UNIVERSITÀ DEGLI STUDI DI PADOVA

ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

UNIVERSITÀ DEGLI STUDI DI PADOVA
Department of Biomedical Science

Interuniversity Bachelor's degree programme
in Biology of Human and Environmental Health

Final dissertation

DEVELOPMENT OF AN ONCOLYTIC AGENT BASED ON THE HERPES SIMPLEX VIRUS TYPE 1 EXPRESSING THE KILLER RED PROTEIN TARGETED TO THE PLASMA MEMBRANE

Supervisor
Professor Arianna Calistri
Department of Molecular Medicine

Candidate
Maddalena Femminella
Student ID number: 2067434

Co-supervisor
Dott. Alessandra Rossetto

ACADEMIC YEAR 2024/2025

Table of Contents

Abstract 3

Introduction 4

Virotherapy as a new cancer treatment 4

Oncolytic Viruses 4

Herpes Simplex Virus type 1 5

Photodynamic therapy: Killer Red protein 6

Killer Red protein targeting the plasma membrane 7

Purpose of the study 8

Materials and Methods 9

Plasmid pCeu-memKR 9

BAC pHSV1(17+)Lox-sacB-ΔICP34.5Zeo-ΔICP47-mir124res 11

Electroporation of GS1783 cells 13

Red Recombination 13

Selection of GS1783 bacterial clones expressing the *KanR* gene 14

Verification PCRs for the selection of GS1783 bacterial clones not expressing the *KanR* gene but the *mem-KR* gene 14

Resolution of recombinant BACs in selected GS1783 colonies 15

Selection of resolved GS1783 colonies by replica picking 16

Resolved BAC DNA extraction 16

oHSV-1 *mem-KR* reconstitution via cell transfection in Vero CCL81 cells using Lipofectamine 3000 17

Viral stock amplification in Vero cells CCL81 18

Viral particles extraction and purification 19

Results 20

Plasmid pCeu-memKR digestion 20

BAC pHSV1(17+)Lox-memKR-INT-ΔICP34.5Zeo-ΔICP47-mir124 20

GS1783 bacterial colonies bearing the resolved BAC BAC pHSV1(17+)Lox-memKR-RES-ΔICP34.5Zeo-ΔICP47-mir124 solely in medium lacking Kanamycin 21

Bacterial colonies bearing the resolved BAC pHSV1(17+)Lox-memKR-RES-ΔICP34.5Zeo-ΔICP47-mir124 produce amplicons of dimensions inferior to the colonies containing the unresolved ones 22

Transfection efficiency was assessed through Killer Red expression and cytopathic effects onset 22

Efficiency of viral amplification in Vero CCL81 cells was highlighted by cytopathic effects and Killer Red expression 23

Conclusion ***24***

Bibliography ***25***

Abstract

Cancer virotherapy is a promising approach for the treatment of tumours and solid neoplasms, especially those with poor prognosis and limited effectiveness of conventional therapeutic approaches (surgical resection, chemotherapy and radiotherapy).

The underlying mechanism of oncolytic virotherapy is based on oncolytic viruses (OV), viruses capable of infecting and replicating selectively in cancer cells. These viruses can act with two distinct mechanisms of action: they can directly induce cancer cell lysis, as a result of the cytopathic activity of viral particles, and/or trigger an immune response in the body.

In this study, an oncolytic virus based on the Herpes Simplex Virus type 1 (oHSV-1) was developed, genetically engineered to express the Killer Red protein targeted to the plasma membrane. Killer Red is a red fluorescent protein which, when photoactivated at a certain wavelength, stimulates the production of reactive oxygen species (ROS) within cancer cells, triggering the process of "photobleaching". Among the different isoforms of the protein, the membrane isoform was selected as being, as indicated in the literature, more effective than the mitochondrial counterpart in exercising the phototoxic action.

The virus produced, combining its cytopathic and immunotherapeutic activity with the phototoxic activity of the KR protein, is therefore proposed as a promising therapy for treating several forms of particularly aggressive cancers.

Introduction

Virotherapy as a new cancer treatment

Tumours are the result of an abnormal growth of body tissues. These anomalies typically arise from genetic mutations, either inherited or acquired, that disrupt the regulatory pathways within the affected cells, ultimately leading to uncontrolled cell behaviour. The mutations, primarily found in genes that govern cell growth, division, and programmed death, contribute to the formation of masses and the establishment of a tumour microenvironment, often followed by the invasion of surrounding tissues. As a result, affected cells become immortal, invasive, and resistant to physiological signals due to their loss of responsiveness. Cancer is therefore recognised as a multifaceted disease, involving a complex interplay of molecular and pathological mechanisms rooted in both genetic and environmental factors. Consequently, cancer treatment encompasses a diverse range of approaches, from surgical resection of the tumour to the use of targeted drugs and treatments designed to eliminate or manage the disease.

Despite the variety of available treatment options, there’s an urgent need to develop therapies that are significantly more effective than chemotherapy, less intrusive than surgery, or safer than radiotherapy. In response to these challenges, research efforts have increasingly focused on innovative strategies, particularly virotherapy, which offers promising solutions to some of the limitations posed by current treatments. (1)

Virotherapy, which involves the use of viruses, natural or genetically modified, to specifically target and destroy cancer cells through replication, has demonstrated versatility as an effective treatment for numerous cancers, particularly in those cases in which conventional therapies have limited success.

Oncolytic Viruses

Oncolytic viruses (OV) represent a promising approach for the treatment of different forms of cancer. Their potential as therapeutic agents lies in their ability to replicate preferentially within cancerous cells, thereby limiting their effect on healthy cells or tissues. This characteristic is the result of different factors, including abnormalities in antiviral pathways of tumour cells, which favour and promote viral replication. Another key factor is the localized suppression of the immune responses within the tumour microenvironment, which

allows viruses to infect cancer cells more easily. Additionally, the high metabolic activity of cancer cells, needed to sustain tumour proliferation, creates an ideal environment for viral replication, supplying the virus with all the resources and the machinery it needs to efficiently carry out its lifecycle. (2)

OV can harm the cells using two mechanisms, direct and indirect, intrinsic to their nature as pathogens. Firstly, the virus infects and kills the cell while replicating, as part of its replication cycle. Secondarily, as a consequence of the damage caused, cytokines, danger-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) are released from the lysis of the cells, as well as tumour-associated antigens (TAAs). In this way, the immune system is alarmed and triggered to induce a systemic response, directed not only towards the infected cells but also to the uninfected tumour cells, amplifying the overall therapeutic efficacy of these agents and highlighting their potential as powerful tools in cancer therapy, including metastatic diseases. (3)

Different types of viruses have been used as models to produce oncolytic agents; among these, the most popular ones are Adenoviruses and Herpes Simplex Virus type 1 (HSV-1). These strains both lend themselves well to oncolytic therapy thanks to their large genome size, which allows for the insertion of modifications, and their strong cytolytic effect. The viruses undergoing clinical trials are many, though just four of them were approved for treatment: *Tlimogene laherparepvec* (T-VEC), an oncolytic virus based on Herpes Simplex Virus Type 1 used for the treatment of not resectable melanoma, *H101, Teserpaturev*, and lastly *Rigvir*. It is evident, therefore, that oncolytic viruses require further advancements to be developed into the stronger therapeutic agents they have the potential to become. (4)

Herpes Simplex Virus type 1

The Herpesviridae family is a large family of viruses divided into three major subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. The viruses belonging to the first subfamilies are Herpes Simplex type 1 (HSV-1) and type 2, and Varicella Zoster. They are all characterised by large double-stranded DNA genomes, ranging in length from 125 to 240 kbp.

More specifically, HSV-1 is an encapsulated virus, with a DNA which encodes 83 genes, and of length of around 150 kbp, involved in different viral processes: replication, virulence and host cell modulation. In its genome we can identify two components, L (long) and S (short), that divide the molecule into the regions U_L and U_S, made both of unique sequences

containing inverted repeated elements, terminal (TR_L and TR_S) and internal (IR_L and IR_S). (5)

Figure 1 Representation of the HSV-1 genome*, from Expasy (6).*

The 30 kbp sequences that are non-essential for viral infection, combined with the lack of genome integration, its ability to infect a broad range of different cells, and its strong lysis property, make HSV-1 a promising candidate for the development of oncolytic viruses. (4) Usually, HSV-1 replicates in epithelial cells of the oral mucosa, causing cold sores; from this region, through a retrograde axonal transport, it reaches the body of different neurons (trigeminal ganglia and others) where it undergoes a latent lifecycle. However, the ability of HSV-1 to invade the central nervous system can induce the development of several neuronal complications, such as viral neuroenchephalitis. Therefore, when it is used as OV, it is important to remove the genes responsible for its neuroinvasive capabilities. In particular, to obtain its neuroattenuation, two genes are deleted: *Us12* and *γ34.5*. The *γ34.5* gene encodes for a protein, ICP34.5, which interferes with host cell processes, such as protein translation and anti-viral response, necessary for HSV-1 replication; while the *Us12* encoded protein mediates immune evasion via inhibition of antigen presentation. Both these removals limit HSV-1's ability to cause neurotoxic effects and enhance their safety in healthy tissues. (7)

Photodynamic therapy: Killer Red protein

Photodynamic therapy has the potential to be a novel treatment for cancer and other diseases, as a less invasive approach compared to the already existing ones. It uses three main components: light, oxygen and a photosensitizer (PS). Photosensitizers are molecules that, when excited at a specific wavelength, emit energy and transfer it to substances around, mostly oxygen, leading to the formation of reactive oxygen species (ROS). This process can take place through two different mechanisms: type I and type II reactions. During the former ones, the PSs interact with surrounding biomolecules, producing mainly

superoxide ion and hydroxyl radicals; in the latter ones, instead, the transfer of electrons reaches molecular oxygen directly, forming singlet oxygen $^1O_2$. (8)

Killer Red protein is an example of a photosensitizer, genetically encoded and derived via mutagenesis from anm2CP, a non-fluorescent chromophore found in jellyfish. It is a red fluorescent protein, absorbing at the spectrum of yellow-green light [540-580nm] and emitting at a maximum of 610nm, in the red region. (9)

It is the first fluorescent protein designed to have high phototoxic activity, able to express toxicity and effectively kill cells only when excited, and not in the dark. When activated, it produces ROS through type I reactions, another feature that makes this protein suitable for cancer therapy. Indeed, type I reactions, compared to type II, can be carried out even in hypoxic conditions, usually typical of tumour environments. Moreover, the ROS produced not only damage cancer cells but are responsible for protein degradation of Killer Red itself, triggering a process called "Photobleaching", which is used as an indicator of the efficacy of the therapy: the chromophore produced reactive oxygen species, while emitting fluorescence, which diminishes gradually as PS activity increases. This ensures a controlled action of the therapy, without causing adverse toxicity to the surrounding tissue. So, it is a mechanism doctors can use as a predictive marker of the treatment progress. (10)

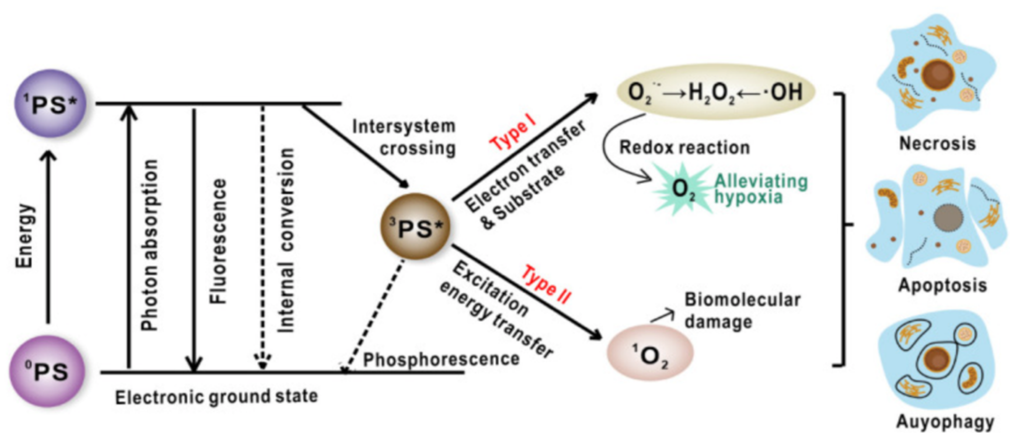

Figure 2 Mechanism of ROS generation in photodynamic therapy*, from J. Liu et al., 2021(8)*

Killer Red protein targeting the plasma membrane

Another important feature to make PDT even more efficient is establishing the location of the photosensitiser. Especially when using a genetically encoded PS like Killer Red, targeting its position to a specific cellular compartment is easily achievable through the addition, to the aminoacidic sequence, of signalling peptides. A good target for a

photosensitiser can be the plasma membrane, which is the structure responsible for protecting cell integrity and for its survival. The damage caused by the production of reactive oxygen species at the cell membrane leads to the peroxidation of the lipids forming its bilayer, disrupting their conformation and inducing the rupture of the plasma membrane, ultimately resulting in cell necrosis. (8)

Purpose of the study

In this study, we developed an oncolytic agent based on Herpes Simplex Virus type 1, modified to express the photosensitiser Killer Red targeted to the plasma membrane. The virus obtained could be effective in the treatment of tumours, exploiting its lytic activity and the phototoxicity caused by the action of Killer Red.

Materials and Methods

Plasmid pCeu-memKR

The plasmid pCeu-memKR [Figure 3] is a plasmid of length 5555 bp, designed to contain the gene encoding the membrane isoform of the Killer Red protein, our gene of interest. The plasmid serves to obtain the fragment containing both the sequence of Killer Red protein and also *KanR*, kanamycin resistance gene. It is defined by genetic elements specific to it:

- Gene sequence of membrane Killer Red protein (*mem-KR*): it is a gene of length 774 bp, encoding for the membrane isoform of the Killer Red photosensitiser;
- Human Cytomegalovirus (CMV) promoter regulating *mem-KR* expression: it is an immediate-early viral promoter, widely used in laboratories thanks to its strong constitutive activity and ubiquitous expression in many different tissues, producing a stable and lasting synthesis of the protein it regulates;
- Gene sequence for resistance to Kanamycin (*KanR*): Kanamycin is an aminoglycoside antibiotic, bactericidal against Gram-negative bacteria. It works by inhibiting the bacterial protein synthesis, interfering with their ribosomes and therefore blocking the translation process prematurely. The resistance gene encodes for *APH*, *aminoglycoside phosphotransferase*, an enzyme needed to inactivate kanamycin activity, by the addition of a phosphate group to modify its structure. When adding the gene *KanR*, the expression is used as a selection marker, discriminating between bacteria containing the fragment and those that do not;
- 2 restriction sites for *I-CeuI*: *I-CeuI* is a restriction homing endonuclease recognising non-palindromic sequences of length around 26 bp (5′-TAACTATAACGGTCCTAAGGTAGCGT-3′), making it very specific and useful for the digestion of large fragments. Indeed, the cut obtained from these two sites will allow us to get a plasmidic fragment of 2898 bp, including both the genes of *mem-KR* and *KanR*;
- One restriction site for *AhdI*, a type II restriction enzyme: it recognises the sequence 5'-GACNNNNNGTC-3', a palindromic sequence, cutting symmetrically at a central position (N), generating blunt ends.

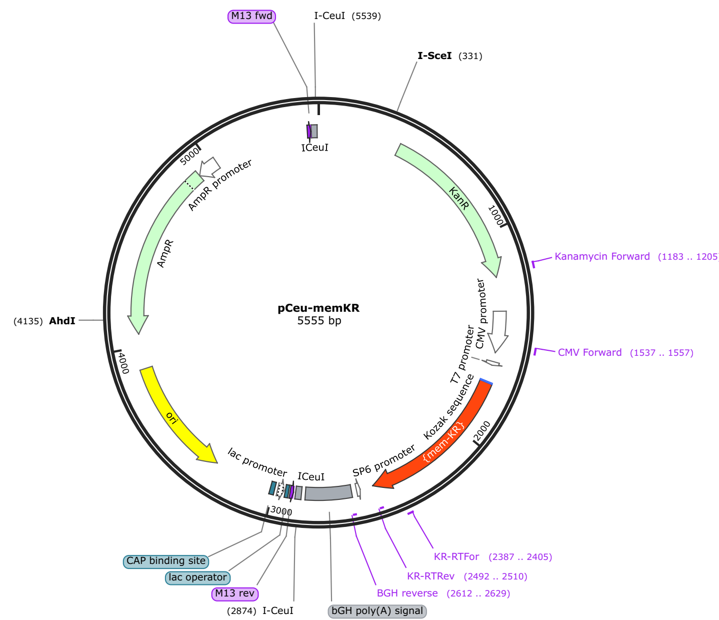

***Figure 3 Map of the plasmid pCeu-memKR**, Snapgene (https://www.snapgene.com/).*

Plasmid pCeu-memKR was utilised in the first phases of the experiment. Its digestion was carried out using two different restriction enzymes, *I-CeuI* and *AhdI*, obtaining a fragment of around 2898 base pairs containing the genes *mem-KR* and *KanR*. The reaction was conducted at a temperature of 37 °C for 3 hours, followed by 2 hours of enzymatic reinforcement at 37 °C. The two products were then separated and analysed using gel electrophoresis on a gel containing 1% agarose.

Subsequently, the fragment of our interest was extracted from the gel and isolated following the protocols described in the *GeneJET™ Gel Extraction Kit*.

By using solely *I-CeuI*, the plasmid would have been digested into two fragments of too similar length; therefore, the addition of the second enzyme, *AhdI*, was necessary for the correct analysis of the products. In this way, the second fragment obtained was further processed into two smaller pieces, clearly distinguishable on the gel.

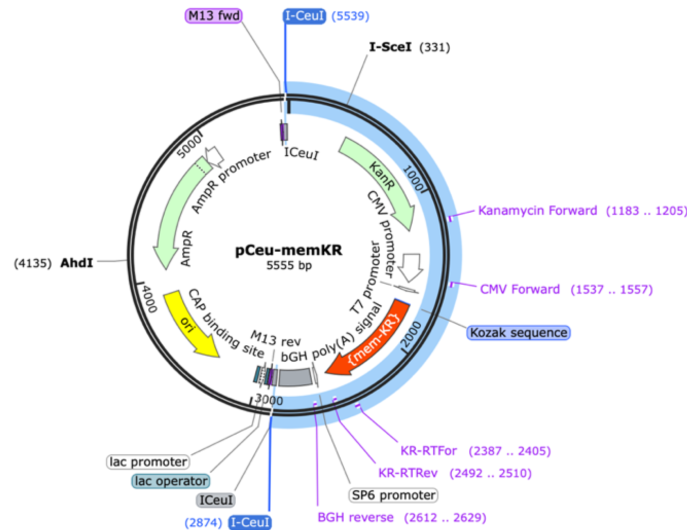

***Figure 4 Plasmidic fragment obtained with the digestion by I-CeuI and Ahd**, Snapgene (https://www.snapgene.com/).*

BAC pHSV1(17+)Lox-sacB-ΔICP34.5Zeo-ΔICP47-mir124res

BACs, also known as bacterial artificial chromosomes, are cloning vectors that can harbour large DNA sequences and, being derived from the F plasmid of E. Coli, can be easily manipulated using established mutagenesis techniques (e.g. Red Recombination). For these reasons, they are widely used to clone viral genomes: they are especially suitable for many herpes viruses, which possess a genome of around 150 kbp.

The BAC pHSV1(17+)Lox-sacB-Δγ34.5Zeo-ΔUs12-mir124res, indeed, contains the whole genome of HSV-1, including several modifications introduced to optimise its function as an oncolytic agent, and at the same time to attenuate its neuropathic activity. In particular, it presents:

- Deletion of both copies of gene *γ34.5*: this modification is widely used in the manipulation of the Herpes Simplex Virus genome, in order to block the synthesis of neurovirulence factors;
- Deletion of the gene *Us12*, encoding for the ICP47 protein, involved in immune response evasion through inhibition of antigen presentation by infected cancer cells;
- Insertion of the gene sequence of *SacB* in the region *UL55-UL56*, under the control of the immediate early CMV promoter. *SacB* is a selectable marker which encodes *levansucrase*, a toxic molecule for Gram-negative bacteria cultured in the presence of sucrose. This region is particularly interesting to our experiment, being the insertion site of the Killer Red protein gene;

- Insertion of binding sites for *mir124*, a microRNA which is expressed in neurons but is low or absent in tumorigenic ones, to downregulate the expression of *UL29* gene. This gene codifies for ICP8 protein, a molecule that is involved in viral genome replication. The addition of *mir124* is made to reduce viral replication in healthy neurons; indeed, it binds to the *UL29* viral transcript and suppresses its translation, blocking the protein synthesis. It helps to decrease its neurotoxicity;
- Addition of a cassette containing the gene for *chloramphenicol* (CHL) *resistance*, used as an additional selection marker. It synthesises an enzyme called *CAT*, *chloramphenicol acetyl transferase*, which inhibits the antibiotic function of blocking bacterial ribosomes by adding acetyl groups from Acetyl Coa (co-substrate of this reaction) to *CHL* hydroxyl residues, making it inactive. It also carries the sequence for Cre recombinases, enzymes which perform site-specific recombination, placed under the control of an eukaryotic promoter. The whole cassette is delimited by two *LoxP* loci, allowing for its spontaneous removal inside eukaryotic cells.

This BAC was previously inserted into GS1783 cells, a strain of E. Coli characterised by the presence of genes involved in the process of Red Recombination, and by the sequence coding for I-SceI, a homing endonuclease known for creating double-strand breaks, placed under the control of an arabinose inducible promoter.

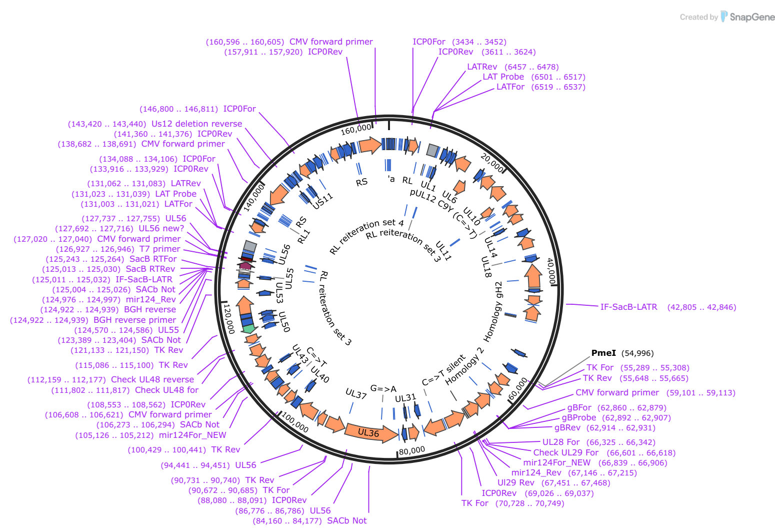

***Figure 5 Map of BAC pHSV1(17+)Lox-sacB-ΔICP34.5Zeo-ΔICP47-mir124res**, Snapgene (https://www.snapgene.com/).*

Electroporation of GS1783 cells

Once obtained the fragment containing the sequence encoding Killer Red protein and the gene conferring Kanamycin resistance through digestion of pCeu-memKR with I-CeuI, we proceeded to incorporate it inside the BAC pHSV1(17+)Lox-sacB-ΔICP34.5Zeo-ΔICP47-mir124res. This recombination event happened subsequent to the electroporation of GS1783 cells. Electroporation is a procedure which allows the break-through of the plasma membrane of competent cells by creating reversible pores in the lipid bilayers through the application of electrical pulses. Through these pores, the molecules of DNA enter bacteria and reach the cytoplasm, where recombination occurs.

The reaction took place by adding the DNA extracted directly to the aliquote of GS1783 cells, and using 2mm cuvettes at 2,5 Volts, 25 μF. Following the protocol, we also performed a shaking step, which lasted one hour at 32 °C, necessary to ensure that the molecules of DNA and the bacteria are evenly distributed in the solution. The bacteria were finally incubated at 32 °C in LBA (*Luria Bertani* Agar) plates supplemented with *Kanamycin* and *Chloramphenicol*.

Red Recombination

The fragment that had been electroporated was then integrated into the aforementioned BAC pHSV1(17+)Lox-sacB-ΔICP34.5Zeo-ΔICP47-mir124res, via the mechanism of Red Recombination.

The Red recombination system is a phage-derived genetic engineering tool used to mediate homologous recombination in *E. coli*. It involves two main components: the exonuclease λ, *λ exo,* and the single-strand annealing protein, *Redβ*. The process of recombination starts with the assembly of a ring shaped homotrimer by *λ exo*, which binds dsDNA ends and proceeds to degrade the 5' strand. Subsequently, a protein-protein interaction takes place: *λ exo* engages with *Redβ,* facilitating its direct attachment to the 3' overhang formed during the previous enzymatic reaction. Lastly, the resultant SSAP orchestrates the annealing with a complementary strand from another DNA molecule, in a homologous recombination manner.

This technique demonstrates high efficiency, thanks to its reliance on short homologous regions, rather than unique restriction sites; therefore, it is particularly effective for the insertion and cloning of larger DNA constructs. For instance, it permits the insertion of segments using homologies as short as 35-50 bp, which could be easily encoded using

synthetic PCR primers, increasing its versatility and ensuring fidelity of the recombination event.

Red recombination has become a key tool in BAC modification, gene knockout studies, and CRISPR template construction. Additionally, it has been shown that the technique could function with the insertion of single-strand oligonucleotides, a hopeful solution to correct single-point mutations or indels. (11)

In our case, the BAC pHSV1(17+)Lox-sacB-ΔICP34.5Zeo-ΔICP47-mir124res successfully incorporated *KanR* and *mem-KR* genes in the region UL55-56, which effectively replaced the expression cassette of *SacB*. The event was permitted by common homology sequences present in both DNA molecules.

Selection of GS1783 bacterial clones expressing the *KanR* gene

To select bacteria in which the electroporation was successful, we cultured the bacterial cells in *Luria Bertani* Agar (LBA) medium, supplemented with the antibiotics *Kanamycin* and *Chloramphenicol.* GS1783 clones were expected to survive the *Chloramphenicol* in this medium due to the resistance gene *CHLR* already present in the BAC. In contrast, solely bacterial cells that have undergone successful recombination can survive *Kanamycin*, as the resistance gene is encoded within the incorporated DNA fragment. Moreover, the medium also contained 10% sucrose, to exclude the bacteria that weren't successfully transformed. Indeed, these ones still retained the *SacB* gene, a gene encoding an enzyme which converts sucrose into a toxic substance for the cells.

Verification PCRs for the selection of GS1783 bacterial clones not expressing the *KanR* gene but the *mem-KR* gene

To further validate the selection procedure of only colonies containing the electroporated fragment, two verification PCRs were performed, subsequent to the extraction of plasmidic DNA from 10 selected colonies through a home-made procedure.

Polymerase chain reaction (PCR) is a method commonly employed for amplification or verification purposes on a determined sequence, making use of primers specific for this DNA molecule. The procedure is composed of 3 main phases, characterised by different temperatures:

1. DNA denaturation: it consists of the disruption of hydrogen bonds holding complementary bases of the two strands together;

2. Annealing: in this step, primers anneal to their complementary sequences on the denatured DNA strands. Annealing temperature is primer-specific and depends on its nucleotide composition;
3. DNA synthesis: it's the extension phase, in which the molecule of DNA is synthesised in the 5'->3' direction. This step is repeated several times to allow the fragment to be amplified.

For the PCR experiments conducted in this study, *AmpliTaq Gold DNA polymerase* was employed to ensure great fidelity of the process.

Regarding this BAC, the first PCR was carried out to amplify the sequence containing the gene *SacB*, verifying this was no longer present following the recombination event. The second PCR served as proof to make sure that these colonies, in which *SacB* was efficiently removed, contained *mem-KR* gene. In order to correctly perform these two procedures, specific primers had to be selected, which produced defined amplicons lengths. In particular, we used:

- *SacB-for* and *SacB-rev* to amplify the *SacB* sequence, with sequence 5'-CTTTTCGACCTCATTCTATTAG-3' and 5'-ACATATACCTGCCGTTCA-3' respectively;
- *KR-RtFor* and *KR-RtRev* to amplify the *mem-KR* sequence, with sequence 5'-GACCATCATCACCAAGCAG-3' and 5'-TTAATCCTCGTCGCTACCG-3' respectively.

This methodological approach strengthened the selection process.

Resolution of recombinant BACs in selected GS1783 colonies

Once verified the efficiency of recombination process, we proceeded with the resolution of the previously selected bacterial colonies, in which the *Kanamycin* resistance gene, used before as a selection marker for the recombination, was removed prior to viral reconstitution to ensure biosafety and compliance with therapeutic vector standards. To successfully complete this procedure, we employed the restriction enzyme *I-SceI*, already present in GS1783 cells, whose expression is controlled by an Arabinose-inducible promoter. *I-SceI* recognises a non-palindromic sequence of 18 bp, present upstream of the *KanR* gene; since only one target sequence is present within the BAC, upon creation of a double-strand break at the site, the BAC from circular becomes linearised and the event of recombination occurs. Therefore, we first incubated the bacteria for 1 hour at 32 °C in LB medium enriched with *Chloramphenicol*; subsequently, 1 mL of LB containing Arabinose was added for *I-SceI* production and activation. After another incubation of 1 hour at 32°C, the bacteria were

placed at 42 °C for 20 minutes, in order to induce the functioning of Red Recombination genes; this mechanism is responsible for the actual elimination of *KanR* (flanked by two homologous sequences with the same orientation). Lastly, the bacteria were incubated again at 32 °C for 2-3 hours by shaking, until they reached an adequate optical density (OD), and plated on 1% Arabinose-CHL-Agar plates for 2 days at 32°C.

Selection of resolved GS1783 colonies by replica picking

Once the incubation period had terminated, both solved and unsolved bacteria were able to grow efficiently on LBA medium, enriched with 1% arabinose and *Chloramphenicol*. To identify just the colonies which had undergone a successful resolution, we employed the method of *replica picking.* Specifically, we picked 10 well-spaced bacterial populations from the master plate and transferred them onto two new plates, at specific locations (permitting a clear localisation), and we incubated the plates at 32 °C overnight. These new dishes contained 1% arabinose-*CHL*-LBA, but one of them also had *Kanamycin*, necessary for selection. In particular, the resolved colonies should have grown only in the plate not containing the *Kanamycin*.

To further confirm the absence of the *KanR* gene, the BAC DNA was extracted from 4 colonies (selected among the 10 we individually picked), and a PCR of the region *UL55-UL56* was carried out, with its results analysed on 1% agarose gel.

We discriminated the correct colonies because of the length of their PCR products: resolved fragments were 2121 bp long, rather than the unresolved fragments that were 3099 bp long.

Resolved BAC DNA extraction

One single solved colony was selected for DNA extraction to obtain the BAC containing the HSV-1 genome required for transfection. We began by preparing a maxi inoculum of the bacteria in a volume of 200 mL LB + *CHL* medium to ensure a sufficient DNA yield for subsequent applications, and we harvested it at 32 °C overnight. To extract the resolved BAC from the cells, we followed the protocol reported in QIAGEN® Plasmid Maxi kit, designed for isolating large constructs.

During the process of plasmid purification, we made use of several buffers to carry out the different steps, alternating them with periods of centrifugation to aid the separation process. Notable among these were the Resuspension Buffer, in which the bacterial pellet was resuspended; the Lysis Buffer, which allowed us to lysate the cells and denaturate DNA; the

Neutralisation buffer, used to effectively separate plasmidic and genomic DNA; and lastly the Elution buffer, to release DNA molecules from the resin of specific columns to which the DNA was associated.

Then we quantified the extracted DNA with NanoDrop One/One^{C} Spectrophotometer. Spectrophotometry relies on UV-Vis radiation and the absorbance of different molecules to assess the purity and concentration of nucleic acids. More specifically, the DNA sample was quantified based on its characteristic peak absorbance at 260 nm. The ratio 260/280 nm was also evaluated, where values inferior to 1.8 indicate contamination with proteins or phenolic compounds.

oHSV-1 *mem-KR* reconstitution via cell transfection in Vero CCL81 cells using Lipofectamine 3000

Transfection is the process by which foreign DNA is introduced into eukaryotic cells, especially of mammalian origin, using chemical or physical methods. It represents a very effective approach, which, allowing for the introduction of large nucleic acids constructs, is suitable for BAC clones generation. Additionally, transfection is a non-viral system, feature which contributed to its gain of importance in biotechnology applications as a safer approach compared to gene delivery based on viral vectors.

The extracted BAC was transfected into Vero CCL81 cells to obtain the reconstitution of viral particles. Vero CCL81 is a cell line derived from epithelial cells of African Green Monkey kidneys; they are widely employed in virology thanks to their high permissiveness to viruses and their ease to be cultivated, maintaining a distinct morphology and being able to grow also in conditions of low to no serum. They are particularly suitable for transfection processes with optimisation (e.g., lipofection, electroporation).

Lipofectamine is a lipid-based transfection reagent commonly chosen to facilitate nucleic acid delivery by forming lipidic complexes (lipoplexes) with DNA molecules, permitted by their opposite charges, that fuse with the cell membrane, enabling easier intracellular uptake. The lipoplexes are attracted towards the cell surfaces through electrostatic interactions, established between the cationic lipids of lipofectamine and the cell membrane, enabling their later uptake via endocytosis.

In order to successfully conduct a transfection, cells need to reach 70-90% confluence; we proceeded, therefore, to seed Vero CCL81 cells in a 6-well plate, ensuring we had 500.000 cells per well, and we let them grow until around 80% confluence.

The process itself was then carried out with Invitrogen™ Lipofectamine™ 3000 reagent (ThermoFisher Scientific) according to the manufacturer’s instructions. As the protocol outlines, the reaction takes place in two distinct phases:

- Creation of lipidic-DNA complexes by combining two solutions, one containing the BAC genome and the P3000 reagent, and the second one containing Lipofectamine 3000, both diluted in Opti-MEM medium. Subsequently, an incubation step of 15 minutes at room temperature follows to allow the proper formation of lipoplexes.
- Transfection of Vero CCL81 with the solution of lipoplexes obtained previously, added directly onto the seeded cells.

Lastly, we incubated the cells at 37 °C overnight and the day after the Opti-MEM solution was replaced by DMEM supplemented with 10% FBS (fetal bovine serum), to sustain their growth.

In the following days, the transfection process was analysed with the fluorescence microscope, exploiting the production of the red fluorescent protein Killer Red by the virus.

Viral stock amplification in Vero cells CCL81

After 72 hours post-viral reconstitution, infected Vero CCL81 cells were detached from the 6-well plate and centrifuged in order to remove cellular debris. The supernatant containing viral particles was collected and used for viral stock amplification.

Viral stock amplification is a process that involves performing successive rounds of cell infection to increase the concentration of viral titer.

For this purpose, a T75 flask of Vero CCL81 cells, cultured until confluent, was infected with the supernatant collected previously. To ensure a successful infection, DMEM 10% *v/v* FBS, in which the cells grew in, was removed and, after a wash in PBS, replaced with a mixture of DMEM with no serum and the Vero CCL81 supernatant; the infection was carried out for 1 hour at 37 °C. Subsequently, the supernatant was discarded, and the cells were incubated in medium DMEM 2% *v/v* FBS.

The virus's cytopathic effects were observed and analysed 72 hours after the infection. The cell medium was then collected, and the amplification process was repeated using two T175 seeded with Vero CCL81 cells, in order to furtherly increase the virus yield.

Viral particles extraction and purification

After terminating these rounds of replications, we proceeded with the arrest of the viral amplification and the stock of the virions produced through their extraction. The primary purpose of viral extraction is to isolate and purify the particles of HSV-1, removing interfering substances and other contaminants (e.g. cellular components), to combine them with the supernatant containing virions that were released during viral replication.

For these reasons, Vero CCL81 were initially detached from the flask surface through scraping and centrifuged together with the supernatant for 5 minutes at 1000 x *g*. The supernatant was then collected, filtered and stored aside while the cellular pellet obtained underwent 4 cycles of freezing (in dry ice) and defrosting at 37 °C, to induce the physical rupture of cell membranes. Indeed, the formation of ice crystals during the freezing phase and their contraction in the defrosting phase weaken the plasma membranes and lyse the cells. The lysis was completed with a sonication reaction; this process relies on the employment of ultrasonic waves to disrupt the cells while keeping the sample cold. Lastly, through centrifugation, viral particles were separated from cellular debris and were successfully released in the supernatant, which was collected and combined with the previous one obtained in the first step of centrifugation.

To isolate the viral particles, we performed a further step of centrifugation (conducted for 1 hour at 4 °C and *19.000 rpm*) with the combined supernatants using the *Sorvall* centrifuge (Thermo Fisher Scientific).

Finally, the pellet was resuspended in DMEM 50% FBS overnight, and the solution was aliquoted into Eppendorf tubes, stored at -80 °C for subsequent applications.

Viral titration was then performed on one vial using a plaque assay on Vero CCL81 cell monolayers cultured in 48-well plates.

Results

Plasmid pCeu-memKR digestion

The digestion of pCeu-memKR mediated by the restriction enzyme *I-ceuI*, and indirectly *AdhI*, provided the production of three fragments that have been separated through gel electrophoresis. In particular, as indicated by the figure below [Figure 6], the obtained band pattern resulting from the analysis of the products on 1% agarose gel indicated a successful digestion: plasmid DNA can be visualised with two intense bands, the higher one being the fragment containing *KanR* and *mem-KR* (2890 bp), which has been subsequently inserted into the BAC genome, as previously described.

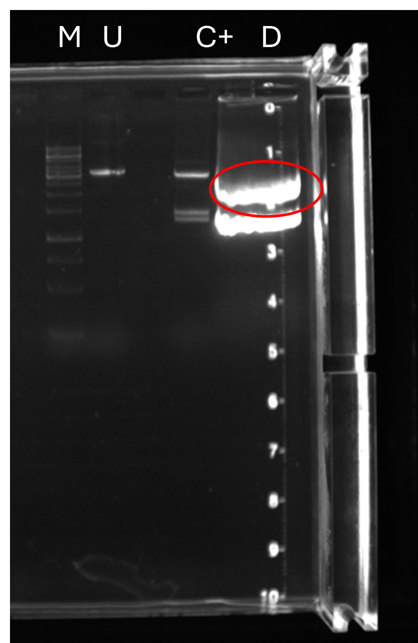

Figure 6 ***Digestion of plasmid pCeu-memKR****. A 1% agarose extraction gel showing the result of the enzymatic digestion of plasmid pCeu-memKR, obtained with the employment of I-ceuI and AdhI restriction enzymes. The image represents the electrophoresis run of Gene Ruler 1 kb marker (M), undigested pCeu-memKR (U), positive control with the correctly digested plasmid (C+), and the digested pCeu-memKR (D).*

BAC pHSV1(17+)Lox-memKR-INT-ΔICP34.5Zeo-ΔICP47-mir124

The recombinant process between BAC pHSV1(17+)Lox-sacB-ΔICP34.5Zeo-ΔICP47-mir124res and the plasmid fragment obtained through the previous digestion of the plasmid pCeu-memKR, using the homology sequences found in both DNA molecules, has determined the formation of the BAC pHSV1(17+)Lox-memKR-INT-ΔICP34.5Zeo-ΔICP47-mir124.

To validate this result, PCRs and a following gel electrophoresis were conducted. We first evaluated the absence of the gene *SacB:* bacteria bearing a successfully recombined BAC lost the gene in order to permit the insertion of the fragment. Through the analysis of the PCR products on the gel [Figure 7 (a)], we observed that none of our colonies produced an amplicon of 252 bp of the gene *SacB*, except for the positive control.

Subsequently, through the second PCR we analysed the presence of the gene *mem-KR*; in our case, only one colony tested positive and produced an amplicon of 124 bp, as shown by the band in the figure below [Figure 7 (b)], indicating the sequence of *mem-KR* was integrated successfully.

In conclusion, only one colony was positive for both the absence of *SacB* and the presence of *mem-KR.*

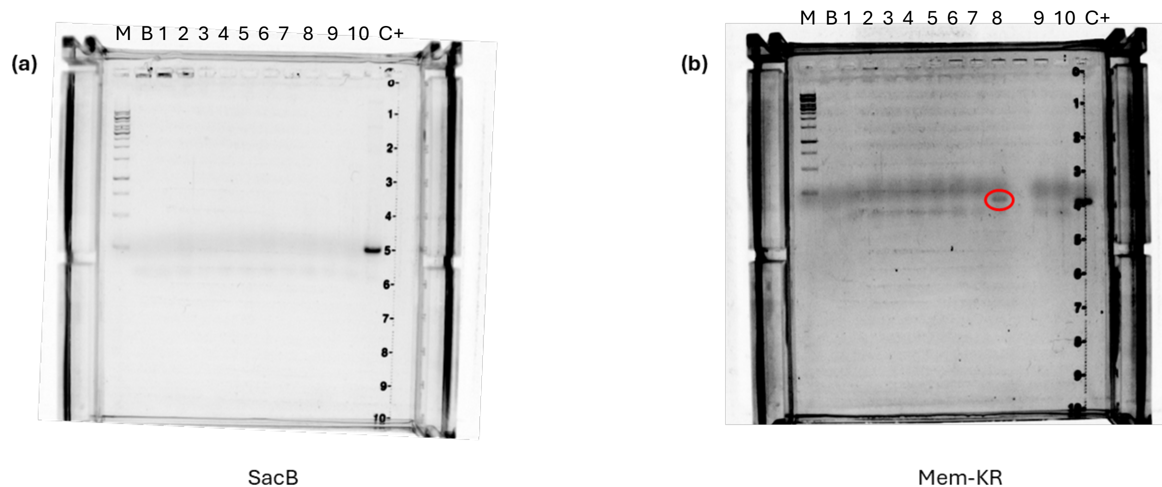

Figure 7 Verification gels of the correct recombinant process which led to the integration of the fragment containing the gene mem-KR. *Gel electrophoresis on 1% agarose gel of the PCR amplicons obtained from electroporated bacterial colonies. The first image (a) represents the electrophoresis run conducted to verify the absence of SacB, with the amplicons obtained from 10 individual colonies (1-10), the Gene Ruler 1 kb marker (M), a positive control containing the amplified SacB sequence (C+), and the PCR blank (B). All the 10 individual colonies tested negative for the presence of the SacB gene. The second image (b) represents the electrophoresis run conducted to verify the presence of the mem-KR gene, with the amplicons obtained from the same 10 individual colonies (1-10), the Gene Ruler 1 kb marker (M), a positive control containing the amplified mem-KR sequence (C+), and the PCR blank (B). Colony 8 tested positive for the presence of mem-KR.*

GS1783 bacterial colonies bearing the resolved BAC pHSV1(17+)Lox-memKR-RES-ΔICP34.5Zeo-ΔICP47-mir124 grow solely in medium lacking Kanamycin

During the *replica picking* experiment, GS1783 bacterial colonies bearing the resolved BAC pHSV1(17+)Lox-memKR-RES-ΔICP34.5Zeo-ΔICP47-mir124 grew exclusively on LB Agar plates supplemented with *Chloramphenicol* but not on those containing both *Chloramphenicol* and *Kanamycin.* This selective growth pattern indicates a successful

resolution of the BAC, which led the colonies to lose resistance to this antibiotic. Therefore, comparison of the two plates enabled clear identification of correctly resolved colonies.

Bacterial colonies bearing the resolved BAC pHSV1(17+)Lox-memKR-RES-ΔICP34.5Zeo-ΔICP47-mir124 produce amplicons of dimensions inferior to the colonies containing the unresolved ones

The GS1783 bacterial colonies bearing the resolved BAC pHSV1(17+)Lox-memKR-RES-ΔICP34.5Zeo-ΔICP47-mir124, following PCR of the region *UL55-UL56*, should produce amplicons of length of 2121 bp, inferior to the 3099 bp long ones obtained from colonies containing the gene *KanR*.

Indeed, on the 1% agarose gel shown below, [Figure 8], the band produced by resolved colonies is found lower than the one obtained from the amplification of the region still containing *KanR*, explained by the fact that linear molecules of dsDNA migrate with inversely proportional speeds compared to the number of base pairs they are composed of.

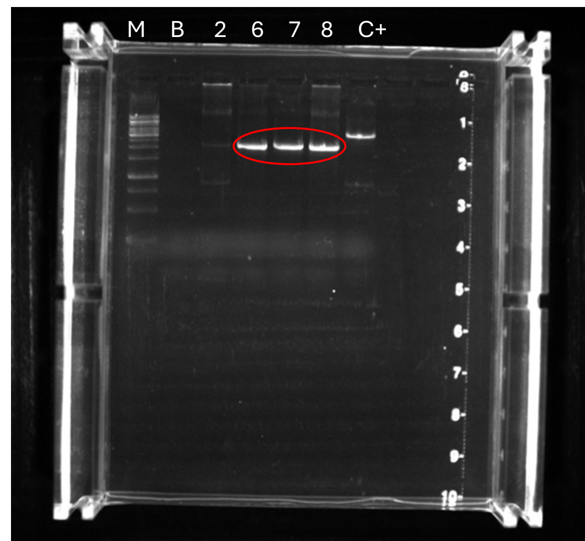

***Figure 8 Verification of the successfully resolved bacterial colonies**. Gel electrophoresis on 1% agarose gel of PCR amplicons from bacterial colonies bearing the resolved BAC pHSV1(17+)Lox-memKR-RES-ΔICP34.5Zeo-ΔICP47-mir124. The image represents the electrophoresis run of the amplicons obtained from colonies selected after the replica picking experiment (2, 6, 7, 8), the Gene Ruler 1 kb marker (M), a positive control containing the amplicon of an unresolved UL55-UL56 region (C+), and the PCR blank (B). Colonies 6, 7 and 8 tested positive for the resolution of the BAC.*

Transfection efficiency was assessed through Killer Red expression and cytopathic effects onset

Three days post transfection of Vero CCL81 cells using the lipofectamine mechanism, we proceeded with the analyses at the fluorescent microscope of these same ones.

As shown below, we detected the expression of the fluorescent-photosensitizer protein Killer Red, clearly visibly located at the plasma membrane of the cells. Moreover, still through the microscope analysis, we have observed some important morphological and structural changes induced by the virus replication in the Vero CCL81 cells. These include the loss of their spread-out morphology and the consequent acquisition of a round or shrunken shape, the loss of adhesion and the detachment from the cell culture dish. [Figure 9 (a)].
Both fluorescence and cytopathic effects typically appeared 24 hours post-infection and become more pronounced over time.
These considerations allowed us to confirm the success of the transfection process, and therefore the viral particles reconstitution. [Figure 9 (b)(c)]

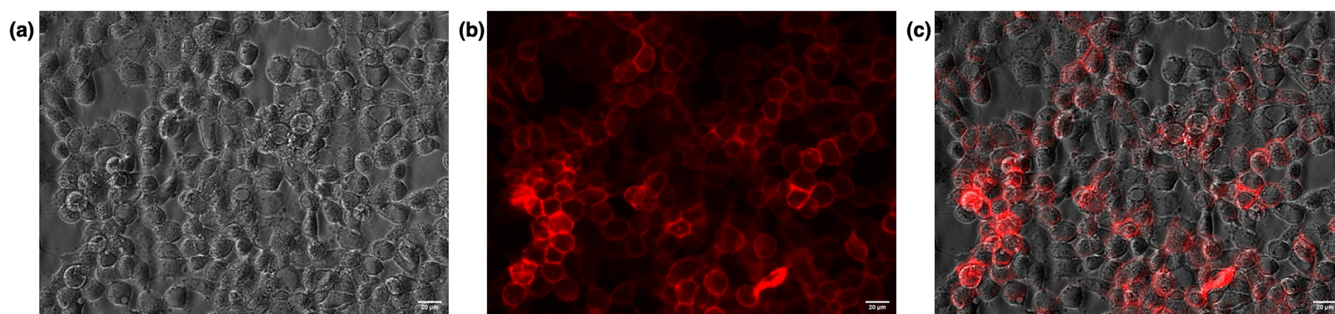

Figure 9 Vero cells CCL81 analysed at 72 hours post transfection. *The viral transfection of Vero CCL81 cells with the BAC pHSV1(17+)Lox-memKR-RES-ΔICP34.5Zeo-ΔICP47-mir124 was assessed by an analysis conducted at the fluorescent microscope of the seeded cells in a 6-well multiwell plate. The images show the analysis with transmitted light (a), the red fluorescence analysis (b), and an average between the two previous images (c). Images were acquired at 40x magnification.*

Efficiency of viral amplification in Vero CCL81 cells was highlighted by cytopathic effects and Killer Red expression

Analogously as described previously for viral reconstitution, Vero CCL81 cells, used for the viral amplification process, were analysed to determine the efficiency of HSV-1 replication. We encountered cytopathic effects and the expression of Killer Red demonstrating the effectiveness of the process in a larger cell culture; indeed, the cells analysed were grown in a T75 cm^2 culture flask, necessary to permit the amplification.
Also in this case, the morphological and structural changes and the protein Killer Red were observable as early as the first day and increased over the following ones.

Conclusion

At the end of this research project, we successfully designed and reconstituted a recombinant HSV-1-based oncolytic agent expressing membrane targeted Killer Red (KR) protein. With this work, we aim to lay the groundwork for a novel therapy combining the oncolytic capabilities of the HSV-1 virus with the spatial and temporal control offered by ROS generation through KR photoactivation.

The results obtained during the project demonstrated that the agent designed is effective in infecting and, subsequently, killing mammalian cells, confirming its strong cytopathic effects. Moreover, it proved to be a perfect delivery system for membrane-localised KR, enabling a wide range of downstream investigations on the advantages of precision phototoxicity.

In future, the viral particles, produced and titrated in Vero CCL81 cells, will be employed to infect cell lines from aggressive cancers, in particular glioblastoma, the most common and lethal form of primary brain tumour. So, with the next experiments we aim to test and evaluate this new virus (oHSV-1 *mem-KR*) for its killing and photoactivable properties in glioblastoma cancer cells, both human and murine.

Even if more analysis is needed, this work (focused on the generation of a genetically engineered HSV-1) provides a critical first step toward a novel cancer treatment, combining oncolytic virotherapy with photodynamic approach.

Bibliography

1. Perspectives on immunotherapy via oncolytic viruses [Internet]. [cited 2025 Jun 18]. Available from: http://ouci.dntb.gov.ua/en/works/4wrwekJ7/

2. Lin D, Shen Y, Liang T. Oncolytic virotherapy: basic principles, recent advances and future directions. Signal Transduct Target Ther. 2023 Apr 11;8(1):156.

3. Geisler A, Hazini A, Heimann L, Kurreck J, Fechner H. Coxsackievirus B3—Its Potential as an Oncolytic Virus. Viruses. 2021 May;13(5):718.

4. Shen Y, Zhang H, Xue M, Zheng C, Chen Q. HSV-1 as a gene delivery platform for cancer gene therapy. Trends Pharmacol Sci. 2025 Apr 1;46(4):324–36.

5. Kukhanova MK, Korovina AN, Kochetkov SN. Human herpes simplex virus: Life cycle and development of inhibitors. Biochem Mosc. 2014 Dec 1;79(13):1635–52.

6. Herpes_genome ~ ViralZone [Internet]. [cited 2025 Jun 24]. Available from: https://viralzone.expasy.org/5756

7. Scanlan H, Coffman Z, Bettencourt J, Shipley T, Bramblett DE. Herpes simplex virus 1 as an oncolytic viral therapy for refractory cancers. Front Oncol. 2022 Jul 27;12:940019.

8. Liu J, Wang F, Qin Y, Feng X. Advances in the Genetically Engineered KillerRed for Photodynamic Therapy Applications. Int J Mol Sci. 2021 Sep 20;22(18):10130.

9. Pletnev S, Gurskaya NG, Pletneva NV, Lukyanov KA, Chudakov DM, Martynov VI, et al. Structural Basis for Phototoxicity of the Genetically Encoded Photosensitizer KillerRed*. J Biol Chem. 2009 Nov 13;284(46):32028–39.

10. Sirotkina MA, Matveev LA, Shirmanova MV, Zaitsev VY, Buyanova NL, Elagin VV, et al. Photodynamic therapy monitoring with optical coherence angiography. Sci Rep. 2017 Feb 2;7(1):41506.

11. Caldwell BJ, Bell CE. Structure and mechanism of the Red recombination system of bacteriophage λ. Prog Biophys Mol Biol. 2019 Oct;147:33–46.