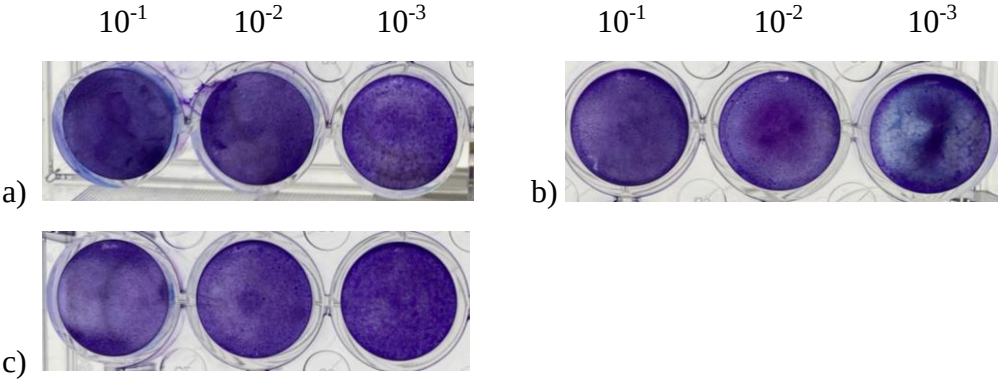


b)

Figure 9. ICW assay of pretreatment of Vero cells with bombinin H2 in dose of μg/mL. Results were obtained by dividing value of fluorescence intensity of viral protein GB (green) with Cell tag (red) followed by reduction of the value (calculated the same way) for the fluorescence value of control – to eliminate control influence on fluorescence. After which values of treated were divided by values of infected to present a percentage of infection

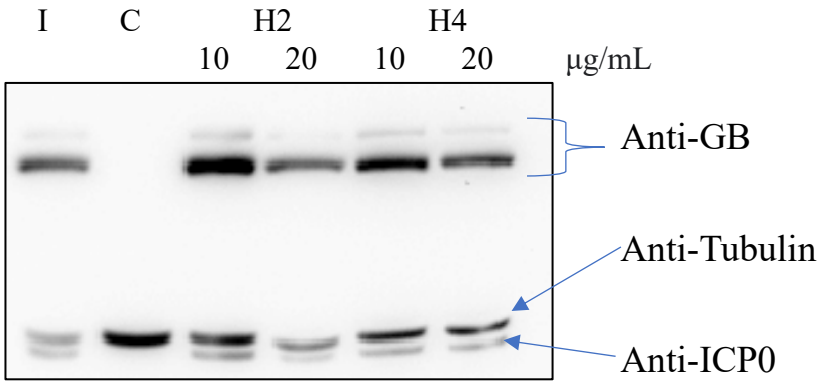


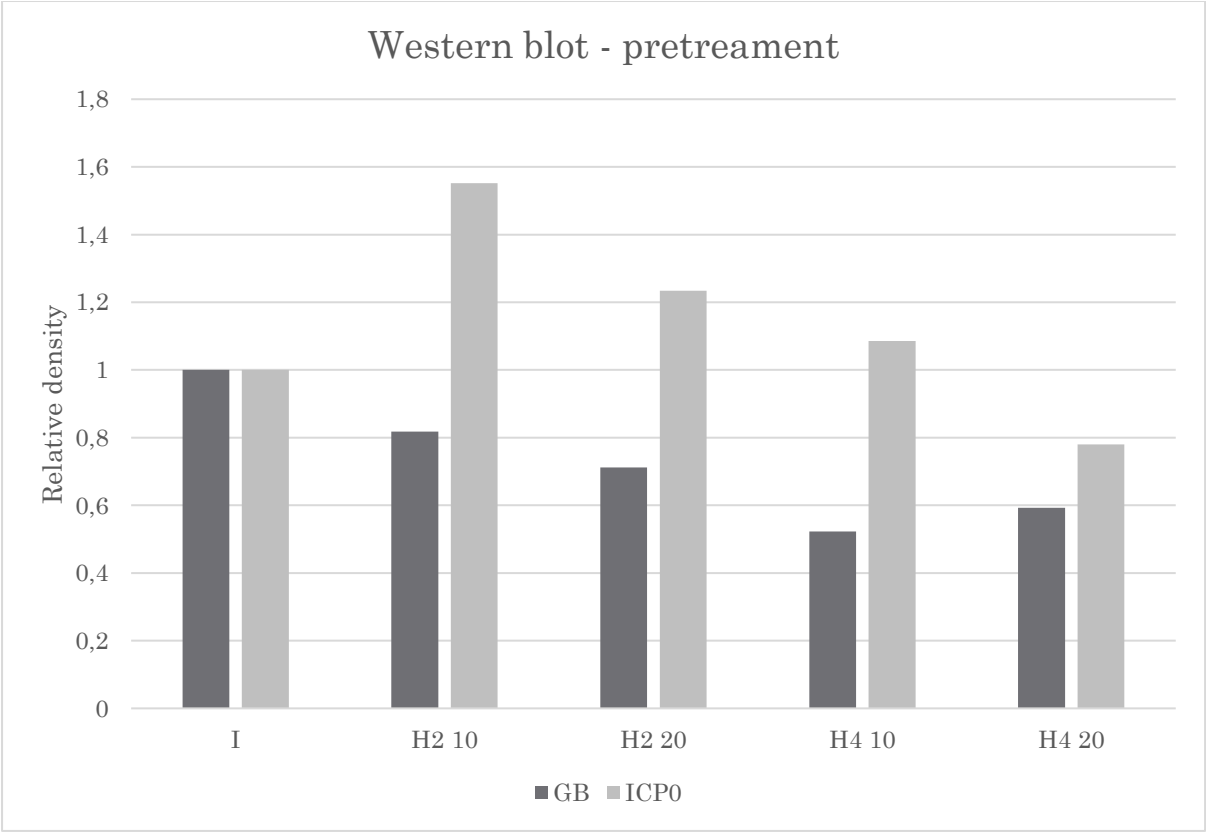
a) b)

c)

Figure 10. Standard plaque assay test of a) infection, b) pre-treated with bombinin H2 dose 20 μg/mL and c) pre-treated with bombinin H4 dose 20 μg/mL. No noticeable plaque reduction.

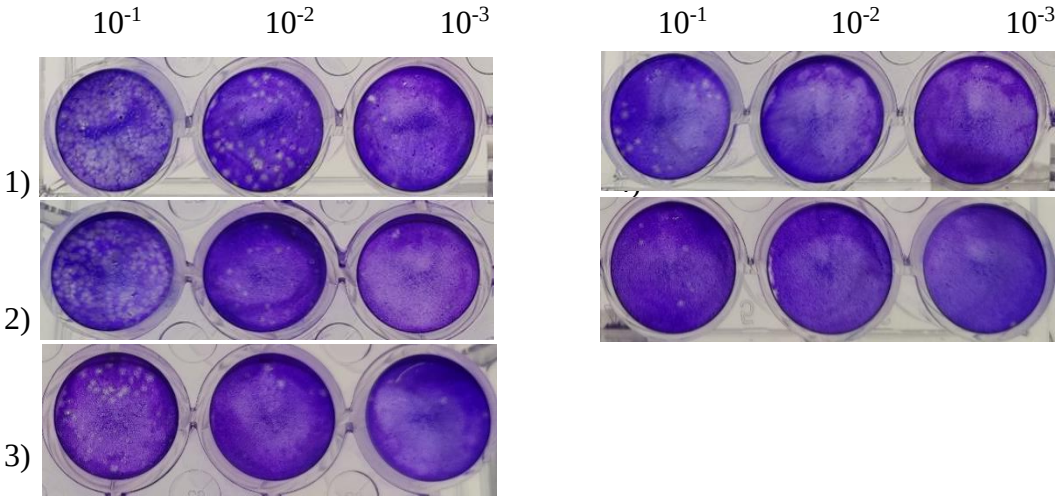
a)





b)

Figure 11. Western blot analysis on cells from experiment – pre-treatment of Vero cells with bombinin H2 and H4 2h before infection with HHV-1.



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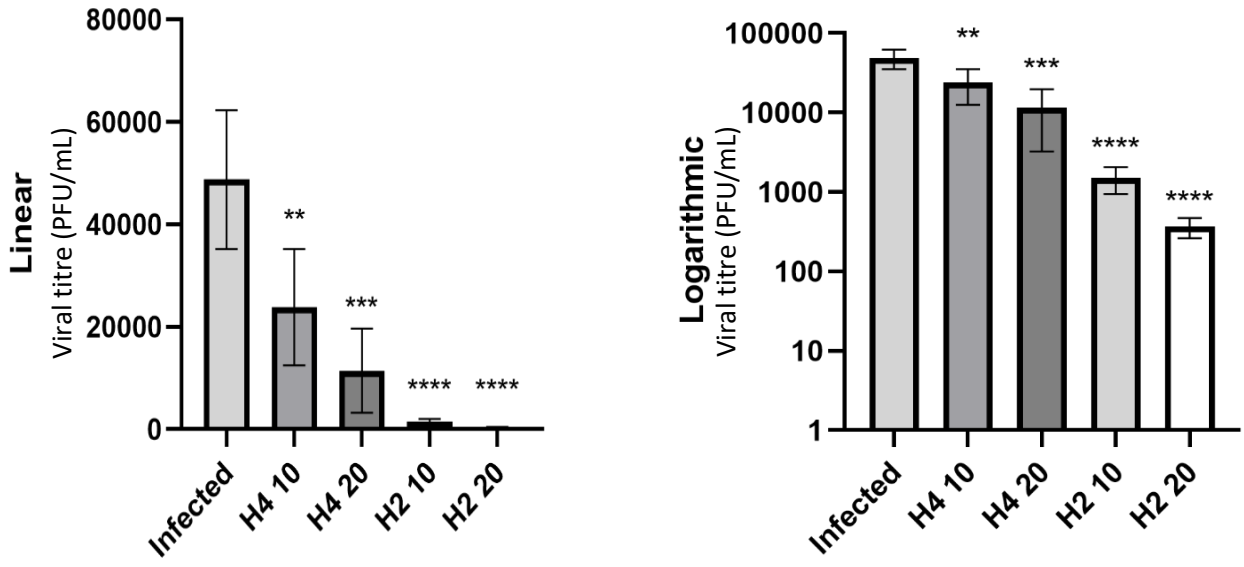
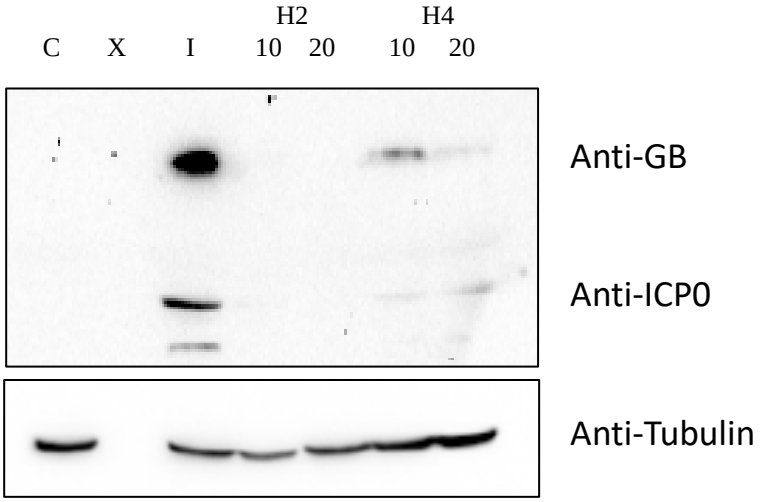


b)

Figure 12. Virucidal effect of bombinin H2 and H4 obtained with plaque assay: pictures of plaques showing 1. infected without treatment, 2. treated with H2 10 μg/mL, 3. treated with H2 20 μg/mL, 4. treated with H4 10 μg/mL, and 5. treated with H4 20 μg/mL; b) two graphs showing same data while on the left side y-axis is linear on the right side it is logarithmic to see a change in the number of viruses more precisely. Values of treated samples are compared to infected samples without treatment **p<0,01, ***p<0,001, ****p<0,0001



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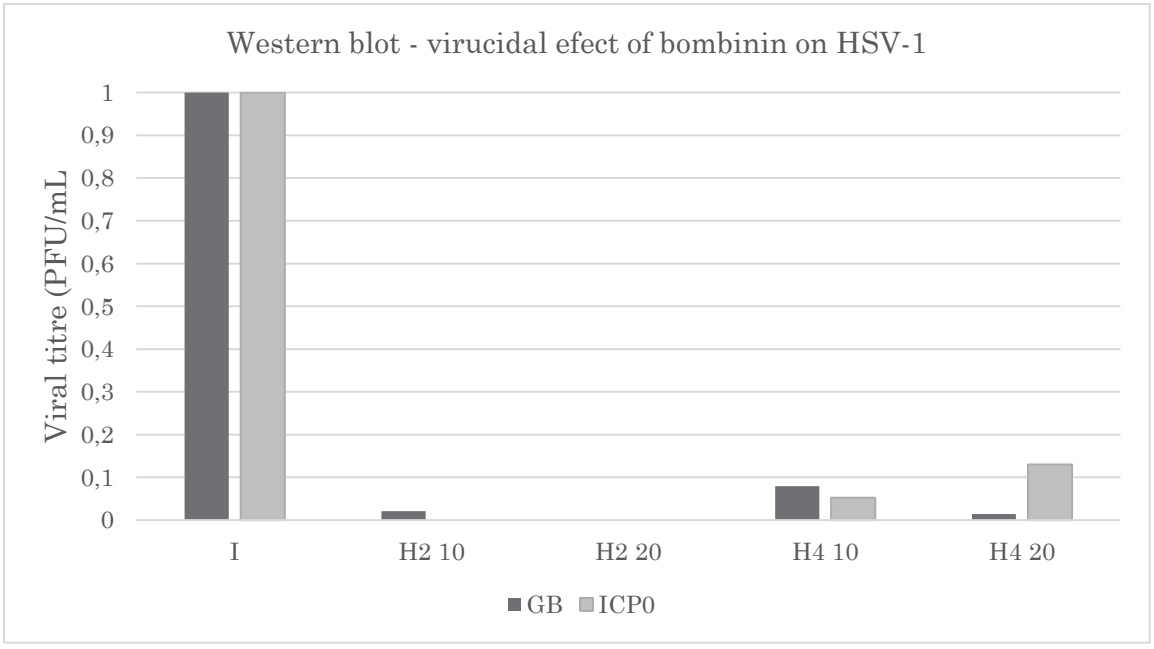

b)

Figure 13. Western blot analysis on cells from the experiment – Virucidal effect on HHV-1, Two viral proteins are shown – GB and ICP0 but also Tubulin a) picture obtained from ChemiDoc, b) Density analysed with QuantityOne. X was without any substance.

Interaction of AMPs – bombinin H2 and temporin G

Previous studies performed in our lab demonstrated that temporin G (Tg) reduces HHV-1 infection when the peptide is added during the viral adsorption phase (data not published). To study the potential synergic effect of bombinins and Tg administration on HHV-1 life cycle, cellular monolayers were infected with virus preincubated with bombinins and were also treated with Tg for 1 h during the virus adsorption to the host cells. After 24 h, supernatants were collected and used to perform a standard plaque assay (Fig.14.). The same experiment was directly repeated in a 96-well plate to perform an Odyssey assay (Fig.15., Fig.16.).

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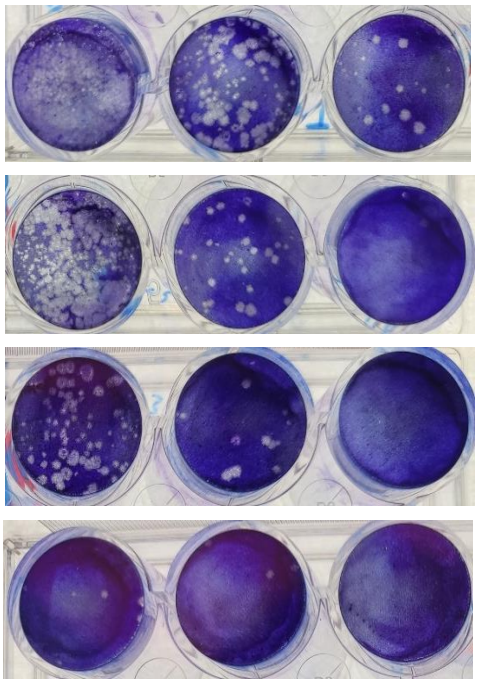

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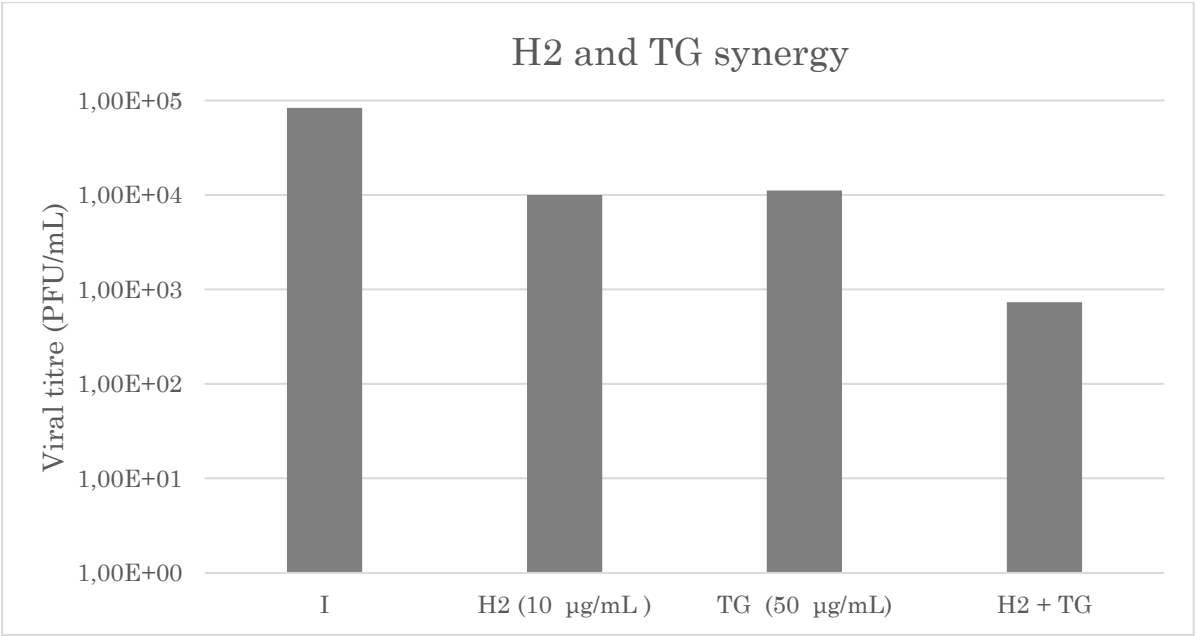
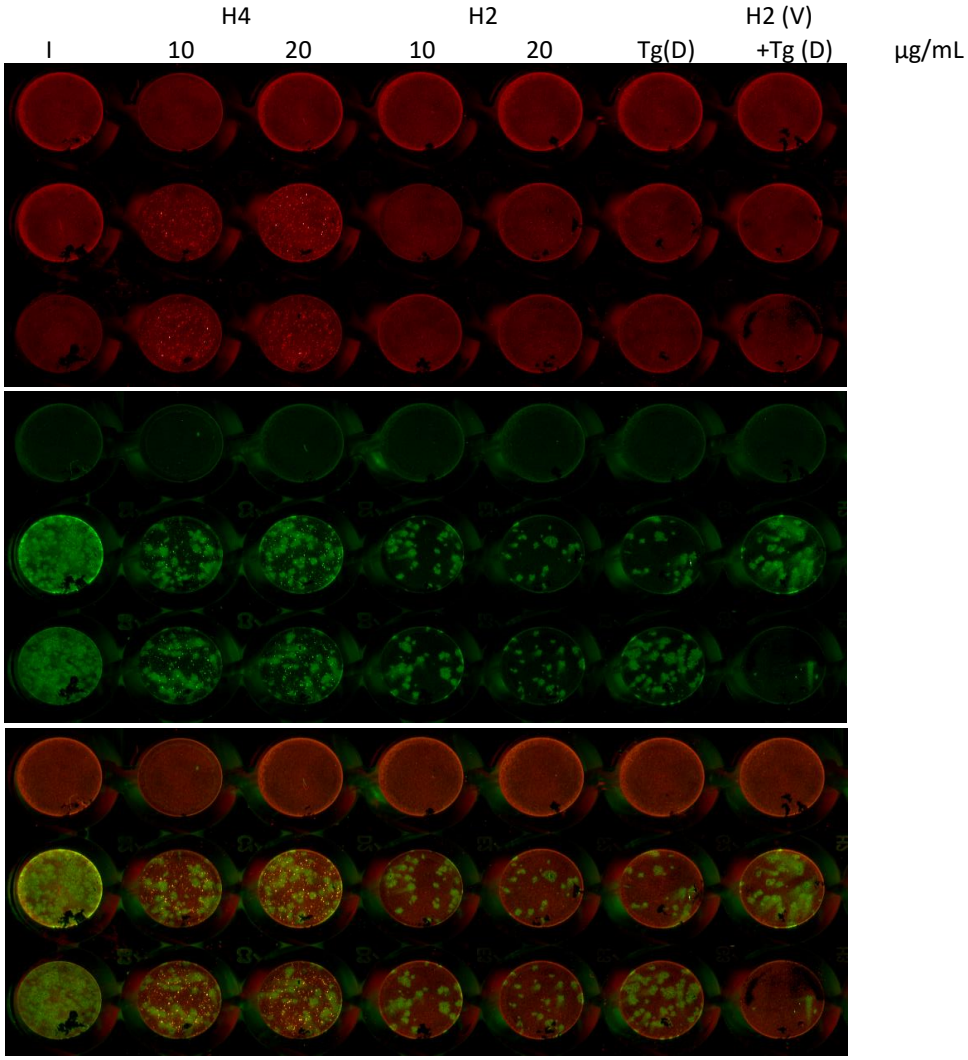


b)

Figure 14. Virucidal effect of bombinin H2 in comparison to treatment with Temporin g (Tg) during adsorption phase and their combination. a) pictures of plaque assay showing 1. Infection 2. Treated with bombinin H2 10 µg/mL 3. Treated with Tg 50 µg/mL 4. Treated with H2 and Tg (same doses as individually) b) graph made from same experiment describing data.



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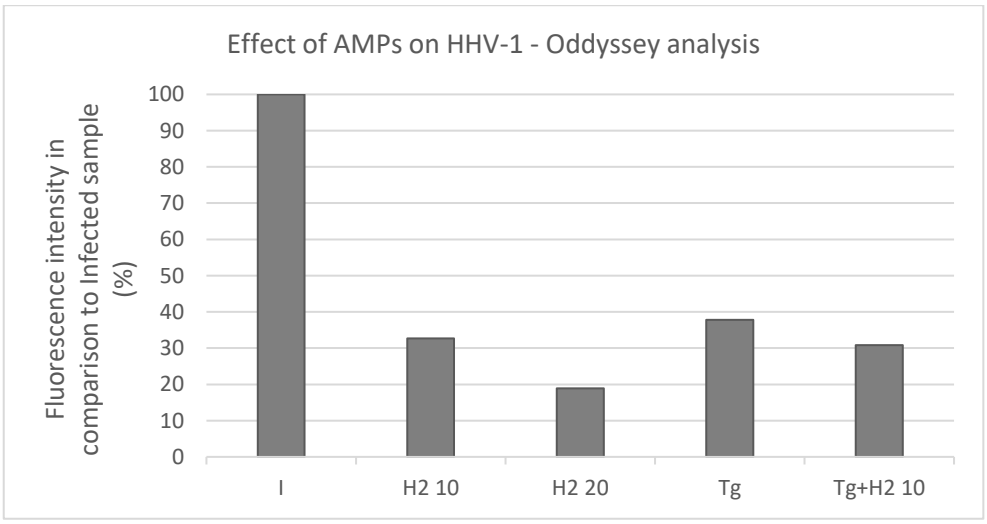


b)

Figure 15. ICW analysis of virucidal effect of bombinin H2 and H4 and effect of temporin G treated during absorption phase of infection directly in 96-well plate. a) picture of Odyssey assay showing fluorescent agent: 1. cell tag which shows density of a cell layer, 2. antibody against viral protein Gb and 3. combination of both. First row is control – cells were not infected nor treated, second and third in every column are in duplicate infected, or infected and treated. b) analysis of fluorescence intensity (doses in µg/ml)

4.2.2. Influenza virus

To conclude are bombinins H2 and H4 virucidal on other viruses than HHV-1, considering the need to improve the portfolio of antiviral molecules against respiratory viruses in the current pandemic of SARS-CoV-2, Influenza A virus was chosen for the experimentation. The influenza virus is an enveloped virus like HHV-1, but its genome is represented by segmented RNA with negative polarity.

Virucidal assays were performed by preincubating influenza virus (final virus concentration in DMEM medium was 1:100) with each bombinin (10 or 20 microg/ml) for 1 hour at 37°C. Then the mixtures were used to infect confluent monolayers of A549 cells. Twenty-four hours later, the cells and supernatants were collected to be used in subsequent Western blot (Fig.18.) and Odyssey assays (Fig.17.), respectively. During the infection, in the well in which virus was treated with bombinin H2 in the dose of 20 μg/mL it was noticed the disruption of monolayer in some zones and some cells were detached (Fig.16.). This is important to note since it could have affected the results in Odyssey assay and WB analysis later. In the case of bombinin H2 at the concentration of 10 μg/mL and bombinin H4 with both doses the cell layer was normal, unaltered, confluent.

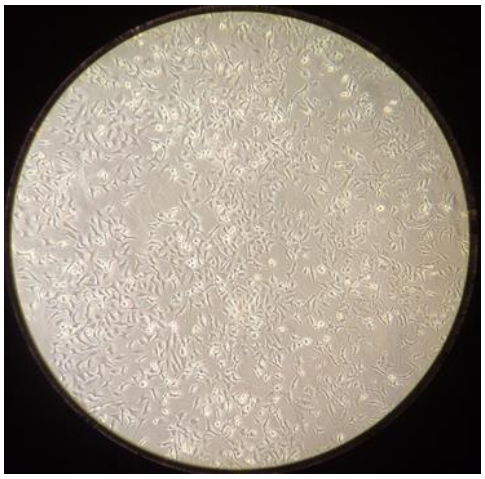
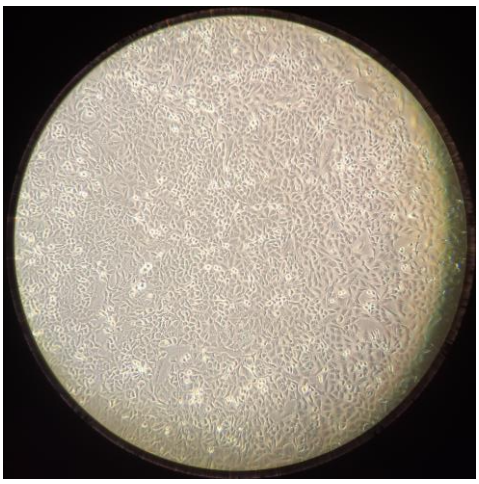
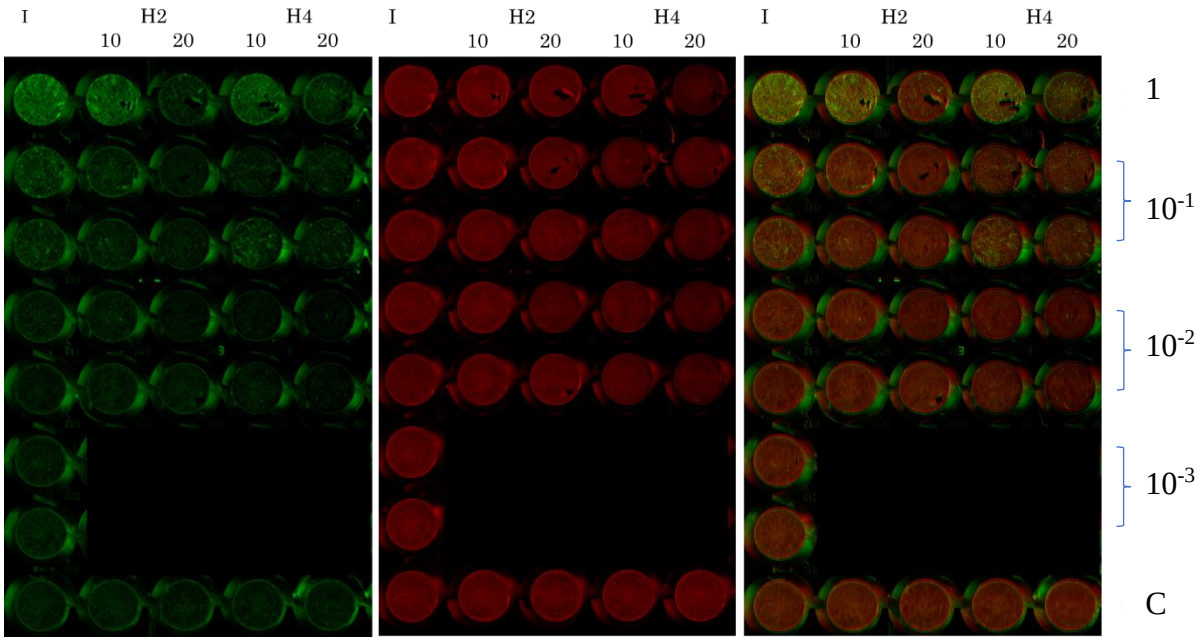
a)  b) 

Figure 16. From virucidal effect of bombinin H2 in a dose of 20 μg/mL on Influenza A virus showed on picture a). Number of A549 cells have detached in comparison to control, b).

Odyssey assays revealed a reduction of IAV infection, showed as HA fluorescence normalized on total cells, in samples treated with H2 at the concentration of 10 μg/ml. This result was confirmed by Western blot analysis, that shows a drastic decrease of HA expression as well as

other IAV proteins. Overall, the investigation demonstrated that bombinin H2 at a concentration of 10 µg/ml has a virucidal effect against influenza A virus.



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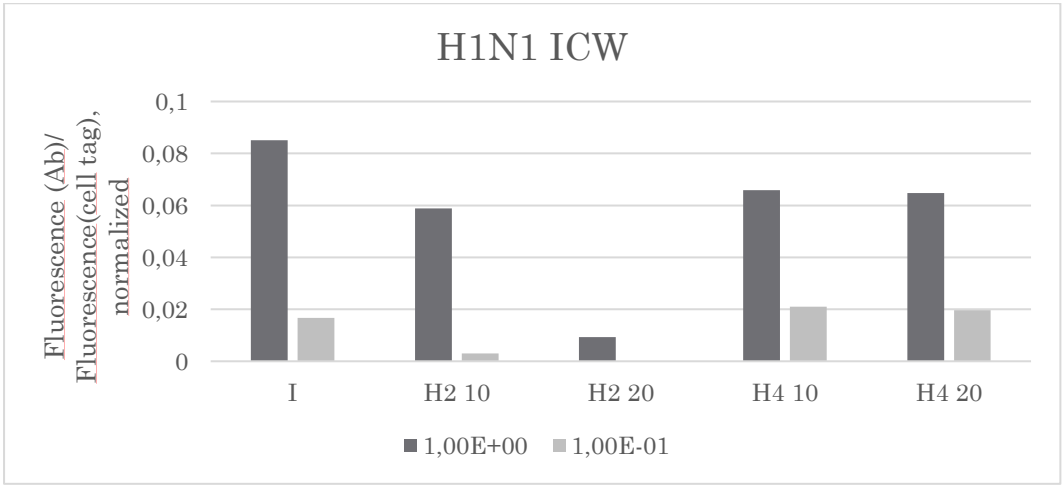
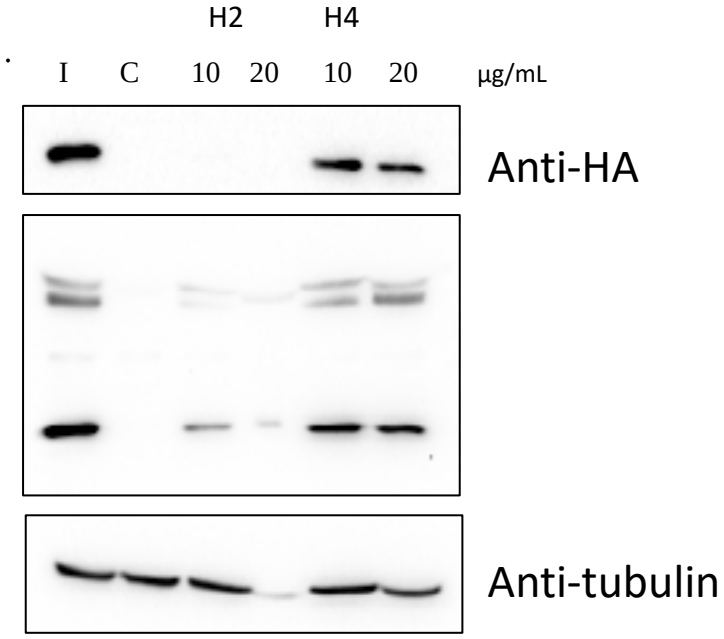


b)

Figure 17. ICW analysis on supernatant of experiment - virucidal effect of bombinin H2 and H4 on IAV H1N1. a) picture of Oddyssey assay showing fluorescent agent: 1. antibody against viral protein HA, 2. cell tag which shows density of a cell layer and 3. combination of both. Supernatant was diluted 10x for 2nd and 3rd row (in duplicate), 100x for 4th and 5th row, and in the case of infection 1000x 6th and 7th row, 8th row is non-infected, non-treated control.



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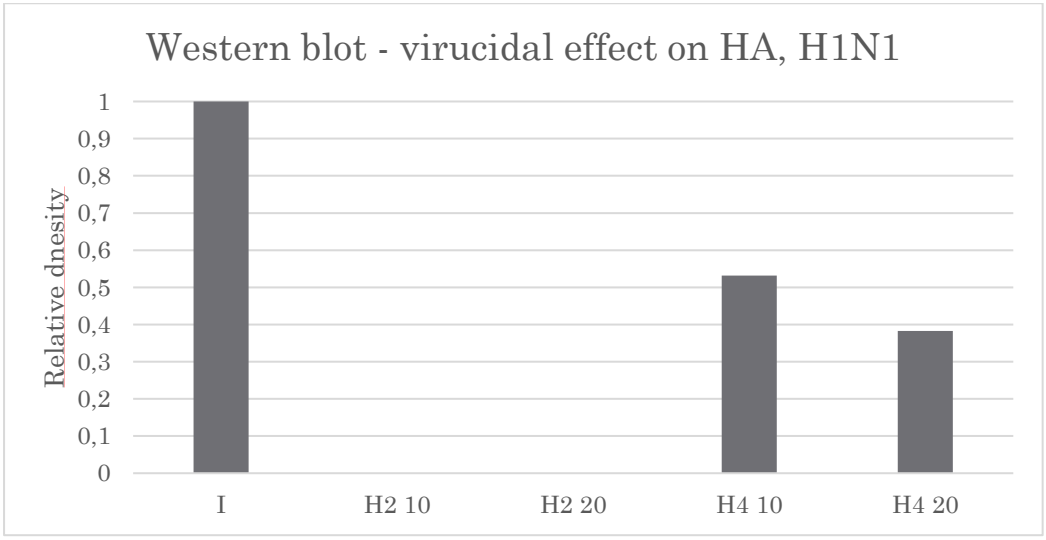


b)

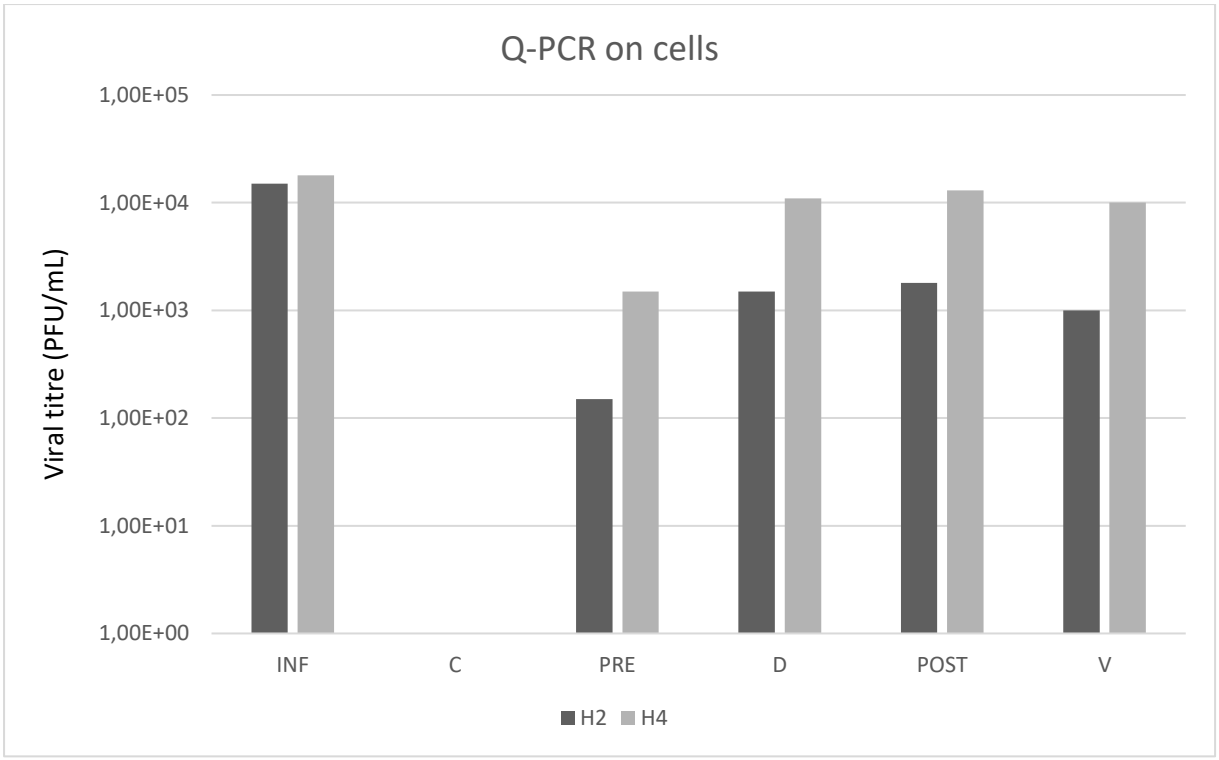
Figure 18. Virucidal effect of bombinins H2 and H4 on IAV H1N1 tested on A549 cells.

4.2.3. Polyomavirus

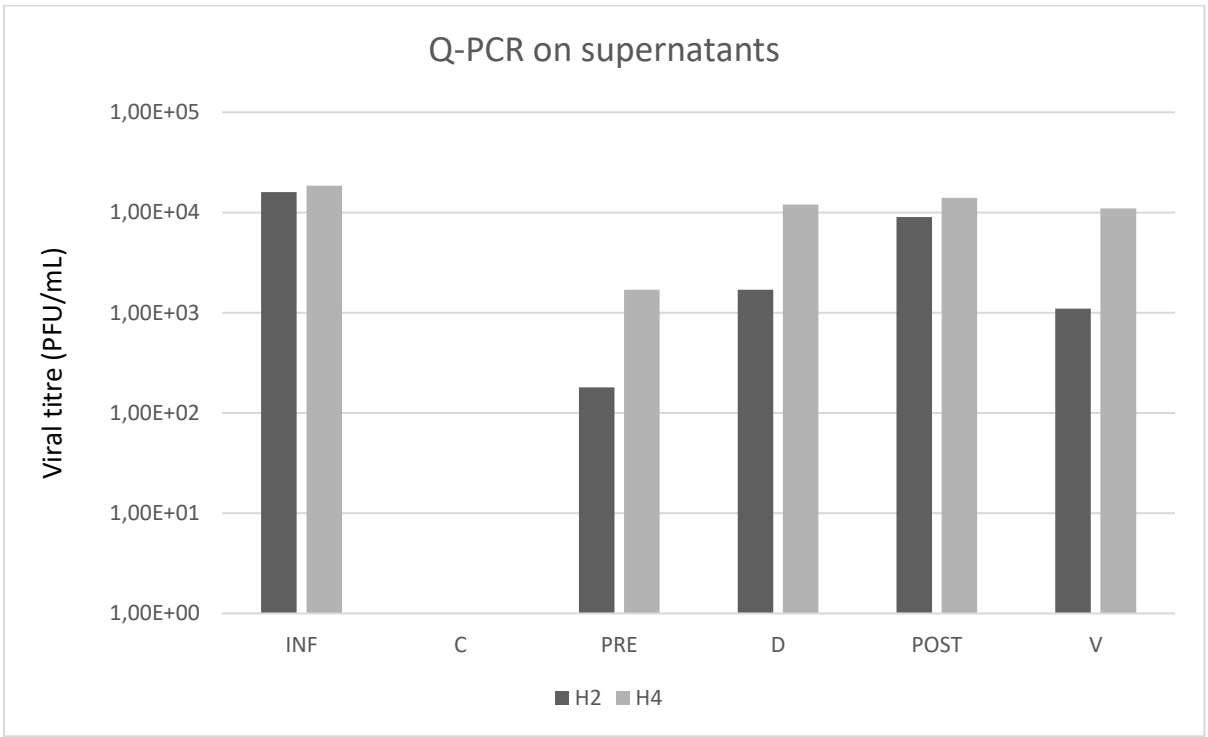
To verify the virucidal properties of bombinins on viral envelope, the next experimentation was addressed to test bombinins against a naked virus. To this aim, JC polyomavirus (JCPyV) was chosen.

SVGp12 cells, highly permissive and sensitive to JCPyV infection, were infected for 48 h with bombinins-pretreated virus, as described earlier for HHV-1 and IAV virucidal tests. To get a broad picture, time-of-addition assay was performed as on HHV-1. No cytotoxicity was observed during these experiments (data do not show). Collected supernatants and DNA extracted from cells were used for Q-PCR analysis. As shown in Fig.19., bombinin H2 at dose 20 μg/mL was surprisingly able to exert a virucidal effect (1-log reduction), even if in virology is not possible considerer a molecule as a good antiviral candidate when the virus reduction is < 2-log. This result prompts us to consider a possible interference of bombinin H2 with the viral capsid proteins, an alternative way to inhibit JCPyV infection than the typical formation of cationic pores on the viral envelope by frog peptides, as seen for temporin B (Marcocci et al, 2018). For this reason, the next step was to perform a time of addition assay, i.e., adding peptide before or during or after the adsorption phase (Fig.20.). Results show a 2-log reduction in pre-treatment of the cells with bombinin H2 at dose 20 μg/mL, and only 1-log reduction when bombinin H2 was administrated during or post the adsorption phase. These data indicate an important interaction between bombinin H2 and a cellular receptor. Other studies should be performed to well characterize this point.

Both the analysis on supernatants and on cells show similar results which is expected if the method is performed correctly.



a)



b)

Figure 19. Q-PCR analysis on a) cells and b) supernatants of testing of antiviral properties of bombinin H2 and H4 on JC Polyomavirus.

5. DISCUSSION

We are now in a new problematic era dealing with a pandemic of SARS-Cov-2 which suddenly shows how big of a problem actually viruses are. Every problematic situation should be used as a study material and as a big lesson which we can learn, and the biggest question is what should be the focus? What are the weak spots in the system? This situation can be used to show people how important research in the field of virology and it shows a possibility of innovative pharmaceutical approaches in antiviral therapy and vaccination. Furthermore, I think that it showed us that it is highly needed to work on the topic before it becomes a problem, in this situation we could see that it is very hard to do research in the middle of a crisis. Many other problems can arise from a pandemic and it is important to be prepared if possible.

Recent evidence highlights the function of antiviral proteinaceous compounds as a defensive barrier, and it is being demonstrated that some antimicrobial peptides may also present activity against a broad range of viruses, thus being called antiviral peptides (AVPs) (Chinchar et al., 2004; Falco et al., 2007; Crack et al., 2012).

These molecules can also be obtained through the utilization of bioinformatic tools which leads to designed or artificial AVPs. Artificial peptides can be tested for interaction against a specific viral target, such as a surface glycoprotein or an important enzyme (Okazaki and Kida, 2004); or obtained in silico using specific software designed for the prediction of peptides (Tiwari et al., 2011; Mooney et al., 2012). Many things are considered, such as the topology, amino acid composition, charge, and many other chemical and structural characteristics that may influence the antiviral activity of a peptide (Maccari et al., 2013; Sharma et al., 2014). The study of AVPs has been the focus of numerous research projects in recent years, and the structures and mechanisms of action of such molecules have been previously reviewed and even compiled in online databases such as the antiviral peptide database As with most molecules, a database was made (AVPdb) which contains 2683 entries of experimentally tested peptides (Barlow et al., 2014; Mulder et al., 2013; Qureshi et al., 2014) . Regarding their mechanism of action (Fig.20.), these are mostly called virucidal when they act directly by inhibiting the viral particle; or by competing for the protein link site in the host cell membrane, interfering in their interaction and consequent adsorption (Galdiero et al., 2013). However, they may also act in other stages of the viral cycle, causing, for example, the suppression of viral gene expression (Qureshi et al., 2013; Zapata et al., 2016).

Skin secretions of the European frog *Bombina variegata* contain a family of hydrophobic peptides, called bombinins H, which probably play a role in the defence against microbes (Zangger et al., 2008). These peptides have been tested and showed prominent results on bacteria (Mangoni et al., 2006), but to my knowledge their effect on viruses has not been tested until now. It has been reported that H2 and H4 were more potent against Gram-positive than Gram-negative bacterial strains but also that bombinin H4 is more potent than H2. An interesting information is that membrane permeabilization experiments indicate that H2/H4 cause large damage at the membrane level, allowing for protein leakage (Mangoni et al., 2000). Generaly speaking the mostly recognized as the well-accepted mechanism to describe the action of cationic AMPs is the membrane permeability. These cationic AMPs can bind and interact with the negatively charged bacterial cell membranes, leading to the change of the electrochemical potential on bacterial cell membranes, inducing cell membrane damage and the permeation of larger molecules such as proteins, destroying cell morphology (Lei et al., 2019). Interaction between AMPs and eukaryotic cell membranes (causing toxicity to host cells) are possible and it would limit their clinical application. But it is important to note that the prokaryotic and eukaryotic cell membranes have different structures and functions. Inherent distinction in lipid composition provides a basis for the preference of AMPs toward a specific membrane and provides researchers with an opportunity to find or design peptides with strong specificity. Prokaryotic membranes have a high negative net charge and are predominantly composed of phosphatidylglycerol (PG), cardiolipin (CL), or phosphatidylserine (PS). In contrast, mammalian membranes are enriched in the zwitterionic phospholipids (neutral net charge) phosphatidylethanolamine (PE), phosphatidylcholine (PC) or sphingomyelin (SM) (Yeaman and Yount, 2003; Giuliani et al., 2007). Moreover, the mammalian cell membrane contains cholesterol, while the prokaryote membrane does not (Tytler et al., 1995). It has been reported that cholesterol can dramatically reduce the activity of AMPs by stabilizing the lipid bilayer or by directly interacting and neutralizing AMPs (Matsuzaki, 1999).

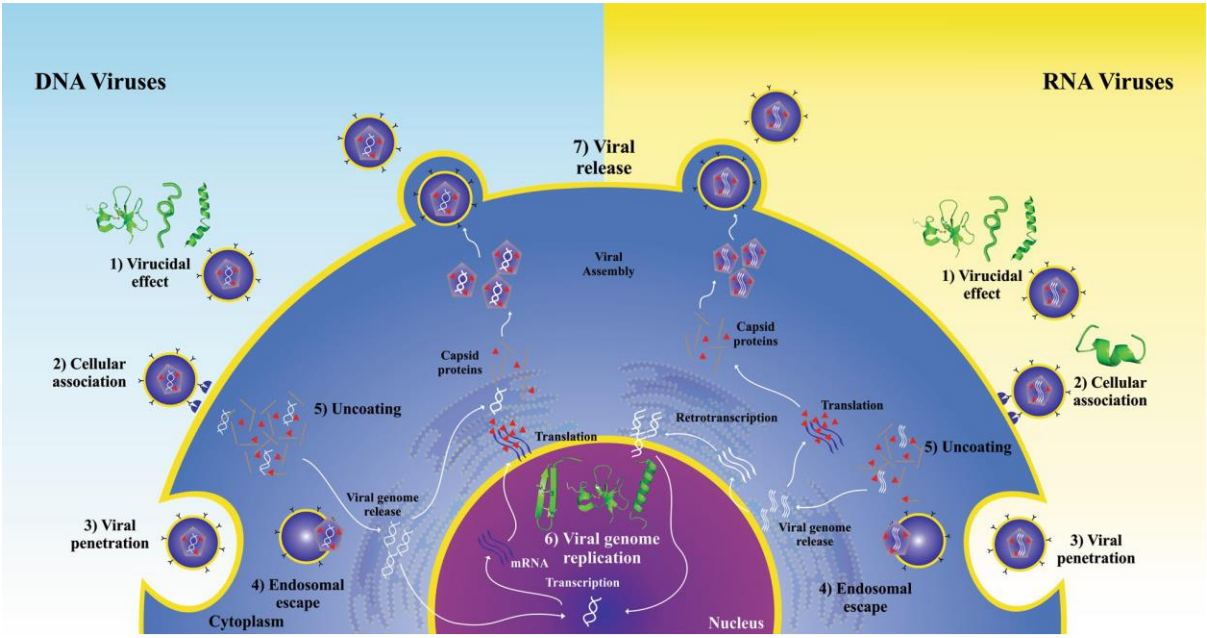


Figure 20. Antiviral peptide inhibition sites on viral replication cycle. The antiviral peptides with a described mechanism of action were placed in their inhibition sites as follows: 1) virion inhibition, 2) adsorption, 3) viral penetration, 4) endosomal escape, 5) viral uncoating, 6) viral genome replication and 7) release of mature virions, from (Vilas Boas et al., 2019)

In this thesis, MTT assay was used to evaluate the effect of bombinin H2 and H4 on cell viability. It showed that administration of the bombinin H2 at concentrations ≤20 µg/ml induces approximately less than 5% cell death in Vero and A549 cells. In the case of bombinin H4 it showed more than 20% decrease in cell viability even at the dose of 10 µg/ml on Vero cells but cell viability of A549 cells was not changed much. In the higher concentrations of bombinin H2 a decrease in cells viability was noticed. However, in contrast to these results, sometimes we observed that Bombinin H2 shows a higher cytotoxicity on A549 cells. In an earlier publication on normal lung cells and cancer lung cells showed that when bombinin H2 was applied to the A549 cell line, a significant cell death has been observed between the untreated cells and the treated cells at concentrations ranging from 12.5 µM to 50 µM ($p \leq 0.05$). Furthermore, bombinin H4 proved to be highly toxic to the A549 cell line after 24-hour exposure, with significant cell death being observed from 1.5 µM to 100 µM ($p<0.05$). Interestingly, whilst bombinin H4 did not produce a cytotoxic effect on erythrocytes, levels of haemolysis were significantly less than untreated cells ($p<0.001$), suggesting that bombinin H4 may have a protective effect on erythrocytes viability (Swithenbank et al., 2020).

Contrary to this publication, results of this thesis have shown that CC50 for bombinin H4 is higher than for H2 which leads to a conclusion that bombinin H4 is less toxic what is contrary to earlier reported results. Interesting to add is that results with bombinin H4 gave an

unpredictable trend line which leads to an opinion that it can create problems in use, the substance must be well predictable, stable and reliable. These outcomes indicate that it should be tested again on human lung cells but also on more types of cells to have a clearer picture.

Different doses were tried out according to the MTT assay and doses 10 and 20 μg/mL were chosen of which the dose of 20 μg/mL was the most efficient. In the case of HHV-1 the most significant effect was a virucidal effect. It can be speculated that bombinin H2 acts directly on viruses' envelope, disrupting it and killing the virus. This hypothesis has to be backed up by other experiments (e.g. electron microscopy test) and results. It was chosen to work with another two viruses, one enveloped (IAV) and the other non-enveloped (JCPyV). A strong virucidal effect was noticed against IAV This may be a confirmation that a key target in antiviral mechanism of bombinin is envelope. However, these experiments showed some aspect of safety problems. During the experimentation, detachment of cells was noticed when treated with H2, dose 20 μg/mL, suggesting that bombinins-damaged virus releases enzymes and peptides which could be toxic for the cell. These tests were conducted on A549 cells which are adenocarcinomic human alveolar basal epithelial cells and it would be important in further research to check how does the peptide act on normal human lung cells. Detachment could have influenced the results to be more reduced than they really are, but it can be easily seen that a high virucidal effect can be spotted also in lower dose of H2, and some reduction in the case of H4 without detachment in all cases. This confirms the similarity in antiviral activity of bombinins H2 and H4 between HHV-1 and Influenza A virus.

To verify other bombinins' action mechanisms and/or targets, time-of-addition assays were performed, and no significant decrease of HHV-1 infection was observed when bombinins were administrated before, during, post or during and post adsorption phase. On the contrary, pretreatment of cells with bombinin H2 reduces infection with JCPyV for 2-log, suggesting a possible interaction between peptide and cellular receptors. In fact, if a substance acts in pre-infection treatment it could point to a mechanism of blocking cell receptors which doesn't allow virus to bind to it. Consequently, replication of the virus and infection rate is reduced which leads to a decrease in a total count of virus and viral DNA. It was recognized that a specific JCPyV recognition motif, present on host glycoproteins and glycolipids, is a linear sialylated pentasaccharide, also called lactoseries tetrasaccharide c (LSTc), with the sequence NeuNAc-α2,6-Gal-β1,4-GlcNAc-β1,3-Gal-β1,4-Glc. The crystal structure of the JCPyV capsid protein VP1 was solved alone and in complex with LSTc. It discloses extensive

interactions with the terminal sialic acid of the LSTc motif and specific recognition of an extended conformation of LSTc (Fig.21.) Furthermore, mutations in the JCPyV oligosaccharide binding sites abolish cell attachment, viral spread, and infectivity, which further confirms the importance of this interaction (Neu et al., 2010; Tsai and Inoue, 2010).

As it was mentioned in the introduction of this thesis the structure of bombinin H resembles the influenza hemagglutinin fusion peptide (Han and Tamm, 2000) and the helical conformer of Alzheimer peptide Ab (Shao et al., 1999). A very important information to highlight is the fact that influenza hemagglutinin peptide recognizes the sialic acid (SIA) receptors expressed on the surface of host cells in the respiratory tract and is responsible for viral attachment and entry. The connection between HA binding SIA, bombinin H2 possessing properties of HA, also bombinin acting in the pre-infection treatment and the fact that a key receptor for JCPyV is a glycoprotein with SIA imposes the idea that bombinin H2 could block cell LSTc receptors by binding to sialic acid. This hypothesis could justify the 1-log reduction of JCPyV infection during virucidal test with H2, probably due to the ability of the peptide to bind the cell receptor quicker than viruses. On the other hand, the possibility that H2 binds the protein component of the JCPyV capsid cannot be excluded. Further studies are needed.

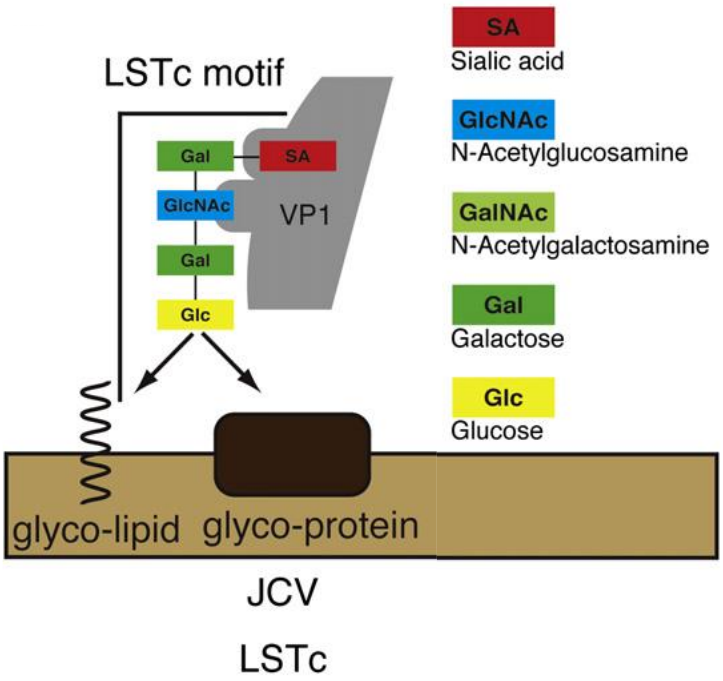


Figure 21. Picture is showing LSTc motif important for binding of JCPyV with VP1.

6. CONCLUSION

Results of experiments confirmed an antiviral effect of peptides found in frog skin; bombinin H2 and bombinin H4. With different methods results confirmed the hypothesys.

- bombinin H2 and H4 were used in doses of 10 and 20 μg/mL which do not show toxic effect on Vero cells
- used doses of bombinin H4 did not show toxic effect on A549 cells
- some cytotoxicity was noticed on A549 cells while virucidal effect was performed with 20 μg/mL of bombinin H2
- dose of 10 μg/mL of bombinin H2 did not show any toxicity
- bombinin H2 and H4 have virucidal effect on enveloped viruses HHV-1 and IAV H1N1
- bombinin H2 has a stronger virucidal effect than H4
- 20 μg/mL of bombinin H2 causes 99% decrease of infection when HHV-1 is treated directly
- no significant decrease of HHV-1 infection was observed when used during, post or during and post adsorption phase, nor when used as pretreatment
- natural peptides, bombinin H2 (added in virus solution 1h before infection) and temporin Tg (added during adsorption phase), in the doses of 10 and 50 μg/mL, respectively, have additive effect reduction of HHV-1 infection
-cells pretreated with 20 μg/mL of bombinin H2 resolve in 99% reduction of JCPyV infection

To our knowledge this is a first report on antiviral activity of bombinin H2 and H4.

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8. SUMMARY

Antiviral effect of antimicrobial peptides bombinin H2 and H4 originated from frog skin (Bombina variegata) was tested on enveloped, dsDNA, Human aplhaherpesvirus-1 (HHV-1) and for comparison other viruses were used to understand the mechanism of peptides better, including H1N1 influenza virus which is an enveloped RNA virus and JC Polyomavirus which is non-enveloped dsDNA virus. MTT assay was used to test possible cytotoxicity of bombinin H2 and H4 as well as to choose appropriate concentrations for further experiments. To test antiviral effect of bombinin H2 and H4 on HHV-1 time of addition assays were performed. Cells were infected and treated with H2 or H4 (10 and 20 μg/ml) at different times of infection: before (PRE), during (D) or after (P) the virus adsorption to the host cells or during and after (D+P, double dose) the adsorption phase and for the following 24 h. Moreover, to verify the potentially direct effect of bombinins on virions (a virucidal action), virus was preincubated with each peptide for 1 h at 37°C, and then the mixtures were used to infect the cells. From these experiments cells were collected and later used for Western blot and supernatants later used for plaque assay. Previous studies performed in our lab demonstrated that temporin G (Tg) reduces HHV-1 infection when the peptide is added during the viral adsorption phase (data not published). To study the potential synergic effect of bombinins and Tg administration on HHV-1 life cycle, cellular monolayers were infected with virus preincubated with bombinins and were also treated with Tg for 1 h during the virus adsorption to the host cells. After 24 h, supernatants were collected and used to perform a standard plaque assay. The same experiment was directly repeated in a 96-well plate to perform an Odyssey assay. To conclude are bombinins H2 and H4 virucidal on other viruses than HHV-1 Influenza A virus was tested in the same manner as for HHV-1 but on A549 cells. Instead of plaque assay used method was ICW and cells were used for Western blot. To verify the virucidal properties of bombinins on viral envelope, the next experimentation was addressed to test bombinins against a non-enveloped virus. To this aim, JC polyomavirus (JCPyV) was chosen. SVGp12 cells, were infected for 48 h with bombinins-pretreated virus, as described earlier for HHV-1 and IAV virucidal tests. To get a broad picture, time-of-addition assay was performed as on HHV-1. Collected supernatants and DNA extracted from cells were used for Q-PCR analysis. Bombinin H2 and H4 were used in doses of 10 and 20 μg/mL which do not show toxic effect during MTT assay. Results obtained from plaque assay and Western blot show virucidal effect of bombinin H2 in doses of 10 μg/ml ($p<0,001$) and 20 μg/ml ($p<0,0001$) μg/ml and H4 in doses of 10

µg/ml ($p<0,01$) and 20 µg/ml ($p<0,001$) on HHV-1. Bombinin H2 shows stronger virucidal effect of about 2-log decrease in comparison to infection which corresponds to 99% inhibition of HHV-1 replication. Bombinin H2 added directly to HHV-1 in dose of 10 µg/ml shows additive effect with temporin G added in the dose of 50 µg/ml during adsorption phase. Results from Western blot and ICW show that bombinin H2 is virucidal on H1N1 influenza virus. Results obtained from Q-PCR show that bombinin H2 reduces infection of JC Polyomavirus for 2-log if cells are pretreated with the peptide. Bombinin H2 shows 1-log reduction of infection if added in other stages of infection or directly to the JC Polyomavirus. Bombinin H4 does not show antiviral effect on JC Polyomavirus. To our knowledge this is a first report on antiviral activity of bombinin H2 and H4.

SAŽETAK

Antivirusni učinak antimikrobnih peptida bombinina H2 i H4, porijeklom iz kože žabe (Bombina variegata) testiran je na Herpes simplex virusu-1 (HHV-1), virusu s ovojnicom i dvolančanom DNA, a za usporedbu su korišteni drugi virusi za bolje razumijevanje mehanizma navedenih peptida, uključujući virus influence H1N1 koji je RNA virus s ovojnicom i JC Poliomavirus koji je dsDNA virus bez ovojnice. Potencijalni citotoksični učinak bombinina H2 i H4 ispitan je MTT testom. Na temelju rezultata MTT testa izabrane su koncentracije za pokuse u ovom istraživanju. Antivirusni učinak bombinina H2 i H4 na HHV-1 ispitan je "time-of-addition" testom. Naime, stanice su bile inficirane i tretirane s H2 ili H4 (10 i 20 μg/ml) u različito vrijeme infekcije: prije (PRE), tijekom (D) ili nakon (P) adsorpcije virusa na stanice domaćina ili tijekom i nakon (D +P, dvostruka doza) adsorpcijsku fazu i sljedeća 24 sata. Nadalje, radi provjere potencijalnog izravnog učinka bombinina na virione (virucidno djelovanje), virus je prethodno inkubiran sa svakim peptidom 1 sat na 37 ° C, a zatim su smjese korištene za inficiranje stanica. Iz ovih pokusa stanice su prikupljene i kasnije korištene za Western blot, a supernatanti su kasnije korišteni za standardno ispitivanje plakova. Prethodne studije provedene u našem laboratoriju pokazale su da temporin G (Tg) smanjuje infekciju HHV-1 kada se peptid doda tijekom faze adsorpcije virusa (podaci nisu objavljeni). Kako bi se proučio potencijalni sinergijski učinak primjene bombinina H2 i Tg na životni ciklus HHV-1, stanični monoslojevi inficirani su virusom prethodno inkubiranim bombininima, a također su tretirani s Tg-om 1 sat tijekom adsorpcije virusa na stanice domaćina. Nakon 24 sata, supernatanti su prikupljeni i korišteni za izvođenje standardnog testa na plak. Isti pokus je izravno ponovljen na ploči s 96 jažica radi izvođenja Unutar staničnog-Western blota (ICW). Peptidi su zatim testirani na H1N1 virusu influence A isti način kao i na HHV-1, ali na stanicama A549. Umjesto testa plaka korištena je metoda ICW, a stanice su korištene za Western blot. Kako bi se potvrdila virucidna svojstva bombinina na ovojnici virusa, sljedeći je eksperiment bio usmjeren na ispitivanje bombinina na virus bez ovojnice. U tu svrhu odabran je JC poliomavirus (JCPyV). Stanice SVGp12, bile su zaražene 48 sati virusom prethodno tretiranim bombininima, kako je ranije opisano za virucidne testove HHV-1 i IAV. Kako bi se dobila šira slika i bolji pregled informacija, provedeno je ispitivanje "time-of-addition" na isti način kao na HHV-1. Prikupljeni supernatanti i DNA, ekstrahirana iz stanica, korišteni su za Q-PCR analizu. Bombinin H2 i H4 korišteni su u dozama 10 i 20 μg/mL koje nisu pokazale toksični učinak tijekom MTT testa. Rezultati testa plaka i Western blot pokazuju virucidni učinak bombinina H2 u dozama od 10 μg/ml ($p < 0,001$) i 20 μg/ml ($p < 0,0001$) μg/ml i H4 u

dozama od 10 µg/ml ($p < 0,01$) i 20 µg/ml ($p < 0,001$) na HHV-1. Bombinin H2 pokazuje snažniji virucidni učinak od oko 2 log smanjenja u usporedbi s infekcijom koja odgovara 99% inhibiciji replikacije HHV-1. Bombinin H2 dodan izravno na HHV-1 u dozi od 10 µg/ml pokazuje aditivni učinak s peptidom temporinom G u dozi od 50 µg/ml tijekom faze adsorpcije. Rezultati Western blota i ICW-a pokazuju da bombinin H2 ima virucidan učinak na H1N1 virus influence. Rezultati dobiveni Q-PCR-om pokazuju da bombinin H2 smanjuje infekciju JC poliomavirusa za 2-log ako su stanice prethodno tretirane peptidom (pre-tretman). Bombinin H2 pokazuje smanjenje infekcije za 1-log ako se doda u drugim stadijima infekcije ili izravno na polimiovirus JC. Bombinin H4 ne pokazuje antivirusni učinak na JC Poliomavirus. Prema našim saznanjima ovo je prvo izvješće o antivirusnom djelovanju bombinina H2 i H4.

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SAŽETAK

Antivirusni učinak antimikrobnih peptida bombinina H2 i H4, porijeklom iz kože žabe (*Bombina variegata)* testiran je na Herpes simplex virusu-1 (HHV-1), virusu s ovojnicom i dvolančanom DNA, a za usporedbu su korišteni drugi virusi za bolje razumijevanje mehanizma navedenih peptida, uključujući virus influence H1N1 koji je RNA virus s ovojnicom i JC Poliomavirus koji je dsDNA virus bez ovojnice. Potencijalni citotoksični učinak bombinina H2 i H4 ispitan je MTT testom. Na temelju rezultata MTT testa izabrane su koncentracije za pokuse u ovom istraživanju. Antivirusni učinak bombinina H2 i H4 na HHV-1 ispitan je "time-of-addition" testom. Naime, stanice su bile inficirane i tretirane s H2 ili H4 (10 i 20 μg/ml) u različito vrijeme infekcije: prije (PRE), tijekom (D) ili nakon (P) adsorpcije virusa na stanice domaćina ili tijekom i nakon (D +P, dvostruka doza) adsorpcijsku fazu i sljedeća 24 sata. Nadalje, radi provjere potencijalnog izravnog učinka bombinina na virione (virucidno djelovanje), virus je prethodno inkubiran sa svakim peptidom 1 sat na 37 ° C, a zatim su smjese korištene za inficiranje stanica. Iz ovih pokusa stanice su prikupljene i kasnije korištene za Western blot, a supernatanti su kasnije korišteni za standardno ispitivanje plakova. Prethodne studije provedene u našem laboratoriju pokazale su da temporin G (Tg) smanjuje infekciju HHV-1 kada se peptid doda tijekom faze adsorpcije virusa (podaci nisu objavljeni). Kako bi se proučio potencijalni sinergijski učinak primjene bombinina H2 i Tg na životni ciklus HHV-1, stanični monoslojevi inficirani su virusom prethodno inkubiranim bombininima, a također su tretirani s Tg-om 1 sat tijekom adsorpcije virusa na stanice domaćina. Nakon 24 sata, supernatanti su prikupljeni i korišteni za izvođenje standardnog testa na plak. Isti pokus je izravno ponovljen na ploči s 96 jažica radi izvođenja Unutar staničnog-Western blota (ICW). Peptidi su zatim testirani na H1N1 virusu influence A isti način kao i na HHV-1, ali na stanicama A549. Umjesto testa plaka korištena je metoda ICW, a stanice su korištene za Western blot. Kako bi se potvrdila virucidna svojstva bombinina na ovojnici virusa, sljedeći je eksperiment bio usmjeren na ispitivanje bombinina na virus bez ovojnice. U tu svrhu odabran je JC poliomavirus (JCPyV). Stanice SVGp12, bile su zaražene 48 sati virusom prethodno tretiranim bombininima, kako je ranije opisano za virucidne testove HHV-1 i IAV. Kako bi se dobila šira slika i bolji pregled informacija, provedeno je ispitivanje "time-of-addition" na isti način kao na HHV-1. Prikupljeni supernatanti i DNA, ekstrahirana iz stanica, korišteni su za Q-PCR analizu. Bombinin H2 i H4 korišteni su u dozama 10 i 20 μg/mL koje nisu pokazale toksični učinak tijekom MTT testa. Rezultati testa plaka i Western blot pokazuju virucidni učinak bombinina H2 u dozama od 10 μg/ml ($p < 0,001$) i 20 μg/ml ($p < 0,0001$) μg/ml i H4 u dozama od 10 μg/ml ($p < 0,01$) i 20 μg/ml ($p < 0,001$) na HHV-1. Bombinin H2 pokazuje snažniji virucidni učinak od oko 2 log smanjenja u usporedbi s infekcijom koja odgovara 99% inhibiciji replikacije HHV-1. Bombinin H2 dodan izravno na HHV-1 u dozi od 10 μg/ml pokazuje aditivni učinak s peptidom temporinom G u dozi od 50 μg/ml tijekom faze adsorpcije. Rezultati Western blota i ICW-a pokazuju da bombinin H2 ima virucidan učinak na H1N1 virus influence. Rezultati dobiveni Q-PCR-om pokazuju da bombinin H2 smanjuje infekciju JC poliomavirusa za 2-log ako su stanice prethodno tretirane peptidom (pre-tretman). Bombinin H2 pokazuje smanjenje infekcije za 1-log ako se doda u drugim stadijima infekcije ili izravno na polimiovirus JC. Bombinin H4 ne pokazuje antivirusni učinak na JC Poliomavirus. Prema našim saznanjima ovo je prvo izvješće o antivirusnom djelovanju bombinina H2 i H4.

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Antiviral effect of natural compounds on Human alphaherpesvirus 1

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SUMMARY

Antiviral effect of antimicrobial peptides bombinin H2 and H4 originated from frog skin (Bombina variegata) was tested on enveloped, dsDNA, Human aplhaherpesvirus-1 (HHV-1) and for comparison other viruses were used to understand the mechanism of peptides better, including H1N1 influenza virus which is an enveloped RNA virus and JC Polyomavirus which is non-enveloped dsDNA virus. MTT assay was used to test possible cytotoxicity of bombinin H2 and H4 as well as to choose appropriate concentrations for further experiments.. To test antiviral effect of bombinin H2 and H4 on HHV-1 time of addition assays were performed. Cells were infected and treated with H2 or H4 (10 and 20 μg/ml) at different times of infection: before (PRE), during (D) or after (P) the virus adsorption to the host cells or during and after (D+P, double dose) the adsorption phase and for the following 24 h. Moreover, to verify the potentially direct effect of bombinins on virions (a virucidal action), virus was preincubated with each peptide for 1 h at 37°C, and then the mixtures were used to infect the cells. From these experiments cells were collected and later used for Western blot and supernatants later used for plaque assay. Previous studies performed in our lab demonstrated that temporin G (Tg) reduces HHV-1 infection when the peptide is added during the viral adsorption phase (data not published). To study the potential synergic effect of bombinins and Tg administration on HHV-1 life cycle, cellular monolayers were infected with virus preincubated with bombinins and were also treated with Tg for 1 h during the virus adsorption to the host cells. After 24 h, supernatants were collected and used to perform a standard plaque assay. The same experiment was directly repeated in a 96-well plate to perform an Odyssey assay. To conclude are bombinins H2 and H4 virucidal on other viruses than HHV-1 Influenza A virus was tested in the same manner as for HHV-1 but on A549 cells. Instead of plaque assay used method was ICW and cells were used for Western blot. To verify the virucidal properties of bombinins on viral envelope, the next experimentation was addressed to test bombinins against a non-enveloped virus. To this aim, JC polyomavirus (JCPyV) was chosen. SVGp12 cells, were infected for 48 h with bombinins-pretreated virus, as described earlier for HHV-1 and IAV virucidal tests. To get a broad picture, time-of-addition assay was performed as on HHV-1. Collected supernatants and DNA extracted from cells were used for Q-PCR analysis. Bombinin H2 and H4 were used in doses of 10 and 20 μg/mL which do not show toxic effect during MTT assay. Results obtained from plaque assay and Western blot show virucidal effect of bombinin H2 in doses of 10 μg/ml ($p<0,001$) and 20 μg/ml ($p<0,0001$) μg/ml and H4 in doses of 10 μg/ml ($p<0,01$) and 20 μg/ml ($p<0,001$) on HHV-1. Bombinin H2 shows stronger virucidal effect of about 2-log decrease in comparison to infection which corresponds to 99% inhibition of HHV-1 replication. Bombinin H2 added directly to HHV-1 in dose of 10 μg/ml shows additive effect with temporin G added in the dose of 50 μg/ml during adsorption phase. Results from Western blot and ICW show that bombinin H2 is virucidal on H1N1 influenza virus. Results obtained from Q-PCR show that bombinin H2 reduces infection of JC Polyomavirus for 2-log if cells are pretreated with the peptide. Bombinin H2 shows 1-log reduction of infection if added in other stages of infection or directly to the JC Polyomavirus. Bombinin H4 does not show antiviral effect on JC Polyomavirus. To our knowledge this is a first report on antiviral activity of bombinin H2 and H4.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: 69 pages, 21 figures, 0 tables and 156 references. Original is in English language.

Keywords: antimicrobial peptides, antiviral effect, bombinin H2, bombinin H4, Human alphaherpesvirus 1, JC Polyomavirus, Influenza virus

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