The molecular mechanism of action of selected immunomodulatory proteins encoded by alphaherpesviruses

Summary of Professional Accomplishments

Dr. Andrea Diana Lipińska

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1. Name.

Andrea Diana Lipińska

2. Diplomas, degrees conferred in specific areas of science or arts, including the name of the institution which conferred the degree, year of degree conferment, title of the PhD dissertation.

- **2006 Ph.D. in Biological Sciences in the field of Biochemistry**

The degree awarded on **July, 5th, 2006**, by the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk based on the dissertation titled "*Identification and characterization of bovine herpesvirus 1 (BHV-1) proteins interfering with the host immune response*". The dissertation was written in English and carried out at the Department of Molecular Virology of the Intercollegiate Faculty of Biotechnology under the supervision of Prof. Krystyna Bieńkowska-Szewczyk.

The dissertation was distinguished (*cum laude*) by the Intercollegiate Faculty of Biotechnology Board.

- **1999 M.Sc. in Biotechnology**

The degree awarded by the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk based on the Master Project titled *„ Expression and analysis of recombinant gE and gI glycoproteins of bovine herpesvirus 1 in baculovirus expression system*" carried out under the supervision of Prof. Bogusław Szewczyk at the Department of Molecular Virology of the Intercollegiate Faculty of Biotechnology.

3. Information on employment in research institutes or faculties/departments or school of arts.

2008 - present

Adiunkt (associate professor), Laboratory of Virus Molecular Biology, Intercollegiate Faculty of Biotechnology, University of Gdańsk.

2006 - 2008

Research assistant, Department of Molecular Virology, Intercollegiate Faculty of Biotechnology, University of Gdańsk.

2004 - 2005

Senior technician, Department of Molecular Virology, Intercollegiate Faculty of Biotechnology, University of Gdańsk

4. 4. Description of the achievements, set out in art. 219 para 1 point 2 of the Act.

a) A title of scientific achievement

The scientific achievement presented in this summary is based on a series of **7 scientific publications**, with a common title: **"The molecular mechanism of action of selected immunomodulatory proteins encoded by alphaherpesviruses"**.

b) Scientific publications constituting the scientific achievement:

The following original experimental publications were published in the years 2011-2023:

1. Verweij M.C., **Lipińska A.D.**, Koppers-Lalic D., van Leeuwen W.F., Cohen J.I., Kinchington P.R., Messaoudi I., Bienkowska-Szewczyk K., Ressing M.E., Rijsewijk F.A., Wiertz E.J. (2011) The capacity of UL49.5 proteins to inhibit TAP is widely distributed among members of the genus *Varicellovirus*.

   Journal of Virology, 85(5), 2351-2363. DOI: 10.1128/JVI.01621-10.
   IF_{2011}=5,402; ministerial points $MNiSW_{2010}$=32; ministerial points $MNiSW_{2012}$=40 (no report in 2011), number of citations: 33.

2. Verweij M.C.*, **Lipińska A.D.***, Koppers-Lalic D., Quinten E., Funke J., van Leeuwen H.C., Bieńkowska-Szewczyk K., Koch J., Ressing M.E., Wiertz E.J. (2011) Structural and functional analysis of the TAP-inhibiting UL49.5 proteins of varicelloviruses.
   * *equally contributing*

   Molecular Immunology, 48(15-16), 2038-2051.
   DOI: 10.1016/j.molimm.2011.06.438.
   IF_{2011}=2,897; ministerial points $MNiSW_{2010}$=32; ministerial points $MNiSW_{2012}$=25 (no report in 2011), number of citations: 29.

3. Graul M., Kisielnicka E., Rychłowski M., Verweij M.C., Tobler K., Ackermann M., Wiertz E.J.H.J., Bieńkowska-Szewczyk K., **Lipińska A.D.*** (2019) Transmembrane regions of bovine herpesvirus 1-encoded UL49.5 and glycoprotein M regulate complex maturation and ER-Golgi trafficking.
   * *corresponding author*

   Journal of General Virology, 100(3):497-510. DOI 10.1099/jgv.0.001224.
   IF_{2019}=3,376; ministerial points $MNiSW_{2019}$=70; number of citations: 5.

4. Karska N., Graul M., Sikorska E., Zhukov I., Ślusarz M.J., Kasprzykowski F., **Lipińska A.D.***, Rodziewicz-Motowidło S.* (2019) Structure determination of UL49.5 transmembrane protein from bovine herpesvirus 1 by NMR spectroscopy and molecular dynamics.
   * *equally contributing supervising authors*

   ***Biochimica et Biophysica Acta* (BBA) Biomembranes**, 1861(5), 926-938.
   DOI 10.1016/j.bbamem.2019.02.005.
   IF_{2019}=3,411; punkty $MNiSW_{2019}$=100; liczba cytowań: 7.

5. Wąchalska M., Graul M., Praest P., Luteijn R.D., Babnis A.W., Wiertz E.J.H.J., Bieńkowska-Szewczyk K., **Lipińska A.D.*** (2019) Fluorescent TAP as a platform for virus-induced degradation of the antigenic peptide transporter.
   corresponding author

   Cells, 8(12):1590. DOI: 10.3390/cells8121590.
   IF_{2019}=5,656; ministerial points $MNiSW_{2019}$=140; number of citations: 7.

6. Karska N., Graul M., Sikorska E., Ślusarz M.J., Zhukov I., Kasprzykowski F., Kubiś A., **Lipińska A.D.**, Rodziewicz-Motowidło S. (2021) Investigation of the effects of primary structure modifications within the RRE motif on the conformation of synthetic bovine herpesvirus 1-encoded UL49.5 protein fragments.

   Chemistry & Biodiversity, 18(2):e2000883. DOI: 10.1002/cbdv.202000883.
   IF_{2021}=2,745; ministerial points MNiSW2019=70; number of citations: 4.

7. Graul M., Karska N., Wachalska M., Krupa P., Ślusarz M.J., Lubocki M., Bieńkowska-Szewczyk K., Rodziewicz-Motowidło S., Sieradzan A.K.*, **Lipińska A.D.***, (2023) The N-terminal proline hinge motif controls the structure of bovine herpesvirus 1-encoded inhibitor of the transporter associated with antigen processing required for its immunomodulatory function.
   * *corresponding authors*

   Journal of Molecular Biology, 13;435(5):167964. DOI: 10.1016/j.jmb.2023.167964.
   IF_{2023}=6,151; ministerial points MEiN=140; number of citations: 1.

The impact factor (IF) and ministerial points for the publications are according to the year of publication record; the number of citations according to Google Scholar.

The total impact factor (IF) for the publications: **29,638**

The total number of citations according to Google Scholar is **86**;
according to Web of Science: **57**

The Hirsch Index of the author according to Google Scholar – **12**

The Hirsch Index according to Web of Science – **11**

ORCID:**0000-0002-8115-9499**

The co-author statements specifying the individual contributions of each author to the respective publication are included in **Attachment 3.1**. Dr. Lipińska's statements regarding her contribution to the publications can be found in **Attachment 3.2.**

c) Summary of the scientific aim and accomplished results of the scientific achievement.

The series of publications mentioned above presents the results of my research work carried out at the Department of Molecular Virology (transformed into the Laboratory of Molecular Biology of Viruses in 2012) of the Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk.

These studies originated from my main scientific interest in the interactions of viruses with the host immune system, particularly focusing on immunomodulatory strategies leading to immune evasion. Viruses, in order to survive through evolution, have acquired the ability to hide from components of the immune system or actively inhibit them. Herpesviruses - large viruses with double-stranded DNA genomes, encoding a large number of immunogenic proteins - hold the title of "masters of immune evasion" as they stand out for the diverse range of viral proteins and small non-coding RNAs involved in immunomodulation. Additionally, herpesviral immunomodulatory proteins act through various mechanisms, targeting both innate immune pathways and antigen presentation pathways responsible for activating B and T lymphocytes. Inhibition of antigen presentation is particularly important due to the latency used by herpesviruses. Latency is a state of concealment in specific host tissues (the nervous system for alphaherpesviruses) – followed by cyclic reactivations from this state, which exposes the viruses to components of the previously activated immune system.

My postdoctoral studies stem from my doctoral thesis, which was carried out at the Department of Molecular Virology in the years 2000 - 2006. During this time, I participated in the **identification of immunomodulatory properties of UL49.5 orthologs** from four alphaherpesviruses: **bovine herpesvirus 1 (BoHV-1), pseudorabies virus (PRV), and equine herpesviruses 1 and 4 (EHV-1, EHV-4)**. Additionally, I determined that the activity of the BoHV-1 UL49.5 protein as an inhibitor of the **antigenic peptide transporter complex (TAP) is negatively regulated** by its interaction with another viral protein, **glycoprotein M (gM)** (Attachment 4, p.II, positions 26-28 of the list of scientific achievements). The formation of the UL49.5/gM heterodimer during BoHV-1 infection allows for the maturation of both proteins in the endoplasmic reticulum (ER) and the release of the complex from the ER. UL49.5 bound to gM does not interact with the cellular TAP transporter.

The research during my doctoral studies left me with a significant longing for more detailed knowledge in this field, especially regarding the molecular aspects of protein interactions and their structures. An important catalyst for further research was **the international research internship at the laboratory of Prof. Emmanuel Wiertz at Leiden University Medical Center in the Netherlands** (2002-2004, a total of 13 months). During this time, I not only finalized experiments for my doctoral thesis (resulting in dual affiliation in publication at the position 28 in the list of scientific achievements), but also triggered new research directions concerning the UL49.5/gM complex to understand, at the molecular level, how both proteins function in selected alphaherpesviruses. This internship also initiated a long-lasting international collaboration with Prof. Wiertz's lab, which resulted in four joint publications after obtaining my doctoral degree.

To determine the molecular mechanism of action of the UL49.5/glycoprotein M complex, I posed four main questions that I sought to answer during my research:

1. How conserved are the immunomodulatory properties of the UL49.5 protein among alphaherpesviruses?
2. What is the mechanism of inhibition of the TAP transporter?
3. How is the binding between UL49.5 and glycoprotein M facilitated, which regulates the immunomodulatory properties of UL49.5, and which motifs are responsible for intracellular transport of UL49.5/gM?

4. What is the mechanism of TAP transporter degradation in the case of the UL49.5 ortholog of bovine herpesvirus 1?

Ad.1.

The antigenic peptide transporter (officially known as **the transporter associated with antigen processing, TAP**) is a crucial protein for **the major histocompatibility complex (MHC) class I-dependent antigen presentation** pathway. Its role is to activate cytotoxic T lymphocytes and their immune responses, including antiviral responses. TAP does not have a functional substitute and has evolved as a heterodimeric protein complex, consisting of **TAP1** and **TAP2**, located in the endoplasmic reticulum (ER) membrane. The TAP1/TAP2 genes have been identified in various vertebrates, including fish, amphibians (*Xenopus levis*), reptiles, birds, and mammals. Structural aspects are of great importance for TAP, as its entire transporter activity is based on a cycle of conformational rearrangements. TAP belongs to the ATP-binding cassette (ABC) transporter family, which harnesses energy from ATP binding and hydrolysis to transport various substrates. Its substrate is a peptide of specific length (typically 8-16 amino acid residues) and preferred sequence, known as an **antigenic peptide**, generated through antigenic protein processing by the proteasome. TAP possesses a total of 19 transmembrane helices, with 9 in TAP1 and 10 in TAP2, forming the transmembrane domain (TMD). Peptide transport from the cytoplasm to the ER lumen occurs through a complex cycle of conformational changes, including a resting open conformation facing the cytoplasm, an intermediate closed conformation upon ATP and peptide binding, and its translocation between the transmembrane helices of the transporter, leading to the opening from the ER lumen side and peptide release (in the conformation open to the ER lumen). The antigenic peptide is then captured by MHC class I molecules with the assistance of chaperone proteins of the peptide-loading complex. Full ATP hydrolysis triggers changes that restore the resting conformation. The binding of the antigenic peptide allows the MHC class I-peptide complex to exit the ER via the secretory pathway to the cell membrane, where it interacts with receptors on cytotoxic lymphocytes.

The *UL49.5* gene (earning its unusual name due to its location between previously discovered *UL49* and *UL50* genes in the unique long (*UL*) region of the genome) is present in all herpesviruses and belongs to the so-called "*core genes*," indicating their important role. In some members of the *Herpesviridae* family, the UL49.5 protein becomes N- or O-glycosylated and is referred to as **glycoprotein N (gN)**. Typically, the role of this protein is to assist glycoprotein M (gM) as part of a covalently linked via a disulfide bridge heterodimer. This complex localizes in the viral envelope and participates in the virus spread between host cells.

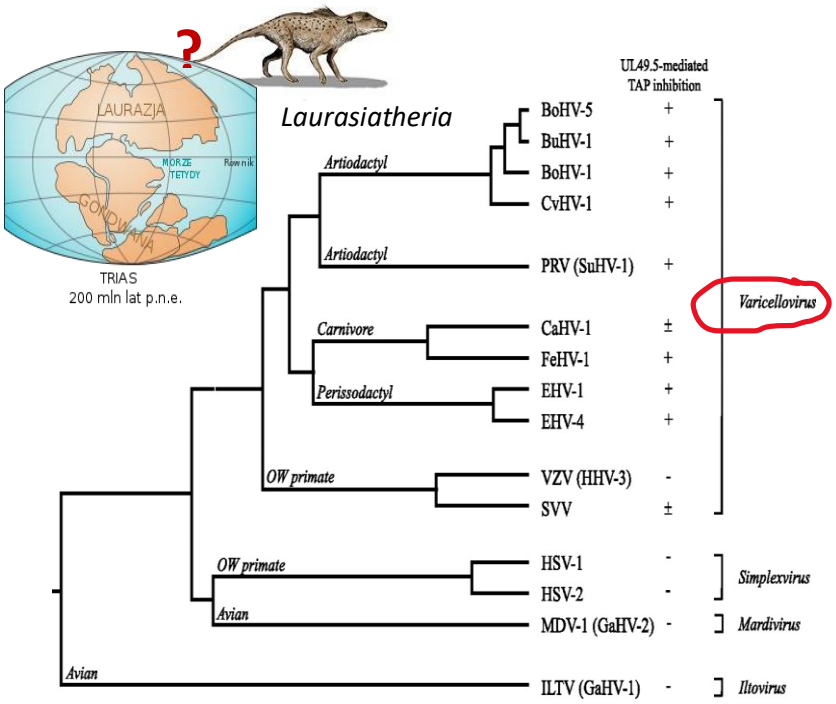
During my doctoral studies, I contributed to the identification of UL49.5 orthologs from **BoHV1, PRV**, and **EHV1/4** as TAP-binding proteins that block its structural rearrangements necessary for peptide translocation. This is a common feature of their mechanism of action. Additionally, UL49.5 from EHV1/4 inhibits ATP binding, while UL49.5 from BoHV-1 induces proteasome-dependent degradation of TAP1 and TAP2 proteins. In one of the most important human alphaherpesvirus pathogens, **herpes simplex virus 1 (HSV-1)**, UL49.5 does not possess TAP-binding ability. Instead, this virus has evolved another TAP inhibitor called **ICP47**, which acts through a different mechanism. The level of protein sequence conservation of TAP among mammals is high, while the degree of amino acid sequence similarity between UL49.5 homologs is low. However, they share a general architecture, including the longest N-terminal

domain, a single transmembrane domain, and a short cytoplasmic region at the C-terminus. Another common feature of UL49.5 among herpesviruses is the formation of a heterodimer with gM.

The natural first research question that I posed was the conservation level of immunomodulatory properties of UL49.5 among alphaherpesviruses. I hypothesized that expanding the characterization of UL49.5's ability to inhibit TAP in other alphaherpesviruses would allow for a comparison of amino acid sequences between UL49.5 proteins that act as inhibitors and those that lack such properties. The results of these studies are described in the first publication of this research series, Verweij *et al.*, JV 2011. I am the second author of this publication because, as part of international collaboration with Prof. E. Wiertz's team and with **Dr. Frans Rijsewijk** from the **Institute for Animal Health in Lelystad, the Netherlands**, research on individual UL49.5 homologs was divided. My contribution involved confirming the presence of a TAP inhibitor in members of the *Varicellovirus* genus, specifically **feline and canine herpesviruses (FeHV-1, CHV-1)**, isolating the *UL49.5* gene from these viruses, establishing human melanoma cell lines and host-specific cell lines (feline and canine) with constitutive expression of *UL49.5* from these viruses, and confirming their immunomodulatory properties. The human melanoma cell line became the most commonly used model in my research due to its established reputation in antigen presentation pathway studies (the cell line produces both MHC class I and class II molecules) and its permissiveness to veterinary viruses, specifically BoHV-1 and PRV.

The UL49.5 homologs I examined **did not affect the levels of human TAP proteins but strongly inhibited the activity of human and host-specific TAP transporters.** Using a similar methodology, I investigated UL49.5 homologs from avian alphaherpesviruses, including **infectious laryngotracheitis virus (ILTV)**, **Marek's disease virus type 1 (MDV-1)**, and **herpesvirus of turkeys (HVT)**. I found that these homologs did not possess the ability to inhibit TAP and MHC class I in either human MJS or avian cell lines. This project required me to gain experience in the laboratory with new species of veterinary alphaherpesviruses and their host-specific cell lines or primary cell cultures, such as those isolated from chicken embryos, which I learned during a research visit to the **National Veterinary Research Institute** in Pulawy, Poland.

The results confirming my contribution to this publication are presented in Figures 3, 4, 7, and 8 of the paper (four out of the seven figures describing experimental data). I also contributed to the conceptualization of the research, analysis of the results, and their graphical representation. I participated in the preparation of the original manuscript version. As part of the team in this publication, Dr. Rijsewijk confirmed the ability of TAP inhibition and also transporter degradation by viruses closely related to BoHV-1, including **BoHV-5 (bovine herpesvirus type 5), BuHV-1 (bubaline (buffalo) herpesvirus type 1), and CvHV-1 (cervid herpesvirus type 1).** On the other hand, in Prof. Wiertz's team, the activity of the UL49.5 gene from the prototype varicellovirus, **varicella-zoster virus (VZV)**, causing chickenpox and shingles in humans, and closely related **simian varicella virus (SVV)** was investigated, **without showing their ability to inhibit the transporter** (**Figure 1**).



Rys. 1. The phylogenetic tree of selected alphaherpesviruses, including the evolution of their hosts. The presence of UL49.5 with immunomodulatory properties was marked as (+), its absence was marked as (-). Based on Fig. 9 published in Verwei et al.., JV 2011.

For these reasons, I consider the aspect of the molecular evolution of alphaherpesviruses in the context of TAP complex inhibitory proteins as very interesting from a scientific point of view. It suggests that during herpesvirus co-evolution with their hosts, their UL49.5 likely acquired the ability to bind to TAP at the stage of a virus pathogenic to the mammalian ancestor of predators and ruminants at the supercontinent of Laurasia. Herpes simplex virus 1 (HSV-1), the human cytomegalovirus from the betaherpesvirus subfamily, and a gammaherpesvirus Epstein-Barr virus possess other genes whose products are TAP inhibitors (as examples of convergent evolution). However, their UL49.5 homologs do not serve this function. To continue the research on the evolutionary context of alphaherpesviruses, I am currently studying the ability of **phocid herpesvirus type 1 (PhHV-1**, closely related to feline and canine herpesviruses) to inhibit the TAP transporter. I am also interested in **bovine herpesvirus type 2** (BoHV-2), which is more closely related to HSV-1 but does not possess the orthologous gene for its TAP inhibitor, ICP47. Does this mean that BoHV-2 is unable to inhibit antigenic peptide transport?

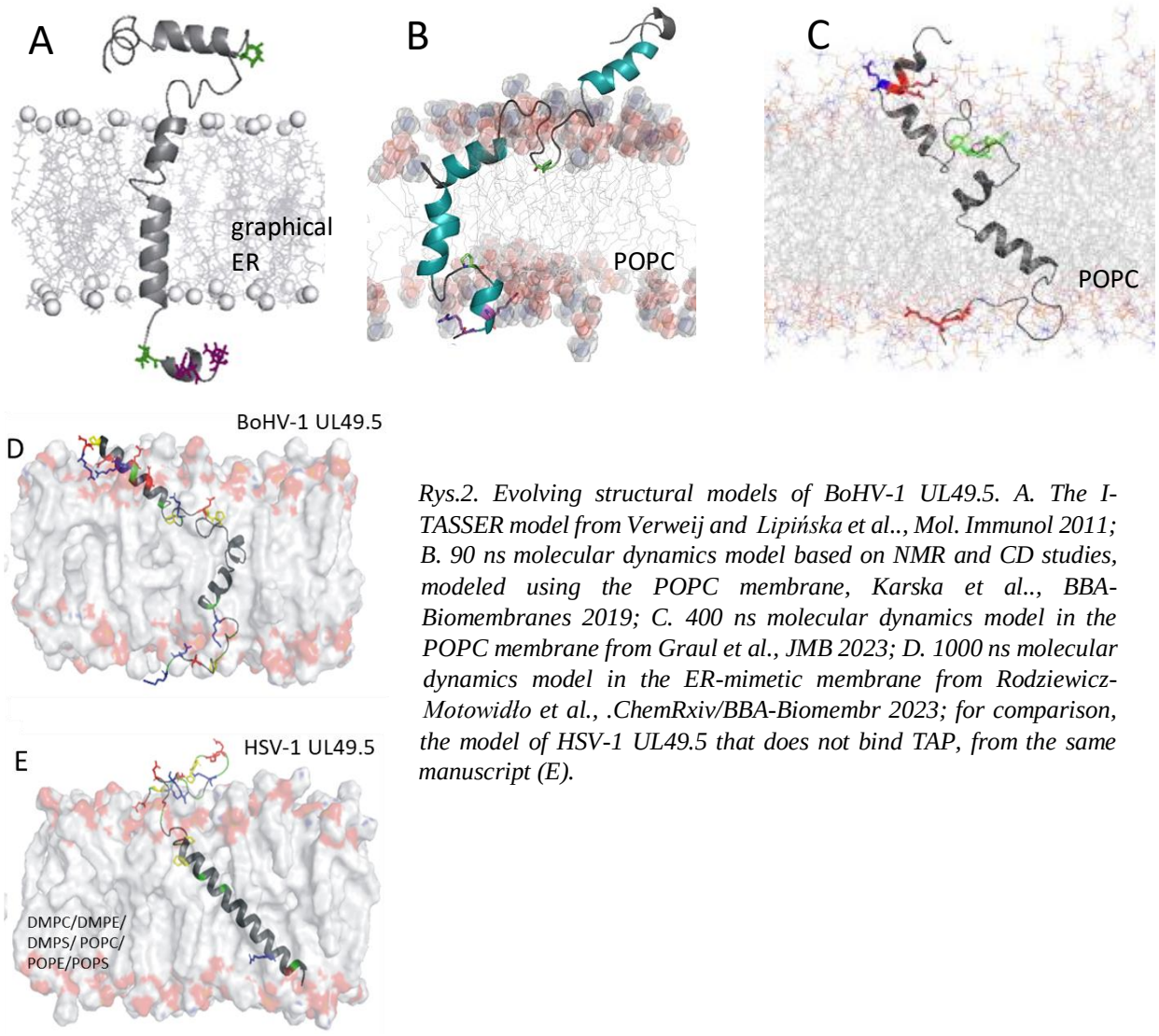
The results of this publication allowed me to compare the amino acid sequences of the investigated UL49.5 homologs in order to attempt to identify conserved residues or motifs that could distinguish UL49.5 with immunomodulatory properties. There were not many such sequences. I decided to investigate the role of these regions and specific residues using site-directed mutagenesis and "domain-swapping" techniques, moving on to the second research question.

Ad.2.

The comparison of amino acid sequences of UL49.5 homologs from alphaherpesviruses allowed for the identification of specific regions for further investigation, primarily **the RGRG** region (four additional residues at the C-terminus present in UL49.5 orthologs capable of TAP degradation), the **KK/SS motif** (potential ubiquitination sites that could affect the ability to

degrade the transporter), and **proline** residues in the N-terminal domain (which could impact structural similarity). The outcome of these studies is the second publication in the series, Verweij and Lipińska *et al.*, Mol. Immunol 2011, where I am a first co-author along with **Dr. Marieke Verweij** from Prof. Wiertz's team. My role involved the design and performing of site-directed mutagenesis and PCR-based constructs with point mutations in the UL49.5 gene of BoHV-1 in different combinations. I generated MJS cell lines with constitutive expression of the obtained variants and assessed their impact on the levels of MHC class I molecules and TAP1/TAP2 proteins. Additionally, Dr. Verweij prepared constructs encoding proteins with swapped domains, obtained by fusion PCR combining the coding sequence for the N-terminal domain of UL49.5 from BoHV-1 with the transmembrane (TM) region of non-inhibitory UL49.5 from VZV and vice versa: the N-terminal domain of UL49.5 from VZV with the TM region from UL49.5 of BoHV-1 ("domain swapping"). Transmembrane regions of cellular proteins, CD3δ and toll-like receptor 2, were also used. This methodological approach aimed to identify at least the domain of UL49.5 responsible for its activity against TAP. The results of these studies indicated that the N-terminal domain of UL49.5 from BoHV-1, anchored in the ER membrane (it can utilize the TM region from another protein for anchoring), was required for TAP inhibition, while the C-terminal RGRG motif, particularly the basic residues within it, was necessary for TAP degradation. Interestingly, the lysine and serine residues, which initially seemed to be the most likely sites for ubiquitination of UL49.5, were determined as unnecessary for TAP degradation.

The results confirming my contribution to this publication are presented in Figures 7, 8, and 9 of the paper (three out of the eight figures describing experimental data). I also contributed to the conceptualization of the research, analysis of the results, their graphical representation, and participated in the preparation of the original and final manuscript versions. Additionally, this study yielded **the first structural model of the UL49.5 protein** from BoHV-1, prepared by **Dr. Hans van Leeuwen** from Leiden University Medical Center in the Netherlands, using the I-TASSER platform (based on the so-called threading method), presented in the manuscript as Figure 10. This model served as the starting point for the "structural approach" to studying UL49.5 protein and evolved throughout subsequent years of my postdoctoral research (**Figure 2**).



Rys.2. Evolving structural models of BoHV-1 UL49.5. A. The I-TASSER model from Verweij and Lipińska et al.., Mol. Immunol 2011; B. 90 ns molecular dynamics model based on NMR and CD studies, modeled using the POPC membrane, Karska et al.., BBA-Biomembranes 2019; C. 400 ns molecular dynamics model in the POPC membrane from Graul et al., JMB 2023; D. 1000 ns molecular dynamics model in the ER-mimetic membrane from Rodziewicz-Motowidło et al., .ChemRxiv/BBA-Biomembr 2023; for comparison, the model of HSV-1 UL49.5 that does not bind TAP, from the same manuscript (E).

The "structural approach" resulted from the conclusion drawn at this stage of the research that the immunomodulatory activity of UL49.5 may rely not only on specific amino acid residues but (also) on structural motifs. Understanding the structure of UL49.5 could provide insights into the mechanism of action of the inhibitor. Additionally, introducing point mutations without knowing their impact on the protein structure raised my concerns. I was uncertain whether the changes would impair the site of interaction with TAP directly or affect the overall protein structure, thereby indirectly altering its activity. As the I-TASSER model of UL49.5 appeared to me as less accurate, I asked for collaboration **Prof. Adam Liwo** and his team from **the Laboratory of Molecular Modeling** at the Faculty of Chemistry, University of Gdańsk, who suggested experimental studies to gain a better understanding of the BoHV-1 UL49.5 structure. I was directed to **Prof. Sylwia Rodziewicz-Motowidło** from the **Department of Biomedical Chemistry, Faculty of Chemistry**, at the University of Gdańsk, initiating a long-term collaboration that resulted in four joint manuscripts and four grant applications submitted to the National Science Centre.

The structural studies utilized NMR spectroscopy and circular dichroism (CD) to investigate fragments of the BoHV-1UL49.5 protein and its variants with substitutions in

specific amino acid residues. The results of these studies were presented in two subsequent publications: Karska *et al.*, BBA-Biomembranes 2019, and Karska *et al.*, Chemistry & Biodiversity 2021. In the first publication, I co-supervised the work with Prof. Rodziewicz-Motowidło, and Dr. Natalia Karska, a post-doctoral researcher in my research team at the time, was the first author. Dr. Karska later joined the research team at the Department of Biomedical Chemistry. Since 2012, I provided scientific assistant supervision to **Małgorzata Graul**, who obtained her Ph.D. in 2019 and is also a co-author of these publications.

Within these studies, Dr. Karska performed experimental structural analyses (CD, NMR) of the synthesized UL49.5 BoHV-1 protein fragments. The results served as a basis for developing a more accurate molecular dynamics-based model of the viral protein. *My role in these studies involved assisting in the planning of UL49.5 fragments for chemical synthesis based on existing knowledge of the viral protein and participating in the interpretation of the results, particularly their biological significance*. Most of the point mutants of BoHV-1 UL49.5 were structurally characterized (NMR, CD) and/or modeled, and their activity was assessed by me and Małgorzata Graul in mammalian cell cultures. During these studies, I also realized the importance of incorporating **a proper membrane model** into the structural studies of UL49.5 and its modeling. TAP is a complex residing in the ER, and UL49.5 interacts with TAP in the ER, while the ER membrane differs in its lipid composition and thickness (among other important parameters) from membranes of other cellular compartments. I assumed that UL49.5 amino acid residues might also interact with membrane phospholipids, influencing the protein's structure. The I-TASSER model did not take the presence of a membrane during modeling into consideration. In both publications, CD and NMR studies, as well as modeling, were performed in a biochemically mimicking environment of a eukaryotic membrane (dodecylphosphocholine micelles and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine bilayer). **The experimentally obtained 3D model of the UL49.5 protein** differed in several aspects from the theoretical model generated by the I-TASSER server, although both models shared a common pattern of the protein architecture. Incorporating a phospholipid membrane into the model allowed us to determine that the BoHV-1 UL49.5 protein is mostly embedded within the membrane, with only a fragment of the N-terminal domain contacting the ER lumen and the previously studied RGRG motif primarily interacting from the cytoplasmic side. The unusual arrangement of the transmembrane (TM) regions in the membrane also caught my attention - the TM helix in this protein was clearly bipartite, with a structural break in the middle and both parts of the TM fragment positioned at an angle (defined here as 110°). Other structural motifs that could play an important role in the protein's function were **the N-terminal α-helix** and the **proline-based motif** in the N-terminal domain, which appeared to further anchor this part of the protein in the membrane.

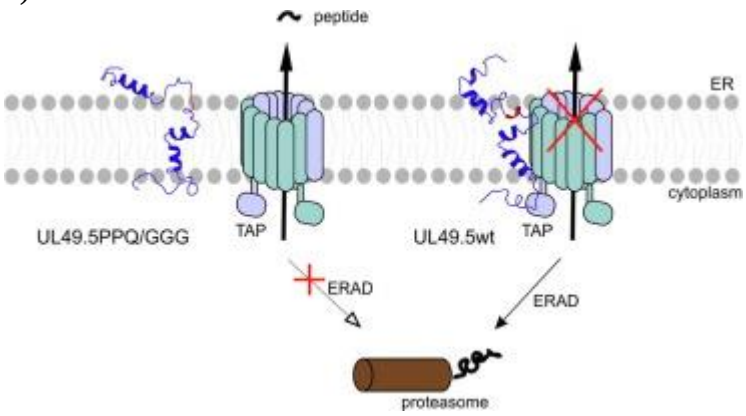
The first validated structural motif was the N-terminal helix of BoHV-1 UL49.5, which, according to secondary structure analysis, was absent in non-TAP-inhibiting alphaherpesvirus homologs. Therefore, in the studies published in Chemistry & Biodiversity, in collaboration with Prof. Rodziewicz-Motowidło, I designed substitutions in the N-terminal domain to disrupt α-helix formation. Although secondary structure predictions suggested such disruptions, the experimental studies (CD and NMR of UL49.5 fragments) revealed that the N-terminal α-helix in the D24-G44 region was relatively resistant to changes, including substitutions in **the RRE(30-32) motif** with glycine or proline residues that were expected to "break" the helix. However, we were able to generate elongated or shortened helices, but without significant

changes in the protein's activity. *My contribution to the publication also involved planning the experimental introduction of mutations into the RRE motif using site-directed mutagenesis, generating MJS cell lines with constitutive expression of the obtained variants, and assessing their impact on the levels of MHC class I molecules and TAP1/TAP2 proteins. I also analyzed the results, participated in the preparation of the original and final manuscript versions, and supervised the research progress of Małgorzata Graul*. Investigating the role of the RRE motif served as a positive control for studying other UL49.5 variants, for comparison, as this motif had been previously described as crucial for the protein's activity by **Dr. Shafiqul Chowdhury's** team at Louisiana State University (DOI: 10.1371/journal.pone.0025742). However, the same sequence in our hands showed activity very similar to the "wild-type" protein. RRE mutations resulted also in only minor changes in the protein structure, although I can realize that even subtle structural changes can result in functional alterations. Therefore, this result was unexpected and challenging to explain at this stage of the research.

As a continuation of the study, in the publication authored by Graul *et al.*, JMB 2023, together with Dr. Małgorzata Graul from my research team, we introduced mutations into the RRE motif **in the genome of the BoHV-1 virus (BoHV-1 UL49.5 RRE/AAA mutant)** to examine how the mutant's activity changes during infection. Nevertheless, the main objective of this publication was to determine the role of another structural motif in UL49.5 BoHV-1, based on the proline residues 52-53, known as the **proline hinge motif** (52PPQ53). This motif seemed to be a distinctive element of the BoHV-1 protein. In this publication, I am the last author and corresponding author, as the work comprised two parts: molecular modeling using two methods, "all-atom" (performed by **Dr. Paweł Krupa** from the Institute of Physics, Polish Academy of Sciences, and Dr. Natalia Karska) and "UNited RESidue – UNRES," which was supervised by **Dr. Adam Sieradzan from Prof. Liwo's team**. I also participated in the biological studies, where my role involved planning the experiments for constructing a series of UL49.5 variants with mutations in the investigated region, constructing retroviral vectors for transferring these variants to establish stable cell lines expressing UL49.5, and the design of the construction using homologous recombination of the cassette for mutagenesis into the **BoHV-1 UL49.5 RRE/AAA** and **BoHV-1 UL49.5 PPQ/GGG** mutants.

In this part of the study, in addition to contributing to the research concept, I performed the analysis of the results and prepared their graphical representation. In the structural part, I participated in the analysis and interpretation of the results and served as the primary author responsible for preparing the original and final manuscript versions. This publication presented an evolving **third 3D model of the UL49.5 BoHV-1 protein**. This time, we performed modeling using two methods, which yielded consistent results, and extended the simulation time. The model revealed that **the proline motif was responsible for additional anchoring of the N-terminal domain of the protein**. Thus, we added this role to the list of possible functions of proline-based motifs in proteins. Analysis of the structural dynamics over time also helped to understand how this atypical membrane anchor regulates the mobility of individual protein regions and the accessibility of the protein's C-terminus in the cytoplasm. The model also highlighted the role of the bend between the two parts of the transmembrane region, which reached almost 88° and exhibited distinct changes in the mutated UL49.5 variants. All these structural changes correlated with significant alterations in the activity of UL49.5 with the impaired PPQ motif. **The PPG/AAA or PPQ/GGG mutants were functionally the most impaired UL49.5 variants I have studied so far**. Their ability to inhibit TAP transport

decreased by 60% in the stable cell line with the mutant gene variant and by 43% in cells infected with the BoHV-1 PPQ/GGG mutant virus. This change in activity was likely due to much weaker interaction with the TAP complex, as demonstrated by immunoprecipitation. The PPQ motif may directly interact with the transporter or indirectly regulate the structure required for TAP "freezing". Interestingly, although the PPQ motif is located in the N-terminal domain of the protein, it is also essential for inducing transporter degradation. This role is attributed to the C-terminal RGRG motif, whose availability for ERAD machinery likely depends on the proline anchor (in those molecules that still interact with TAP, albeit to a lesser extent) (**Figure 3**).



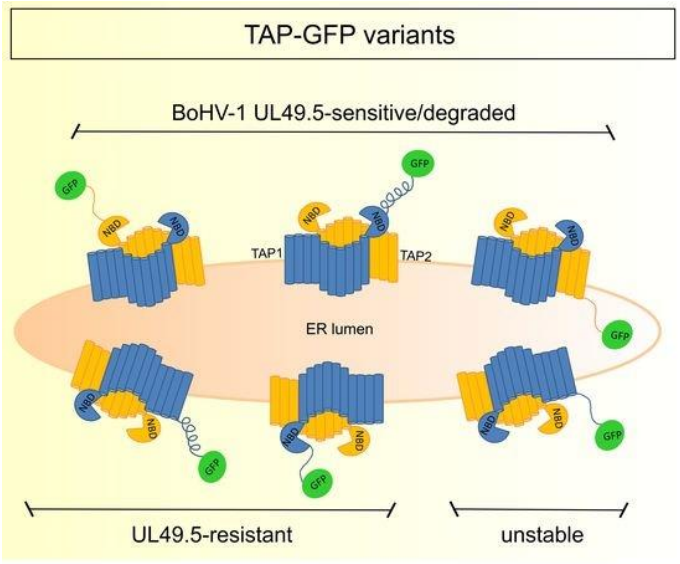
Rys. 3. The graphical abstract from Graul et al.., JMB 2023 summarizing the molecular properties of BoHV-1 UL49.5 with substitutions in the N-terminal PPQ motif („proline hinge").

In the studies, the BoHV-1 RRE/AAA mutant was found to only slightly impair the ability to inhibit antigen peptide transport, and it bound to the transporter and induced its degradation just like the wild-type virus. My discussion with Shafiq Chowdhury at the European Congress of Virology in Gdańsk in May 2023 shed some light on this discrepancy, and I came up with a **microhypothesis** that could partially explain the observed differences in mutant activity in the RRE motif. Besides methodological differences in the studies, the mutant created in the USA is based on a different strain of the virus - Cooper. This strain is not genetically distant from the Dutch Lam strain we used, but according to Dr. Chowdhury, it has a completely different kinetics of UL495-gM gene expression and the ability of UL49.5 to interact with other viral proteins. This does not explain the differences observed in stable cell lines, where the only viral element is the UL49.5 gene with an identical sequence in these strains. However, I have decided to verify the microhypothesis about different virus strains in the future by obtaining the reference Cooper BoHV-1 strain through collaboration with the National Veterinary Institute - National Research Institute in Puławy.

I will continue the structural studies of UL49.5 in the future. The protein model keeps evolving, and its next version is presented in the paper referenced as No. 1 in the Attachment 4 (**Rodziewicz-Motowidło *et al.*, ChemRxiv/BBA-Biomembranes 2023**). In this model, an even longer molecular dynamics simulation was applied, and most importantly, an ER membrane model with phospholipid composition resembling a biological membrane was developed for *in silico* studies (by Dr. Paweł Krupa from the Institute of Physics, Polish Academy of Sciences). This work also involved structural examination of the HSV-1 UL49.5 homolog by Dr. Karska, indicating significant structural differences between these proteins, primarily in the TM region (bent in BoHV-1/straight in HSV-1) and the N-terminal domain (less rigidly structured, with a PPQ motif in BoHV-1/unstructured, flexible, without additional anchoring in HSV-1). In this study, the obtained preliminary TAP model was also docked to BoHV-1 UL49.5, resulting in a preliminary model suggesting that the PPQ motif and the TM region of UL49.5 BoHV-1 may play a major role in interactions. This model requires

optimization and experimental validation, encouraging us to submit a grant application to the National Science Center for this research, with Dr. Krupa and the team of Prof. Rodziewicz-Motowidło. Dr. Natalia Karska has already obtained funding from the National Science Center to obtain structures of other viral TAP inhibitors.

For years, we struggled with the lack of commercially available good anti-TAP1 and anti-TAP2 antibodies while studying the interaction of TAP with UL49.5 variants. Therefore, I decided to develop a **cell-based platform** for research based on a **fluorescently labeled transporter**, in the form of a fusion with green fluorescent protein (GFP). The results of these studies are presented in another publication from the series: Wachalska *et al.*, Cells 2019. In 2014, **Magda Wąchalska** joined my team as a Ph.D. student, and I guided her as an assistant supervisor (doctorate obtained in 2022). For construction of the **TAP-GFP platform** in MJS cells and human leukemia U937 cells, we used cell lines with a CRISPR/Cas9-generated "knock-out" mutation - deletion in the TAP1 or TAP2 gene (MJS) or both genes (U937). This approach allowed us to replace the endogenous TAP subunit with an exogenous version fused with GFP. I obtained the TAP1KO/TAP2KO lines thanks to the collaboration with the team of Prof. Wiertz from the Netherlands. Earlier studies on BoHV-1 UL49.5 had showed that the fusion of GFP to TAP could result in its resistance to degradation. Therefore, in this study, I planned a series of variants by attaching GFP to either the N- or C-terminus of TAP1 or TAP2, using different linkers (**Figure 4**). Two of the constructs showed the most optimal activity and susceptibility to UL49.5, so I used them in further studies. In the Cells paper, we also introduced genes encoding other TAP inhibitors – HSV-1 ICP47, US6 from human cytomegalovirus, and CPXV012 from cowpox virus - into the obtained TAP-GFP system to compare their activities. We found that UL49.5 reduced the level of MHC class I on the cell surface as effectively as the ICP47 protein but was the only one capable of degrading the transporter. I also achieved co-expression of inhibitor genes in pairs, which is a common approach used to determine the TAP conformation bound by inhibitory proteins. According to the data we have obtained, still awaiting publication, UL49.5 binds to a later conformation than ICP47 but earlier than CPXV012, which is consistent with the hypothesis that the intermediate conformation with a bound peptide is the one blocked by UL49.5.



Rys.4. The graphical abstract from Wąchalska et al., Cells 2019, visualizing the constructed TAP-GFP variants tested as a cell-based platform for further studies of the activity of UL49.5 and other virus inhibitory gene products.

Ad.3

The role of heterodimer formation with the viral glycoprotein M is a crucial mechanism regulating the activity of UL49.5 during BoHV-1 infection, as I demonstrated in my doctoral research (publication in attachment 4, p. II, No 27). To understand the molecular aspects of UL49.5 functioning, I sought to investigate the interactions with glycoprotein M at the

molecular level, identifying the protein domains, motifs, or amino acid residues responsible for this binding. I employed an approach involving deletions in *UL49.5* and the *UL10* gene, which encodes gM, as well as site-specific mutagenesis. The research results were compiled in another publication from the series: Graul *et al.*, JGV 2019.

In this publication, I am the last author and corresponding author, and I supervised the team work during the research, including Małgorzata Graul as part of her doctoral work. *I was responsible for the research concept, data analysis, visualization, and preparation of the original manuscript version*. In this study, we demonstrated that **the cytoplasmic domains of both proteins are redundant** for the formation of the UL49.5-gM complex and its transport to the cell membrane. Instead, the transmembrane domain (gM has 8 transmembrane helices) and proper folding of the proteins seem to be crucial, occurring only when UL49.5 and gM form a complex. The cytoplasmic domain of gM is necessary for the endocytosis of the complex from the cell surface and its incorporation into BoHV-1 virions (endocytosis is considered a mechanism in alphaherpesviruses through which envelope proteins are delivered to sites of virion assembly). These studies required mastering the construction of viral mutants using site-specific DNA recombination in the form of a **bacterial artificial chromosome (BAC)**, a large plasmid maintained in *E. coli*. This technology is particularly useful for genetic modifications of the viral genome that impair its replication. I acquired the BAC technology through collaboration with **Prof. Mathias Ackermann from the University of Zurich in Switzerland**, where the doctoral student completed a research internship.

The redundancy of the cytoplasmic domain of gM for protein maturation and its positive validation by the ER quality control system was quite unexpected. The cytoplasmic domain of the glycoprotein, consisting of 32 amino acid residues, appeared as the most likely source of motifs responsible for retention in the ER or release from the ER. During these studies, scientific reports emerged regarding the role of **glycine motifs/glycine zippers**. A glycine zipper is a double glycine motif **GxxxGxxxG**, where the glycine residue can be replaced by another small residue such as alanine or serine. Therefore, we decided to investigate the sequences of the 8 transmembrane regions of gM and the TM region of UL49.5 using bioinformatics methods to search for potential glycine-like motifs. We identified three such motifs in gM and one in the TM region of UL49.5, which could explain the mechanism of interaction between both BoHV-1 proteins. The sequences encoding these motifs were subjected to site-specific mutagenesis, resulting in the replacement of both glycine or alanine residues in the motif with leucine, which is a standard method for these motifs. It turned out that changes within the **5th** (particularly) and **7th glycine motif** of gM significantly impaired the formation of the UL49.5 complex and its protective function, which involves competition with TAP. Since mutagenesis of the potential motif in UL49.5 did not affect complex formation, it appears that glycine motifs are responsible for maintaining the conformation of the gM transmembrane domain necessary for UL49.5 binding. During these studies, we also decided to use UL49.5 constructs with swapped domains from the VZV UL49.5 homolog published in Verweij, Lipińska *et al.*, Mol. Immunol. 2011. Additionally, using the sequence encoding the TM region of the **H5N1 influenza hemagglutinin** isolated in our laboratory from an infected swan, I prepared a construct allowing for the membrane anchoring of the N-terminal domain of BoHV-1 UL49.5 via the TM region of VZV UL or influenza HA. These constructs enabled us to determine that heterodimer formation between gM and the chimeric protein with the N-terminal domain of BoHV-1 UL49.5 anchored in the membrane by the TM region of VZV UL

or HA is possible, but only the region from the native BoHV-1 protein ensures proper maturation and intracellular transport of the heterodimer. Therefore, the role of the UL49.5 TM region seems to be more crucial for interaction with gM than with TAP. In the JMB paper, we also demonstrated that the PPQ region of UL49.5 is not necessary for binding to gM and maturation of the complex, unlike its interaction with TAP.

Ad.4

In the publication on the role of the PPQ motif, we demonstrated that the fluorescent TAP model can be used to study transporter degradation in the presence of UL49.5. **UL49.5 with mutations in the PPQ motif lost the ability to induce TAP degradation**.

Therefore, understanding the mechanism of TAP1/TAP2 degradation in the presence of BoHV-1 UL49.5 (and possibly its orthologs with the RGRG motif at the C-terminus) has become an important research task. Degradation does not seem to be a necessary process to inhibit the TAP transporter. Based on years of research, I am inclined towards the hypothesis that it is only the ultimate removal of transporter proteins that are already inhibited by UL49.5 binding. This hypothesis is supported by constructed variants of UL49.5 BoHV-1 with mutations altering salt bridges stabilizing the N-terminal domain in structural studies (NMR): R30DR31D (RR/DD) and D36K, which have unaltered inhibition of antigen transport but do not destabilize the level of TAP proteins. These results are still awaiting publication; we presented them at scientific conferences. I have decided to describe here the results of our studies on the degradation mechanism prepared as two manuscripts, currently under review in scientific journals. In these studies, we used the fluorescent TAP model published by us in Cells.

When planning the research, I considered two research hypotheses regarding UL49.5 - dependent TAP degradation: 1) UL49.5 freezes TAP in a conformation of the transporter that is susceptible to degradation, intensifying TAP removal through the ER quality control system associated with ER-associated degradation (ERAD); 2) UL49.5 serves as an adapter recruiting components of the ERAD pathway that cannot recognize TAP in the absence of the viral protein. To verify the hypotheses, we used **high-throughput screening** assays. The first approach was a screen based on **an interfering RNA (siRNA) library**, made possible through collaboration with **Dr. Roman Szczęsny** from the **Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw**, established at a national conference. In this team, sub-libraries of siRNA and a visualization method based on automated transfection and microscopic imaging of fluorescence changes (in our case - TAP-GFP) after silencing of individual genes from the sub-library defined as "**proteostasis - protein homeostasis**" were available. Luckily, this library, consisting of siRNA for 1920 human genes, included genes for **the E3 ubiquitin ligase**. It is the multisubunit **CRL2 ligase** based on the **culin-2** platform, along with the **elongin B and C** proteins. CRL2 recognizes various cellular proteins depending on the specific **substrate receptor** protein. Identification of this receptor and other participating proteins was aided by a screen based on a **lentiviral sgRNA library of the CRISPR/Cas9 system**. This study was performed by Magda Wachalska during her stay in **Prof. Ron Kopito's laboratory at Stanford University**. This internship initiated my collaboration with Prof. Kopito, an expert in protein degradation, which will certainly continue in the future to verify the role of other proteins indicated by the screen. Gene deletions in MJS-TAP2-GFP cells using the CRISPR/Cas9 method allowed us to identify the **KLHDC3 (Kelch domain containing**

protein 3) protein as the CRL2 receptor. This protein is involved in the recognition of **C-degrons**, unfavorable amino acid motifs at the C-terminus of proteins that promote their intensive degradation, usually dependent on the proteasome. In our case, KLHDC3 recognizes the RGRG motif of UL49.5, recruiting CRL2, which is a cytoplasmic protein. When the results are published, this will be the first report on the involvement of CRL2 in the ERAD system. The list of known protein substrates of KLHDC3 is short, so we added another one, and the first viral protein. Using structural approaches, **Dr. Magdalena Ślusarz** from Prof. Liwo's team **modeled KLHDC3 with a decapeptide representing the C-terminus of UL49.5 and also the full structure of UL49.5 in the ER membrane with KLHDC3**. These models indicate important interactions of the last glycine residue G95 and arginine residue R93 with KLHDC3 and explain why the KK/SS residues of UL49.5 (whose roles I verified in the Mol. Immunol. publication) are unavailable for CRL2-mediated ubiquitination - they are involved directly in KLHDC3 binding. As a result, TAP (we do not know yet which subunit) becomes the molecular target for ubiquitination and degradation, while another cellular ubiquitin ligase, HRD1 of the ERAD system, is responsible for UL49.5 degradation. I plan to continue experimental studies to verify the interaction with KLHDC3 by site-specific mutagenesis of KLHDC3. We would also try the crystallization of KLHDC3 with the peptide representing the C-terminus of UL49.5. According to *in silico* predictions, KLHDC3 binding may change the positioning of UL49.5 domains in the membrane, which should be verified experimentally - KLHDC3 is in complex with UL49.5 and TAP, but the temporal and spatial aspects of this interaction, the order in which it occurs, is another fascinating scientific question.

In my postdoctoral research, I combine the analyses of structural aspects of target proteins for a better understanding of their molecular mechanisms of action and interactions. I would like to "see" the UL49.5 complex with the TAP transporter and the KLHDC3 protein, as well as the UL49.5 and gM complex on a molecular level. My scientific goal is to attempt to develop an **experimental model for structural studies of the TAP complex** with UL49.5 BoHV-1 using **cryo-electron microscopy (cryo-EM)**. I have started preparations for gene expression of the complex in two eukaryotic systems - the **baculovirus system**, where the production of TAP alone has already been reported, and I have obtained the expression of the UL49.5 gene. However, I expect the usefulness of the protozoan *Leishmania tarentolae* system, where eukaryotic proteins with similar posttranslational modifications to those obtained in human cells can be produced in large quantities and homogeneously (which is a prerequisite for cryo-EM studies). The cryo-EM model may pose a challenge because, in addition to the three studied proteins in the ER membrane (isolated as microsomes), the synthetic antigenic peptide and ATP need to be added to the system. I will attempt to conduct the research in Poland, thanks to the collaboration with **Prof. Michał Szymański** from our faculty, at the National Synchrotron Radiation Center SOLARIS, Jagiellonian University in Krakow. Alternatively, research in the United States with **Dr. Jue Chen's** team at the Howard Hughes Institute is also an option. Dr. Chen has already obtained the cryo-EM structure of TAP with the ICP47protein (the only available experimental structure of TAP), and I have established contact with her while writing a publication on the structure of UL49.5 docked to TAP.

I have received funding for research on the molecular mechanism of UL49.5 action through two research grants. After maternity leaves, in 2011, I received a grant under the 2nd Edition of the POMOST Program of the Foundation for Polish Science ("*Exploring new immune evasion mechanisms of herpesviruses - the search for improvement strategies of anti-herpesviral vaccines and virus-based therapeutics*"), and then in 2015, a grant under the

SONATA BIS4 Program of the National Science Center ("*Molecular mechanism of action of alphaherpesvirus proteins crucial for modulating the immune response*").

As my most important achievements presented in this series of papers, I consider:

- Adding UL49.5 orthologs from FHV-1 and CHV-1 to the list of UL49.5 proteins capable of inhibiting the TAP complex,
- Obtaining a 3D model of the BoHV-1 UL49.5 protein based on experimental studies,
- Indicating the important role of the PPQ motif in BoHV-1 UL49.5 in TAP binding and inhibition, although further research is needed for a complete understanding of the mechanism,
- Demonstrating that glycine zippers in the TM regions of the glycoprotein M play a crucial role in heterodimer formation with UL49.5, regulating the activity of UL49.5 during infection,
- Explaining that UL49.5-gM interactions and ER complex maturation/export are controlled by the structure of the transmembrane domain, not specific signal sequences,
- Development of a cellular model and methodology for studies of virus proteins acting as inhibitors of TAP.

Additionally, outside the series:

- Identifying cellular proteins responsible for TAP degradation induced by UL49.5 and for UL49.5 degradation itself.

Importance of the obtained results for the development of the field of science and the discipline of biotechnology:

Understanding the molecular mechanism of action of alphaherpesviral UL49.5/gM proteins in the context of the immune response is significant on several levels. **1)** It allows for a better understanding of the pathogenesis of specific viruses and indicates how we can biotechnologically utilize this knowledge, for example, by planning improved vaccines or antiviral drugs. My results indicate that BoHV-1 inhibits the TAP complex and induces its degradation in the early phase of infection (5-11 hours), while in later phases, other immunomodulatory proteins (such as the viral ribonuclease vhs, the product of the *UL41* gene) become more important. These results correlate with observations made by other research groups regarding the course of the immune response in infected cattle, where early and very early viral antigens play a crucial role, and later on, TAP no longer effectively performs its role in antigen presentation. As a result, in preparing a grant application to the European veterinary network ERA-NET-ICRAD for the development of BoHV-1 vaccines based on new antigen

expression platforms, I proposed including the immediate-early protein Circ of BoHV-1 as a second antigenic protein.

2) BoHV-1 UL49.5 is used in research on new vectors for **human anticancer therapy**, known as oncolytic vectors. They are based on the HSV-1 virus, in which the gene encoding the immunomodulatory protein ICP47 is replaced with UL49.5. Understanding the mechanism of UL49.5 activity is important in this case to better predict how such vectors will function in therapy.

3) Research tools, such as the TAP-GFP platform for studying the MHC class I-dependent antigen presentation pathway, is quite versatile and can be used to study the immunomodulatory properties of other viruses. The methodology that I developed can be applied in my current research, which involves studying **the immunomodulatory properties of SARS-CoV-2 proteins within the framework of the OPUS NCN project**. I primarily examine the ability of individual coronavirus proteins to inhibit antigen presentation pathways (using TAP-GFP). Many of these viral proteins have multiple transmembrane regions, so I can also benefit from my experience with gM as a transmembrane protein.

4) Viral proteins are often valuable tools that help us understand cellular molecular pathways. Viral proteins usually follow the same rules, pathways, mechanisms, and utilize the same enzymes as cellular proteins. The activity of the ERAD pathway was initially discovered thanks to viral proteins that hijack this machinery for viral propagation and immune evasion, similar to how UL49.5 BoHV-1 hijacks the cellular C-degron pathway and ERAD for TAP removal. The only known structure of TAP to date was obtained in complex with the HSV-1 ICP47 protein, which stabilizes the complex. However, this structure represents only one conformation of TAP. Using UL49.5, we may be able to explore another structural conformation of TAP and confirm the existence of three conformations of this transporter.

5. Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions.

My first international internship took place during my master's studies (a 2-month internship at the Institute for Animal Health in Compton, United Kingdom), followed by a scientific scholarship at the International Agriculture Centre (IAC) in Wageningen (a 3-month internship at the Institute for Animal Health and Science in Lelystad, the Netherlands, in Dr. Frans Rijsewijk's laboratory). From 2002 to 2004, I joined Prof. Emmanuel Wiertz's team at the Department of Medical Microbiology, Leiden University Medical Center in Leiden, the Netherlands, and spent several months there (3 months + 5 months + 5 months) finalizing experiments for my doctoral thesis (resulting in a dual affiliation in the publication No 28 of the total achievements compilation). This internship was funded by two FEBS (Federation of European Biochemical Societies) scholarships that I obtained**: the FEBS Summer Fellowship** and **the FEBS Scholarship for Central and Eastern Europe**. The research in Leiden also initiated a long-term international collaboration with Prof. Wiertz's team, resulting in four joint publications after obtaining my doctoral degree. During my stay in Leiden, I began preparing constructs for herpesviruses other than those described in my thesis and started planning UL49.5 mutants with point mutations to verify their impact on the protein's activity. I also prepared mouse cell lines with BoHV-1 UL49.5 because this protein uniquely blocks as well the mouse transporter. I have attached a statement from Prof. Wiertz confirming the role of this internship in my postdoctoral career as **Attachment 3.3.**

Additionally, the scientific collaboration and friendship established with **Dr. Danijela Koppers-Lalic** (the first author of publication No 28 in the total achievements compilation in Attachment 4) during my time in Prof. Wiertz's team led to further collaboration on the topic of exosomes and three joint publications after my doctorate when Dr. Koppers-Lalic moved to the Erasmus Medical Center in Rotterdam, the Netherlands. I began studying the role of exosomes in alphaherpesvirus infection as part of my SONATA research project, where I was a leader.

6. Presentation of teaching and organizational achievements as well as achievements in popularization of science or art.

Teaching activity has constituted a very important component of my work at the Intercollegiate Faculty of Biotechnology since the beginning of my employment. I continue to develop my teaching skills by participating in pedagogy training provided by the Center for Teaching Excellence and Tutoring at the University of Gdańsk. I also incorporate innovative teaching methods into the classes I teach for students at the Intercollegiate Faculty of Biotechnology (at the Ist and IInd undergraduate levels), the Bioinformatics at the Faculty of Mathematics, Physics, and Informatics (lectures and laboratory exercises for the Cell Biology and Metabolism course at the undergraduate level), the Faculty of Pharmacy at GUMed (lectures and seminars for the "Viruses as Sustainable Drug Targets and Pharmaceutical Platforms" course, conducted in English for the International Master in Sustainable Drug Discovery - S-DISCO program), and the Marine Biotechnology program jointly offered by the Intercollegiate Faculty of Biotechnology and the Faculty of Oceanography and Geography at the University of Gdańsk (an English-language course at the IInd level titled "Principles of Molecular and Cellular Biology"). Additionally, I will supervise master's students in the framework of the **European University of the Seas (SEA-EU)** network, joined by the University of Gdańsk. I was a member of a working group that developed two educational programs: a Bachelor's degree program in Biotechnology based on thematic modules (for this work, the team and I were awarded the First Degree Team Award by the Rector of the University of Gdańsk), and a new English-language program in Marine Biotechnology mentioned earlier. I actively participate as a lecturer, supervisor of master's theses, and supervisor of summer internships for students enrolled in the S-DISCO international program, collaborating in this regard with the Faculty of Pharmacy at GUMed. I have developed curriculum programs for various courses, including the aforementioned English-language programs, the laboratory exercises for the Protein Biochemistry Lab for the Intercollegiate Faculty of Biotechnology students (where I frequently lead English-speaking groups of international exchange Erasmus students, contributing to the internationalization of my university), the Laboratory of Virology (of which I am the initiator), Model Organisms - Mammalian Cells (seminars and laboratory exercises), Applications of Viruses in Biotechnology and Medicine (lectures), Seminar on Experimental Publications in Molecular Biology and Biotechnology (where I also lead English-speaking groups), lectures on Systematics and Taxonomy of Organisms, Overview of Microorganisms (protists, cyanobacteria, and viruses), and Viral Plant Pathogens. I previously conducted methodological seminars on Molecular Biology Methods for the Ist level Biotechnology program. For both laboratory exercises, I am a co-author of Polish and English-language scripts for students. A significant achievement in my teaching career was the nomination for the Academic Teacher of the Year competition organized by Dziennik Bałtycki newspaper in 2021. I am also a co-

author of a teaching-related poster presentation titled "Boosting the Learning of Biotechnology by Concept-Based Teaching" presented at the 45th FEBS Congress "Molecules of Life: Towards New Horizons" in 2021.

In recognition of my scientific and teaching activities, I received **the Medal of the National Education Commission (KEN) in 2022**.

From January 2021 to June 2022, I supervised a scientific **Student Minigrant project** focused on studying SARS-CoV-2 proteins as part of the ProUG project ("PROgram Rozwoju Uniwersytetu Gdańskiego") co-financed by the European Union through the European Social Fund. The Minigrant was carried out by four undergraduate Biotechnology students selected in a competition. I annually supervise specialization laboratories for bachelor's and master's students, supervising their thesis work at the undergraduate level (bachelor's) and guiding laboratory work and thesis projects for master's students. In total, I have supervised around **19 bachelor's theses and 25 master's theses**.

I have served as an **assistant supervisor for three completed doctoral dissertations**: **Magdalena Graul** (completed in 2019), **Kinga Grabowska** (2022), and **Magda Wachalska** (2022). Currently, I remain an assistant supervisor for three ongoing doctoral processes (Dorota Lesiak, Marcin Lubocki, Michalina Michalska) within my research team at the Department of Molecular Biology of Viruses.

To **popularize science**, I regularly participate in the **Baltic Science Festival** and have been a member of the organizing team for this event at the faculty level, for which the whole organizing committee was honored with the First-degree Team Award from the Rector of the University of Gdańsk. I have delivered popular science lectures, including topics such as "No Secret at All: What Do COVID-19 Vaccines 'Hide'?" in 2021 and "About 'Jumping' Viruses - Why They Can Leap and Surprise Us" in 2022, during the science popularization event "**Night of Biologists**." I have also given lectures to secondary schools. In 2018 I was a member of the Main Committee organizing a competition for secondary schools “A Quiz on Vaccines”.

In terms of **organizational activities** at the University of Gdańsk, I am or was a member of several faculty committees (Zespoły), including the Faculty Committee for Ensuring the Quality of Education, the Faculty Committee for Biological Safety and the Use of GMOs and GMMs, the Faculty Committee for the Evaluation of Academic Teachers, the Faculty Committee for Conducting Qualifying Interviews in English for the first year of second-degree studies, and I serve as the chair of examination committees during undergraduate diploma examinations. At the university level, I am or was a member of various commissions (Komisje), including the University Commission for Ensuring the Quality of Education, the University Commission for the Statute of the University of Gdańsk, and in the 2019-2024 period, I hold the position of **Senator** at the University of Gdańsk.

7. *Apart from information set out in 1-6 above, the applicant may include other information about his/her professional career, which he/she deems important.*

a) Other scientific research activities:

In addition to the research that formed the basis of the publication series described in section 5 of this Summary, I served as the Project Leader for research projects on different topics that yielded scientific publications:

A project titled "*Exploring new immune evasion mechanisms of herpesviruses – the search for improvement strategies of antiherpesviral vaccines and virus-based therapeutics*" funded by the **Foundation for Polish Science** within the **POMOST/2010-2/7** program (project duration: July 1, 2011, to September 30, 2014, budget: 554,000 PLN). In addition to studying the molecular mechanism of the UL49.5/gM complex, I investigated the immunomodulatory properties of the serine-threonine kinase US3 of BoHV-1 and PRV viruses, resulting in publications No 14, 20 (Appendix 4); one manuscript is still awaiting publication.

A project titled "*Exploring the role of exosomes in the course of alphaherpesvirus infections*" within the **SONATA 2** program of the **National Science Centre** (project duration: August 6, 2012, to December 5, 2015, budget: 759,615 PLN). The project resulted in publications No 7, 8, 17 (Appendix 4). In this project, I investigated the incorporation of proteins from HSV-1, BoHV-1, and PRV viruses into **exosomes** released during infection and in stable cell lines producing homologous **glycoproteins B** of these viruses. Glycoprotein B (gB) is a highly conserved herpesvirus protein responsible mainly for host cell entry. My research contributed to understanding its role in later stages of infection when gB is produced for incorporation into virions. Additionally, I discovered that it is incorporated into extracellular vesicles that include exosomes. Furthermore, its presence in cells expressing MHC class II molecules reduces the surface levels of these MHC. I compared these properties among the three investigated alphaherpesviruses, demonstrating that gB of BoHV-1 exhibits immunomodulatory properties, albeit weaker than that of HSV-1, while gB of PRV did not show such properties. I also demonstrated the incorporation of other viral proteins (e.g., glycoprotein gD) into extracellular vesicles and the ability of these vesicles to interact with antibodies in serum produced in response to BoHV-1 infection, indicating their potential role as decoys affecting the course of infection. This project required expertise in working with exosomes and methods such as size exclusion chromatography, ultracentrifugation (including iodixanol gradient), and transmission electron microscopy (through collaboration with **Dr. Magdalena Narajczyk** from the Electron Microscopy Laboratory at the University of Gdańsk). During this project, thanks to a research internship at Professor Wiertz's laboratory, I established collaboration with **Dr. Irene Bijnsdorp** from the Amsterdam University Medical Center in Amsterdam, the Netherlands, which provided valuable protocols and enabled us to optimize our research methods on urine-derived exosomes.

Currently, I am leading the **OPUS 21 NCN** project titled "*Immunomodulatory properties of SARS-CoV-2 proteins*" (project duration: July 8, 2021, to September 30, 2025, budget: 1,908,360 PLN). In this project, my main focus is to investigate the ability of individual SARS-CoV-2 proteins to inhibit antigen presentation pathways, aiming to identify their immunomodulatory properties.

In addition to my own research projects on different topics, I am happy to collaborate with other research teams, which has so far resulted in publications No 2, 5, 9, 13, 15, 16, 18, 19 (Appendix 4). These collaborations include international ones (e.g., production of antibodies for sigma reovirus protein detection, position No 18 in Appendix 4) and collaborations with other national units and within the faculty (e.g., testing antiviral activity of metal compounds

and other biologically active compounds, cytometric analysis, and stable cell line construction using retroviral vectors in cancer research, position No 19 in Appendix 4).

Reviews in scientific journals:

According to the register of my reviews in the Publons/Web of Science database, I have prepared 23 reviews of articles for international scientific journals such as Nature Communications, Journal of Applied Genetics, Cells, Viruses, Microorganisms, Biomolecules, Pharmaceutics, Membranes, Pharmacological Reports, and Veterinary Research. I am a member of the Reviewer Board for the journal Membranes (MDPI) and, together with **Dr. Magdalena Weidner-Glunde from the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn**, an editor of special issues ("Herpesvirus Manipulation of Cellular Processes" and "Herpesvirus Manipulation of Cellular Processes 2.0") in the journal Viruses (MDPI).

Membership in scientific societies and organizational committees:

I am a member of the Polish Biochemical Society, the European Society of Veterinary Virology, and the International Society of Extracellular Vesicles. In 2013, I was a member of the Organizing Committee for the 36th International Herpesvirus Workshop held in Gdańsk, and in 2023, for the European Virology Congress, also held in Gdańsk, where I also served as a session chair.

Collaboration with the business environment:

During the COVID-19 pandemic, I responded to the need for collaboration with the business environment by testing the virucidal properties of a disinfectant for AVANTI company and evaluating the antiviral properties of a virus-resistant material for potential mask production.

b) Participation and leadership in grants, received scholarships, honors, and scientific awards after obtaining a doctoral degree:

Chronologically, starting with the most recent achievements and events:

2022 - Medal of the National Education Commission (KEN).

Since 2021 - Leading the research project OPUS 21 NCN "*Immunomodulatory properties of SARS-CoV-2 proteins*" as described above.

2019 - First-degree Team Award from the Rector of the University of Gdańsk for the development of a new and innovative Education Program at the Intercollegiate Faculty of Biotechnology.

2016 - Second-degree Team Award from the Rector of the Medical University of Gdańsk for research on the mechanism of interaction between the FGFR2 receptor and RSK2 kinase and their role in the migration of breast cancer epithelial cells.

2015-2021 - Leading the research project SONATA BIS 4 NCN "*Molecular mechanism of action of alphaherpesvirus proteins key to modulation of the immune response*." This was the main project funding the research that formed the basis of the publication series

described in section 4 of the Summary. In addition to studying UL49.5/gM proteins, this project investigated the role of HSV-1 viral microRNAs in infection, introducing the research on viral microRNAs.

2012-2015 - Leading the research project SONATA 2 NCN "*Exploring the role of exosomes in alphaherpesvirus infections*" as described above.

2011-2014 - Leading the research project POMOST FNP "*Exploring new immune evasion mechanisms of herpesviruses – the search for improvement strategies of antiherpesviral vaccines and virus-based therapeutics*" as described above.

2011 - Second-degree Team Award from the Rector of the University of Gdańsk for scientific achievements supported by scientific publications.

2010 - First-degree Team Award from the Rector of the University of Gdańsk for coordination of works within the Science Festival at the Faculty in two past periods.

2010 - Plenary Lecture at the Conference of the Polish Society for Microbiology "Microbiology 100 Years After Robert Koch."

2009 - START Scholarship from the Foundation for Polish Science.

2006 - First-degree Team Award from the Rector of the University of Gdańsk for scientific achievements supported by scientific publications.

2006 - Distinction (*cum laude*) for the doctoral dissertation by the Intercollegiate Faculty of Biotechnology Board.

Signed by /
Podpisano przez:

Andrea Diana
Lipińska
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Date / Data:
2023-07-31 22:37

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(Applicant's signature)