Oncolytic activity of herpes simplex virus type II (oHSV-2) on Colorectal cancer (CRC)

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Abstract

The rising prevalence of cancer worldwide has led to the emergence of several biotechnological advancements, including oncolytic virotherapy, a novel treatment strategy for multiple tumors. Among these approaches, herpes simplex virus (HSV) based vectors have garnered significant attention as extensively researched oncolytic agents on a global scale. In this study, We investigated in vitro the effects of oHSV-2 with singular gene deletion gama 34.5 Expression recombinant HSV-2 virus or infected cell protein 47 Expression recombinant virus (ɣ34.5 ExRV or ICP47 ExRV) and viruses with deletion of both genes ɣ34.5 & ICP47 Expression recombinant virus (Dual ɣ34.5 & ICP47 ExRV), on the LS174T colorectal adenocarcinoma cell line model compare with a normal vero cell line . The infection efficacy and the cell viability studies were carried out on human colorectal carcinoma cells LS174T by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) cell proliferation colorimetric assay. Results show the EC50 for each agent was,(0.413, 0.494 and 0.53) respectively for ɣ34.5 ExRV, ICP47 ExRV, and Dual ɣ34.5 & ICP47 ExRV. However, The positivity of therapeutic selectivity index was calculated for agents with dual gene deletion only (SI = 2.925). WT HSV-2 strain was also included in this study to compare the effect of virus without gene deletion with the effect of recombinant viruses of different modulations, however, results revealed effect with correlation coefficient of dose dependency but with low SI. This reflects the high safety margin of oHSV2 on normal cells and high efficacious effect on malignant cells.

Keywords: oHSV-2, Colorectal cancer (CRC), cell line, MTT cytotoxicity assay.

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Introduction

Colorectal cancer (CRC) is a significant contributor to cancer-related deaths, and a considerable number of patients will experience the spread of the disease to other parts of the body during the course of their treatment. Conventional therapies like surgery, chemotherapy, and radiation often show limited effectiveness in advanced stages, highlighting the need for the development of innovative treatments (LaRocca *et al*., 2023). One promising novel approach is oncolytic virotherapy, which involves using viruses that selectively replicate within tumor cells, sparing normal cells from damage (Chaurasiya *et al*., 2018). In the past, oncolytic virotherapy research primarily focused on naturally occurring viruses, such as West Nile virus,

rabies virus, yellow fever, hepatitis, etc., and their inherent ability to cause tumor destruction. This notion was based on the viruses' lytic characteristics (Kelly and Russell, 2007). However, with advancements in genetic engineering technology approaching the year 2000, it became possible to make various modifications to wild-type viruses. By doing so, modified oncolytic viruses (OVs) could be equipped with specific exogenous genes that exert profound antitumor effects through different mechanisms. Initially, the primary aim of these modifications was to enhance target specificity, selective replication, and oncolysis. Subsequently, researchers appreciated the potential of eliciting an antigen-specific antitumor immune response during tumor lysis, providing an additional advantage of OVs as immunotherapy (Li *et al*., 2020).

The Herpes simplex virus (HSV) has gained significant prominence in the era of Oncolytic Viruses (OVs). Its unique characteristics, such as the ability to infect a wide range of tumor cell lines, well-understood life cycles, genomic structure, and a substantial genome space for transgene insertion, have positioned HSV-1 as an ideal tool for cancer therapy (Argnani *et al*., 2005) HSV-1 exhibits a rapid infection cycle, leading to cell lysis within 10 hours of infection and subsequent release of new viral particles (Speranza *et al*., 2016). Meanwhile, HSV-2 has also been receiving increasing attention and is currently under investigation. A particular oncolytic HSV-2, OH2, has recently entered phase I/II clinical trials for the treatment of solid tumors, although its modification approach remains similar to that of T-VEC (Zhang *et al*., 2021) .ICP34.5, a protein frequently deleted in oncolytic *herpesviruses* due to its potential neurovirulence, plays a crucial role in antiviral immunity. It serves as an antagonist of the type I interferon-mediated innate immune response and inhibits autophagy in neurons by binding to Beclin 1 (Manivanh *et al*., 2017).

As of 2022, there are four approved Oncolytic Viruses (OVs) for human use, with half of them being engineered *herpesviruses*. One of these is talimogene laherparepvec (T-VEC; IMLYGIC), a modified herpes simplex virus type 1 (HSV-1) approved in the United States and Europe in 2015 for the treatment of melanoma. Another approved herpesvirus is DELYTACT (teserpaturev/G47D), an attenuated HSV-1 used in Japan since 2021 to treat primary brain cancer. Both T-VEC and DELYTACT are partially attenuated by deleting ICP34.5; DELYTACT is further attenuated due to the deletion of ICP6 and ICP47, while T-VEC combines ICP47 deletion with the ability to express granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rahman and McFadden, 2021). The inclusion of secretable GM-CSF in these viruses aims to enhance immune activation, although it comes with some risks. GM-CSF may also act as an anti-inflammatory cytokine, promoting a "tolerogenic" dendritic cell phenotype that is associated with an increase in regulatory T cell populations (Bhattacharya *et al*., 2015). Among the approved OVs, IMLYGIC is the only one approved by the United States Food and Drug Administration (FDA). Although it has shown excellent safety in multiple clinical trials, its efficacy as a monotherapy has been somewhat disappointing. However, when administered in combination with an immune checkpoint inhibitor, improved efficacy was observed (Ferrucci *et a*l., 2021). In this study, aim to investigate the oncolytic activity of HSV-2 against LS174T colorectal adenocarcinoma and normal vero cell lines, as well as its ability to induce apoptosis.

Materials and methods

1- Cell lines and virus

The cell lines that used in this study involved African green monkey kidney (Vero),For the propagation and study the cytopathic effect of the wild type HSV-2 ,

this cells Kindly provided by Dr. Ahmed Al-Shammari, College of Veterinary Medicine, University of Baghdad, Iraq. The other type of cell line that used in this study are metastatic , non immunogenic LS-174T- Colorectal Adenocarcinoma. The cells were cultured in growth media contained 10% fetal calf serum (FCS) instead of the original 5%. The pH of both media ranged from 6.8 to 7.1. To prevent bacterial contamination, antibacterial agents, namely streptomycin at a concentration of 100 µg/ml and penicillin G at a concentration of 100 IU/ml, were added to various media and solutions. Additionally, Nystatin, an antifungal agent, was added to achieve a final concentration of 25 IU/ml. the cells incubated at 37 C in a humidified atmosphere of 5% CO2. The wild type HSV-2 isolated from semen samples of the men, who were attended to laboratory of semen analysis in fertility Center of Al-Sader Medical City – Al Najaf) to get medical care, after identification the virus by Real- Time- qPCR , the samples with high viral load of HSV-2, was selected and pretreated with antibiotics, then propagated via cell culture technique using Vero cell line to study the cytopathic effect, then prepared the microtiteration of the propagated virus. The serial dilution of propagated WT HSV-2 stock sample prepared by added 400 µl from WT HSV-2 to 3600 µl from maintenance media, then mix with 100 µl from antibiotic , after this the tube incubate for 30 min. the 10 fold serial dilution prepare by add 1800 µl from maintenance media to each tube then transfer 200 µl from the stock tube to another respectively.

2-Preparation of the Plasmid Construction

Preparation of the ɤ 34.5 sub cloned vector Psp72 and the ICP47 sub cloned vector Psp73

In this study preparation of the sub cloned, expression and dual vectors done according to (Zhao *et al*.,2014) with slightly modification, the method for cloning γ 34.5 gene was performed using Psp72 Vector, in two steps. The purified upstream ɤ 34.5 gene and plasmid vectors (Psp72 Vector) were digested with restriction endonucleases (xbal/ PvuⅡ) and then Ligation of digested DNA fragments was performed using T4 DNA ligase according the manufacture procedure, then insertion the purified downstream ɤ 34.5 gene by digested with (EcoRV/ BglⅡ) restriction endonucleases and Ligation by T4 DNA ligase. The method for cloning ICP47gene was performed using Psp73 Vector, in two steps: digested the purified upstream ICP47gene and plasmid vectors (Psp73 Vector) with restriction endonucleases (Hind III / BglII) and Ligation of digested DNA fragments by using T4 DNA ligase according the manufacture procedure. then insertion the purified downstream ICP47gene by digested with (EcoRV/ BglⅡ) restriction endonucleases and Ligation by T4 DNA ligase.

Preparation of singular HSV-2 ɣ34.5 or ICP47 expression vectors

The pEGFP-N1 expression vector of (Psp72- ɤ 34.5 and Psp73- ICP47 sub cloned vector) prepared by digestion the pEGFP-N1 expression vector and sub cloned vector(Psp72- ɤ 34.5 and Psp73- ICP47), with unique cut restriction enzyme (BglII) according to the Bio lab procedure of this enzyme, which 16 µl of nuclease-free water added to Eppendorf tubes that specific to each vector, then 2 µl of 10X Speedy One buffer added to tubes, then add 2 µl from vectors to tubes that label with the name of specific vector, mix gently and spin down for a few seconds and add 2 µl Speedy Cut BglII with mix, after this Incubated at 37 °C for 60 minutes, then inactivate the enzyme by incubation at 80 °C for 20 minutes to stop the enzymatic reaction.

Preparation of complete HSV-2 expression vectors (Dual cloning).

Preparation of complete HSV-2 expression vectors of dual cloning for both ɣ34.5 and ICP 47 was done by digestion the pEGFP- N1-Psp72- ɤ 34.5 and sub cloned vector Psp73- ICP47 with unique cut restriction enzyme (BglII) and ligation according to the Bio lab procedure.

After the preparation the vectors ,all viral samples , vectors(Psp72- ɤ 34.5 and Psp73- ICP47 sub cloned vectors, expression vectors (pEGFP-N1-Psp72- ɤ 34.5 (ɣ34.5 ExRV),pEGFP-N1-Psp73- ICP47(ICP47 ExRV,) , dual expression vectors (pEGFP-N1-Psp72- ɤ 34.5 - Psp73- ICP47 (Dual ɣ34.5 & ICP47 ExRV) ,treated with 25 µl from antibiotic (streptomycin ,penicillin and nystatin) to each 1 ml from sample, to removing the contamination before application on Vero and colorectal cell lines.

The stock of each vectors prepared by addition of 20 µl from vectors to 2000 µl from maintenance media, then mix with 40 µl from viafect transfection according to viafect kit procedure. Tenfold serial dilution was achieved to give gradient titers of $10^{\wedge-1}$, $10^{\wedge-2}$ and $10^{\wedge-3}$. by the addition of 200 µl of each vector to 1800 µl of maintenance media, mixed well, then transferred subsequently to the other corresponding tubes containing the mentioned titers.

Preparation the oncolytic HSV-2 (OHSV-2)

Oncolytic HSV-2 is an attenuated oncolytic herpes simplex type 2 that was derived from the wild-type HSV-2 that isolated from semen samples, oHSV-2 was constructed by deleting the singular ɤ 34.5 gene and ICP47 gene or dual by using homologous recombination methods. which the Psp72- ɤ 34.5- GFP plasmid replaced the primary ɤ 34.5 gene of wild-type HSV-2 by co transfection into vero cells, and Psp73- ICP47-GFP plasmid replaced the primary ICP47 gene ,also in dual pEGFP-N1-Psp72- ɤ 34.5- Psp73- ICP47 plasmid replaced the both genes of the wild-type HSV-2.

For the construction of oHSV, The 6- well plates cultured with Vero cells were transfected with designed vectors and subsequently infected with wild-type HSV-2. Afterward, recombinant viruses were isolated by plaque purification technique using GFP as a reporter signal by fluorescent microscope. Finally, the isolated recombinant viruses were cultured, titrated and stored at -70 °C for further applications (Zhao *et al*.,2014).

Cytotoxicity assay application (MTT assay)

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to assess cell viability. This method relies on the conversion of the yellow tetrazolium compound, MTT, into a soluble formazan mixture by mitochondrial enzymes. The intensity of the resulting color is directly proportional to cell viability. Live cells that remain attached exhibit a blue color, while dead cells that detach appear pale or colorless, as observed during MTT exposure.

To perform the assay, cell lines were propagated onto 96-well microtiter plates at a density of 2×10^5 cells per ml. Following incubation at 37°C for 48 hours, when a confluent cell monolayer of LS-174T and Vero cells was formed (with a growth of approximately 80-100%), gradient concentrations of wHSV-2, oHSV-2 (with singular and dual γ34.5 and ICP47 gene deletions), were added to the cultured wells. Each well received a volume of 100 µl of the respective substance in triplicate, except for the untreated control wells. Beyond incubation at 37°C for 24 h in 5٪ CO_2, the 96 microtiter plates were prepared out and brought to Biohazard safety-cabinet through an aseptic manipulation to avoid any contaminated factor, all previous media were

launched away, The monolayers of LS-174T and Vero cells were then washed three times by PBS to remove an extra amount of colored composites or standard antitumor agents that may merge with MTT. Then all monolayers including treated , untreated cells and those of blank triplicate, were treated with 100 µl of maintenance media for each well. Every well was supplied by MTT (20 µl) . After 4 h of incubation (37°C, and with 5% CO_2), the molecules were formed by an enzymatic mitochondrial reaction of the LS-174T and Vero cells, these formazan particles were absent in dead cells because its mitochondria was disturbed. The insoluble formazan crystals were then dissolved by dimethylsulfoxide DMSO: isopropanol in a volume ratio of (1:1) on each well including blank wells, by an ELISA reader, at 490 *nm* the absorbance was read with a reference wavelength of *630 nm*. This protocol of MTT assay measurement was mentioned by many reports (Accardo *et al*.,2014; Wang *et al*., 2010).

Results and discussion

The cytotoxic effect of both LS174T colorectal adenocarcinoma and vero cell lines of untreated and treated groups (with the corresponding MOI of 1, 0.1 and 0,01 (pfu/cell) of wHSV2, oHSV2 of singular and dual gene deletion, was assessed by the colorimetric method MTT assay that based on the conversion of the reagent of MTT by dehydrogenase enzyme of viable cells to insoluble formazan particles, and the viability is proportional with the intensity of color as shown in figure (1). After 48 hours incubation, the absorbance at 490nm was measured for each well by ELISA reader and the percentages of growth inhibition (GI %) was determined.

Figure (2) reveals the GI% of each treatment on LS174T colorectal adenocarcinoma cells while figure (3) shows GI% of the same treatments on Vero cells. By using GI % dose response curve for different agent concentrations, we estimate the EC50, regression correlation coefficient (r^2) by plotting GI% versus agent concentration Figure (4). Tables (1) and (2) were also revealing mean± SEM, the half maximal inhibitory concentration (EC50) of oHSV2, level of significance and r^2 for each agent on LS174T CRC cells and Vero cells respectively. The selectivity index (SI) for each agent on LS174T cells was also calculated as mentioned in table (1). SI was estimated by dividing EC50 of agent in VERO cells / IC50 of same agent in LS174T cells. The agents that have SI value greater than two were considered to have a high selectivity towards cancerous cells (Badisa *et al.*,2009; Flis, and Spławiński, 2009). Positive correlation coefficient was obtained via plotting dose versus response of LS174T cells to each viral agent including singular HSV-2 gene deletion (ɣ34.5 ExRV or ICP47 ExRV) and viruses with deletion of both genes (Dual ɣ34.5 & ICP47 ExRV), values of r^2 were listed respectively in table (1), suggesting dose dependency of these recombinant viruses. Results of EC50 for each agent was also calculated,(0.413, 0.494 and 0.53) respectively for ɣ34.5 ExRV, ICP47 ExRV, and Dual ɣ34.5 & ICP47 ExRV. However, The positivity of therapeutic selectivity index was calculated for agents with dual gene deletion only (SI = 2.925). WT HSV-2 strain was also included in this study to compare the effect of virus without gene deletion with the effect of recombinant viruses of different modulations, however, results revealed effect with correlation coefficient of dose dependency but with low SI, results were shown in table (2). This reflects the high safety margin of oHSV2 on normal cells and high efficacious effect on malignant cells.

Table(1) Anticancer Effect of γ34.5 ExRV, ICP47 ExRV and dual γ34.5 & ICP47 ExRV and WT on LS-174T cells, presented by OD490nm Mean±SEM, r^2, EC50 and SI

Doe(MOI)	CRC cells				
	No.	Mean of triplicate absorbance (OD) ± SEM			
		γ34.5 ExRV	ICP47 ExRV	Dual γ34.5 & ICP47 ExRV	w-HSV-2
1	3	0.526±0.048*	0.433±0.027*	0.435±0.021*	0.541±0.022
0.1	3	0.588±0.044	0.573±0.008	0.537±0.040	0.486±0.033*
0.01	3	0.626±0.013	0.594±0.062	0.535±0.054*	0.373±0.0528*
0	3	0.719±0.038	0.719±0.038	0.719±0.038	0.719±0.038
r^2		0.9063	0.9987	0.9912	0.6445
EC50		0.413	0.494	0.53	0.294
SI		1.274	1.041	2.925	0.306

*= at significance positivity of p value less than 0.05

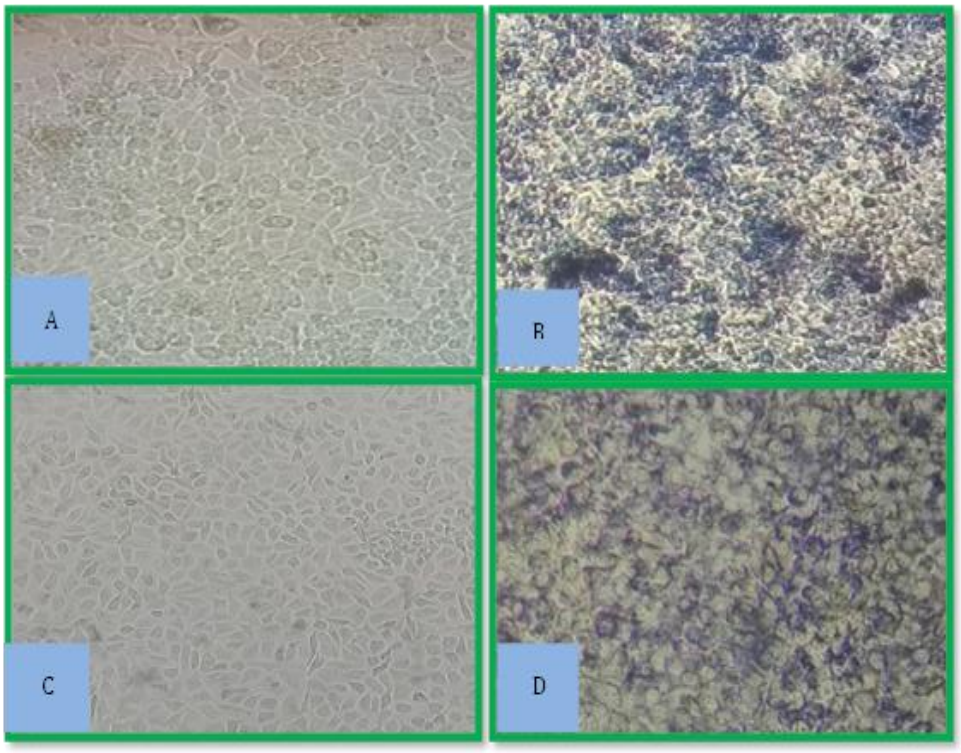

Figure (1): A- LS 174T Cell Line before treatment or addition of MTT reagent; B- Untreated LS 174T cell monolayer after addition of MTT reagent; C& D : untreated VERO cells before and after MTT addition respectively.

Table(2) Cytotoxic effect of γ34.5 ExRV, ICP47 ExRV and dual γ34.5 & ICP47 ExRV and WT on Vero cells, presented by OD490nm Mean±SEMr^2 and EC50

Dose (MOI)	Vero cells				
	No.	Mean of triplicate absorbance (OD) ± SEM			
		γ34.5 ExRV	ICP47 ExRV	Dual γ34.5 & ICP47 ExRV	w-HSV-2
1	3	0.121±0.015*	0.175±0.018*	0.137±0.015*	0.116±0.008*
0.1	3	0.171±0.004	0.196±0.054*	0.168±0.013*	0.114±0.004*
0.01	3	0.171±0.009	0.215±0.007	0.130±0.0003*	0.156±0.020*
0		0.198±0.001	0.198±0.001	0.198±0.001	0.198±0.001
r^2		0.9942	1	0.0698	0.2855
EC50		0.526	0.51	1.55	0.09

*=significant according to t-test (tow tailed)at p< 0.05

The IC50 of oHSV-2 with singular and dual deletion of γ34.5 and ICP 47 was extrapolated from the dose-response graph. The compound concentration that reduced the viability of cells by 50% (IC50 or EC50) was determined by plotting cell growth inhibition percentages versus a concentrations range (Kuppusamy *et al*., 2016). On CRC cells, oHSV2 show titer dependent effect with highly significant difference from control (P value : 0.000).

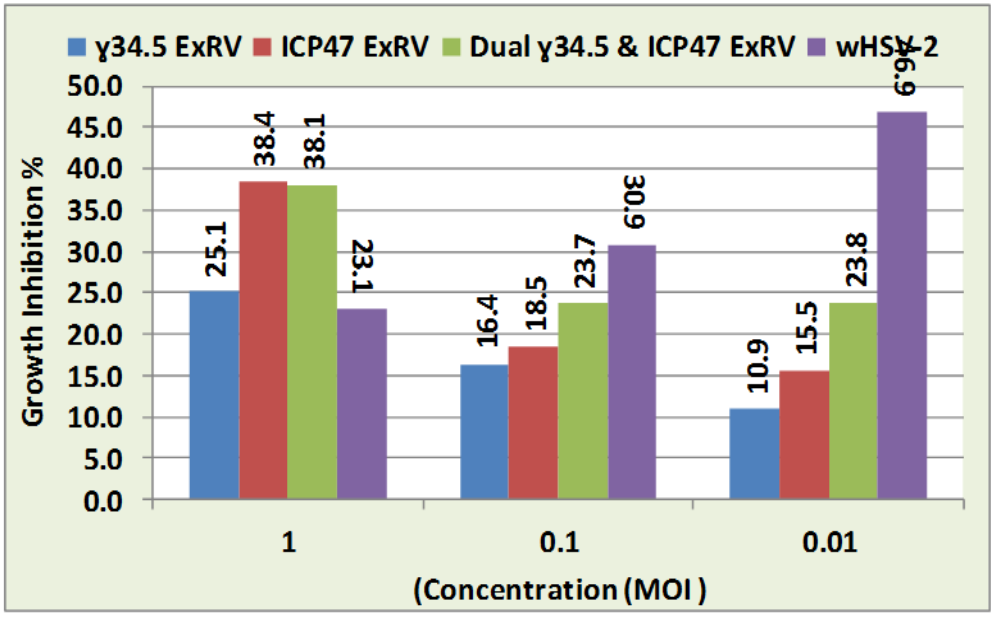

Figure (2): Growth inhibition % of LS174T cells treated with ɣ34.5 ExRV, ICP47 ExRV and dual ɣ34.5 & ICP47 ExRV and WT HSV-2

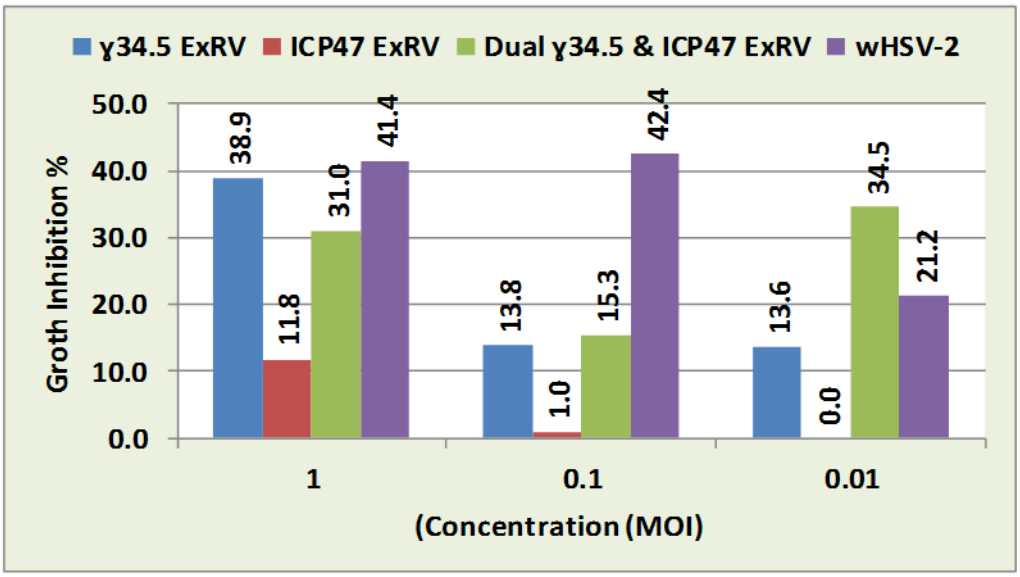

Figure (3): Growth inhibition % of Vero cells treated with ɣ34.5 ExRV, ICP47 ExRV and dual ɣ34.5 & ICP47 ExRV and WT HSV-2

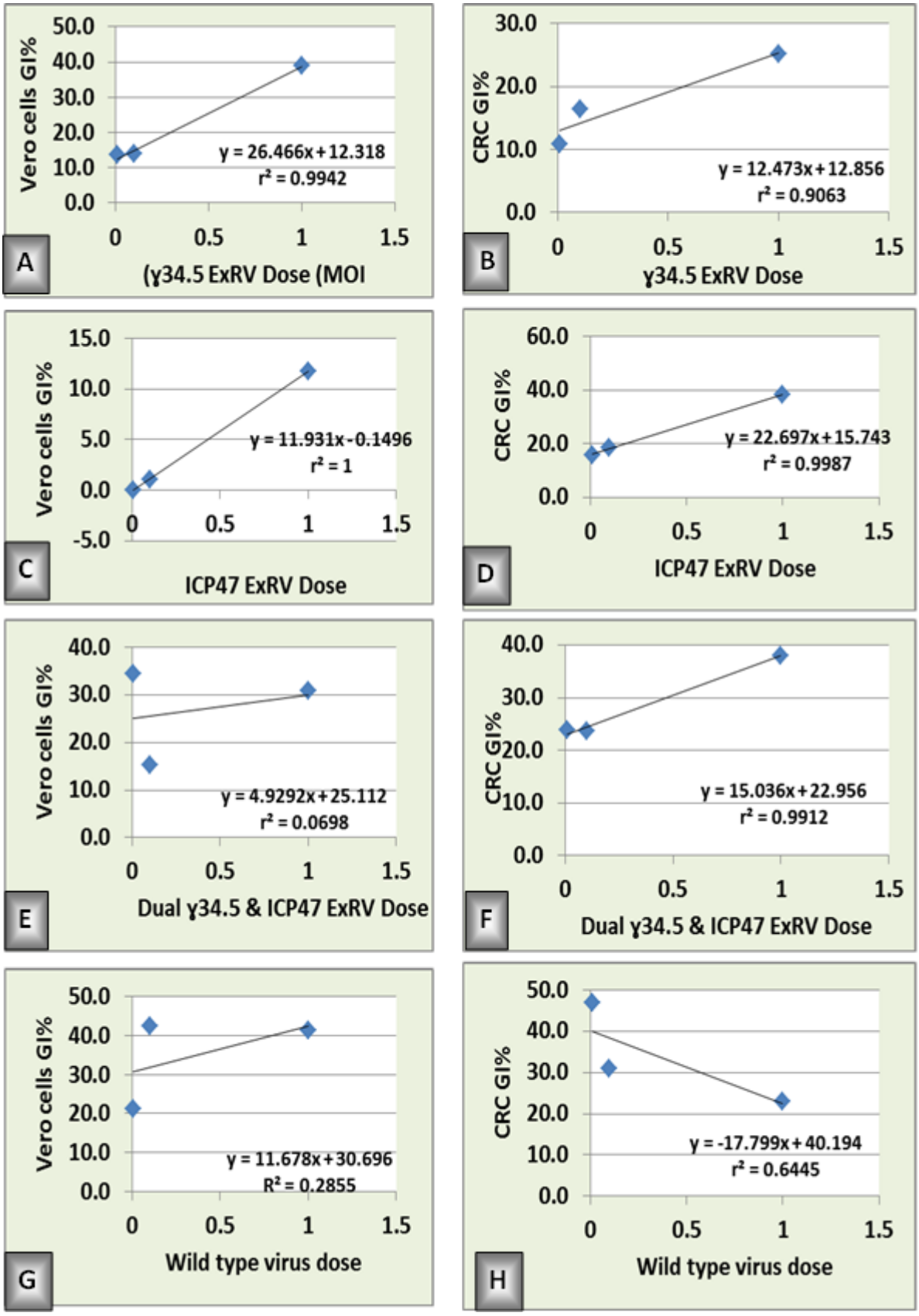

Figure (4): Growth inhibition curve plotted by response vs dose of ɣ34.5 ExRV, ICP47 ExRV and dual ɣ34.5 & ICP47 ExRV and WT HSV-2 on both Vero cell and LS174T CRC cell lines respectively.

Numerous types of cancers remain incurable to date. However, oncolytic virotherapy, an emerging treatment approach, holds significant therapeutic potential. It employs replication-selective viruses that specifically target tumors, leading to tumor cell destruction through cytolysis (Russell *et al*., 2012). After the tumor cells are eliminated, viral progenies are released, enabling them to infect neighboring tumor cells continuously. This property can be inherent to the virus or achieved through genetic engineering (Lin *et al*., 2004). Among the oncolytic viruses, HSV has received considerable attention and has gained approval from the Food and Drug Administration (FDA) for melanoma treatment (Yura, 2017). Our findings align with previous reports indicating that attenuated, replication-competent oncolytic HSVs can effectively infect and destroy various tumor cell lines in vitro (Abdoli *et al*., 2017; Learmonth *et al*., 2015). Viruses must manipulate various cellular organelles involved in defense and immune processes to survive and replicate within cells. Upon entering the host cell, they modulate cell signaling pathways and organelles, including mitochondria, for their own benefit (Anand *et al*., 2013). As reported, the increased metabolic viability observed during infection may be attributed to hyperactive mitochondria or an increase in mitochondrial mass, as the conversion of MTT into formazan primarily occurs in the mitochondria (Rai *et al*., 2018). Consequently, infection of a non-tumor cell line could lead to an apparent rise in mitochondrial activity during oHSV infection, generating a false signal of cell proliferation. On the other hand, a tumor cell line would be affected and ultimately lysed by oHSV, resulting in reduced formazan formation. The results were coming in agreement with the results of many studies that show the effect of oncolytic virus on colorectal cancer cells , Bennett *et al*. showed that NV1023 (a multi-mutant HSV-strain F) could replicate inside and kill CT26 cell in vitro. They concluded that NV1023 has the oncolytic potential to eliminate colon cancer cells (Bennett *et al*.,2001). Esaki et al. showed that the combination of HF10 (a highly attenuated HSV-1) and gemcitabine resulted in a considerable reduction of the growth of CT26 tumor cells in comparison with each of them, separately (Esaki *et al*.,2013). They demonstrated that HSV could infect and lyse the CT26 cell. Abdoli *et al* demonstrated that HSV-GR recombinant virus could efficiently infect and replicates in three mice syngeneic cell lines (4T-1, CT26, and TC-1 cells). (Abdoli *et al*.,2014).

References:

Abdoli ,S .: Roohvand Farzin .:, Teimoori-Toolabi, Ladan .: Shayan, Sara 1 , and Shokrgoza M, Ali.,(2019) Cytotoxic effect of dual fluorescent-labeled oncolytic herpes simplex virus type 1 on mouse tumorigenic cell lines

Abdoli S, Roohvand F, Teimoori-Toolabi L, Shokrgozar MA, Bahrololoumi M, Azadmanesh K. (2017).Construction of Various gamma34.5 Deleted Fluorescent-Expressing Oncolytic herpes Simplex type 1 (oHSV) for Generation and Isolation of HSV-Based Vectors. Iranian biomedical journal.;21(4):206-17.

Accardo, *et al*. (2014) (AMS Collaboration); High Statistics Measurement of the Positron Fraction in Primary Cosmic Rays of 0.5–500 GeV with the Alpha Magnetic Spectrometer on the International Space Station, *Phys. Rev. Lett*, Vol. 113, Iss. 12 — 19, 121101.

Anand, S.K.; Tikoo, S.K. (2013).Viruses as Modulators of Mitochondrial Functions. Adv. Virol.,. [CrossRef] [PubMed]

Argnani R, Lufino M, Manservigi M, Manservigi R. (2005). Replication-competent herpes simplex vectors: design and applications. Gene Ther.;12(S1):S170-S7.

Badisa, R.B.; Darling-Reed, S.F.; Joseph, P.; Cooperwood, J.S.; Latinwo, L.M. and Goodman, C.B. (2009). Selective cytotoxic activities of two novel synthetic drugs on human breast carcinoma MCF-7 cells. Anticancer res, 29(8): 2993-2996.

Bennett JJ, Malhotra S, Wong RJ, Delman K, Zager J, St-Louis M, et al. (2001).Interleukin 12 Secretion Enhances Antitumor Efficacy of Oncolytic Herpes Simplex Viral Therapy for Colorectal Cancer. Annals of Surgery.;233(6):819-26.

Bhattacharya, P., Budnick, I., Singh, M., Thiruppathi, M., Alharshawi, K., Elshabrawy, H., Holterman, M.J., and Prabhakar, B.S. (2015). Dual role of GM-CSF as a pro-inflammatory and a regulatory cytokine: implications for immune therapy. J. Interferon Cytokine Res. 35, 585–599.

Bhattacharya, P., Thiruppathi, M., Elshabrawy, H.A., Alharshawi, K., Kumar, P., and Prabhakar, B.S. (2015). GM-CSF: an immune modulatory cytokine that can suppress autoimmunity. Cytokine 75, 261–271.

Chaurasiya, S., Chen, N. G. & Fong, Y. (2018)Oncolytic viruses and immunity. Curr. Opin. Immunol. 51, 83–90.

Christopher J. LaRocca, Stacey Wilber, Eric Jensen, Subree Subramanian, Clifford Steer, Julia Davydova (2023) Development of an Oncolytic Adenovirus to Treat Metastatic Colorectal Cancer University of Minnesota

Esaki S, Goshima F, Kimura H, Murakami S, Nishiyama Y. (2013)Enhanced antitumoral activity of oncolytic herpes simplex virus with gemcitabine using colorectal tumor models. International Journal of Cancer.;132(7):1592-601.

Ferrucci, P.F., Pala, L., Conforti, F., and Cocorocchio, E. (2021). Talimogene laherparepvec (T-VEC): an intralesional cancer immunotherapy for advanced melanoma. Cancers 13, 1383.

Flis S. and Spławiński J. (2009).Inhibitory Effects of 5-Fluorouracil and Oxaliplatin on Human Colorectal Cancer Cell Survival Are Synergistically Enhanced by Sulindac Sulfide. International Journal Of Cancer Research And Treatment , Jan;29(1):435-41

Kelly, E. & Russell, S. J. (2007)History of oncolytic viruses: genesis to genetic engineering. Mol. Ther. 15, 651–659.

Kuppusamy, P., Ichwan, S. J. A., Al-Zikri, P. N. H., Suriyah, W. H., Soundharrajan, I., Govindan, N., et al (2016) 'In Vitro Anticancer Activity of Au, Ag Nanoparticles Synthesized Using Commelina nudiflora L. Aqueous Extract Against HCT-116 Colon Cancer Cells', Biological Trace Element Research, 173(2), pp. 297–305.

Learmonth K, Braidwood L, Woll P, Conner J. (2015).Immune responses following intrapleural administration of oncolytic SEPREHVIR in patients with malignant pleural mesothelioma. *Journal for immunotherapy of cancer*.;3(Suppl 2):P335.

Li, Y. et al. (2020)Oncolytic virotherapy in hepato-bilio-pancreatic cancer: the key to breaking the log jam? Cancer Med. 9, 2943–2959.

Lin, E.; Nemunaitis, J. Oncolytic viral therapies. Cancer Gene Ther. 2004, 11, 643–664. [CrossRef] [PubMed]

Manivanh, R., Mehrbach, J., Knipe, D.M., and Leib, D.A. (2017). Role of herpes simplex virus 1 g34.5 in the regulation of IRF3 signaling. J. Virol. 91, e01156–e01217.

Rahman, M.M., and McFadden, G. (2021). Oncolytic viruses: newest frontier for cancer immunotherapy. Cancers 13, 5452.

Rai, Y.; Pathak, R.; Kumari, N.; Sah, D.K.; Pandey, S.; Kalra, N.; Soni, R.; Dwarakanath, B.S.; Bhatt, A.N. (2018).Mitochondrial biogenesis and metabolic hyperactivation limits the application of MTT assay in the estimation of radiation induced growth inhibition. Sci. Rep., 8, 1531. [CrossRef] [PubMed]

Russell, S.J.; Peng, K.W.; Bell, J.C.(2012).Oncolytic virotherapy. Nat. Biotechnol., 30, 658–670. [CrossRef] [PubMed]
Speranza MC, Kasai K, Lawler SE. (2016).Preclinical Mouse Models for Analysis of the Therapeutic Potential of Engineered Oncolytic Herpes Viruses. ILAR journal.;57(1):63-72.
Wang H, Cheng H, Wang F, Wei D, Wang X. (2010).An improved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay for evaluating the viability of Escherichia coli cells. *J Microbiol Methods*. Sep; 82(3):330-3.
Yura Y. (2017).Presage of oncolytic virotherapy for oral cancer with herpes simplex virus. The Japanese dental science review.;53(2):53-60.
Zhang, B. et al. (2021).Intratumoral OH2, an oncolytic herpes simplex virus 2, in patients with advanced solid tumors: a multicenter, phase I/II clinical trial. J. Immunother. Cancer 9, e002224.
Zhao Q, Zhang W, Ning Z, Zhuang X, Lu H, et al. (2014) A Novel Oncolytic Herpes Simplex Virus Type 2 Has Potent Anti-Tumor Activity. PLoS ONE 9(3): e93103. doi:10.1371/journal.pone.0093103.