ENHANCEMENT OF ONCOLYTIC HERPES SIMPLEX TYPE 1 VIROTHERAPY

by

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M.Sc., Linkoping University, Sweden, 2010

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate and Postdoctoral Studies

(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2016

Abstract

Despite excellent safety data, antitumor efficacy of the oncolytic herpes simplex virus type 1 (oHSV-1) in glioblastoma (GBM) patients is not satisfactory. A major enhancement of the oncolytic activity of the oHSV-1 is necessary to eradicate the GBM in clinics. To enhance the efficacy and tumour specificity, we first developed a GBM specific triple regulated oHSV-1 amplicon system (SU4-124 HSV-1). Translational regulation was achieved by incorporating five copies of microRNA 124 target sequences into the 3'UTR of the ICP4 gene. Additionally, a 5'UTR of rat fibroblast growth factor -2 was added in front of the viral ICP4 gene open reading frame. The SU4-124 HSV-1 demonstrated enhanced tumour specificity and stronger anti-tumour efficacy compared to the tumour non specific CMV- ICP4 HSV-1 in both in-vitro and in-vivo GBM models. We then examined the effect of a potent STAT inhibitor, which is the key regulator of interferon (IFN) response, nifuroxazide (NF), a prescription anti-diarrheal drug, on the oHSV-1 efficacy. This was done with the aim of reinforcing the anti-tumour efficacy of oHSV-1 and developing an effective combination therapy. Here, we found that NF synergistically augments the anti-tumour efficacy of oHSV-1 by regulating the anti-apoptotic properties of HSV-1 in various tumour cells. Moreover, our data demonstrated that STAT1/3 activation mediated the underlying cellular mechanism of this novel combination. To further improve the efficacy of oHSV-1, possible barriers in the microenvironment of GBM need to be identified. Since previous clinical GBM have documented an abundance of microglia /macrophages, we first investigated the interaction of oHSV-1 and microglia/macrophages in in-vitro and in-vivo GBM models. We found evidence that microglia/macrophages suppress oHSV-1 in glioma mass by generating a physical barrier to the dissemination of oHSV-1. We also observed that the deficiencies in viral replication in microglial cells are associated with the STAT1/3-mediated silence of particular viral genes. We found that an oxindole/imidazole derivative, C16, can aid the viral replication in microglia/macrophages and dramatically increase the therapeutic efficacy of oHSV-1 in GBM animal model. In conclusion, this project outlines possible ways to overcome the barriers involved in oHSV-1 therapy to successfully eradicate clinical GBM.

Preface

The manuscripts reported in this thesis are based on my work performed toward the completion of my Ph.D. program, which is either published or submitted for publication or in preparation now.

A version of Chapter 2 has been published and reproduces under Creative Commons Attribution 3.0 License (Copyright @ 2016 Impact Journals). Tumour-specific triple-regulated oncolytic herpes virus to target glioma. Zahid M. Delwar, Guoyu Liu, Yvonne Kuo, Cleo Lee, Luke Bu,3, Paul S. Rennie, William W. Jia. Oncotarget. 2016 May 10;7(19):28658-69. Most of the in-vitro and in-vivo experiments were performed by myself. Data were interpreted and analyzed by myself. Tissue DNA extraction and qPCR were carried out by Yvonne Kuo and myself. miRNA 124, 143 and 145 expression pattern in mouse and human tissues were performed by Dr. Cleo Lee. Technical support was provided by Guoyu Liu and Luke Bu. The manuscript was written by myself with the help of Dr. William Jia. Conception, design, and revision of the manuscript were performed by Dr. Paul S. Rennie and Dr. William Jia. The study was supervised by Dr. William Jia.

A version of Chapter 3 has been submitted for publication. Zahid M. Delwar, Justin Lardizabal, Yvonne Kuo, Erin Kwa, Gabriel Fung, Honglin Luo, William W. Jia. An antibiotic drug, nifuroxazide synergistically enhances anti-tumour effect of oncolytic herpes simplex virus. Most of the in-vitro and in-vivo experiments were performed by myself. Data were interpreted and analyzed by myself. Technical support for synergy analysis was provided by Justin Lardizabal. Western blot analysis was performed by myself and Yvonne Kuo. Cell death analysis was conducted by myself, Erin Kwa, Dr. Gabriel Fung and Dr. Honglin Luo. The manuscript was written by myself and revision was done by Dr. William Jia. The study was supervised by Dr. William Jia.

A version of Chapter 4 will be submitted for publication. Zahid M Delwar, Yvonne Kuo, Yan Hua Wen, William Jia. Microglia/macrophages mediated inhibition of oncolytic virotherapy is STAT1/3 dependent. Most of the in-vitro and in-vivo experiments were performed by myself. Data were interpreted and analyzed by myself. Immunostaining

was carried out by myself and Yvonne Kuo. The manuscript was written by myself and revision was done by Dr. William Jia. The study was supervised by Dr. William Jia.

A review article is published, based on recent progress on oncolytic virotherapy, including knowledge gained from this project, which is reproduced with permission from ©Nature Publishing Group. Zahid Delwar, Kaixin Zhang, Paul S. Rennie & William Jia. Oncolytic virotherapy for urological cancers. Nature Review Urology. 2016 Jun;13(6):334-52

A collaborative work with Dr. Aziz Gahary was also published. Yunyuan Li,Hans Adomat,Emma Tomlinson Guns,Payman Hojabrpour,Vincent Duronio,Terry-Ann Curran,Reza Baradar Jalili, William Jia, Zahid Delwar,Yun Zhang,Sanam Salimi Elizei, Aziz Ghahary. Identification of a Hematopoietic Cell Dedifferentiation-Inducing Factor. Journal of Cellular Physiology. 2016 Jun;231(6):1350-63

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Acknowledgements

First and foremost, I would like to express my profound gratitude and indebtedness to my supervisor Dr. William Jia for his patience, motivation, and immense knowledge, and being a perennial source of inspiration in all through my Ph.D. study. His guidance helped me a lot to conduct my research and writing this thesis. I could not have imagined having a better mentor for my Ph.D. study. Besides my supervisor, I am also very much grateful to the rest of my supervisory committee: Dr. Paul Rennie, Dr. Honglin Luo and Dr. Christopher Ong, for their patient hearing, insightful comments, encouragement and constructive criticism, which helped me throughout the entire work to complete the study successfully. I thank my fellow labmates, particularly, Dr. Si Zhang, Mr. Justin Lardizabal, Mr. Luke Bu and Ms. Guoyu Liu for their kind help and guidance during my lab work. Last but not the least, I would like to thank my family for their continuous support and inspiration.

To my parents

1 Introduction

1.1 Glioma

Glioma is one of the most common primary brain tumours [1,2] that is rapidly growing and therapy-resistant [3]. Prognosis for high-grade glioma patients is very poor [4,5] and the median survival is only 14.6 months after receiving all the currently available treatments [1]. A new effective therapeutic approach is therefore urgently needed.

1.1.1 Treatment of glioma

Radiation and chemotherapy following maximal surgical resections are the current treatment for newly diagnosed high-grade glioma patients [6,7]. Very few effective chemotherapeutic agents are available to treat glioblastoma. In addition, most of the chemotherapeutic agents show only marginal clinical benefit [4,5]. Chemotherapeutic treatment of glioma is mainly temozolomide (TMZ) based [3], which is an alkylating chemotherapeutic drug [4,5]. TMZ arrests the cell cycle by methylates guanine group in the DNA and therefore, resulting O-6-methylguanine mispairs with thymine upon DNA replication. However, many patients fail to respond to TMZ due to inherent or acquired resistance [3,8,9]. The DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) over-expression is considered as a key mechanism of TMZ resistance. MGMT repairs the lesion by removing the methyl group from guanine to a cysteine residue prior to the formation of the mismatch [10]. Resistance to cytotoxic agents remains the greatest barrier to the successful treatment of glioma [3]. In addition, chemotherapeutic agents induce premature senescence in cancer cells which re-enter the cell cycle and lead to cancer relapse after treatment [11-15].

1.2 Oncolytic Virotherapy

Oncolytic virotherapy has emerged as a promising anti-cancer therapeutic approach recently. The first oncolytic virus T-VEC (herpes virus-based oncolytic virus) was approved last year (2015) by a combined FDA advisory panel for treating melanoma [16,17]. This is a landmark for the success of oncolytic virotherapy and extends its clinical use to other types of cancer. Oncolytic virotherapy uses replication-competent viruses to specifically destroys cancer cells through oncolysis: a killing mechanism characterized by the lysis of cancer cells through the course of virus replication [18]. As oncolytic viruses (OVs) can kill cancer cells via a mechanism distinct from the killing effects of conventional chemotherapy and radiotherapy, OVs are potentially ideal to kill cancer cells that are non-responsive to conventional treatment.

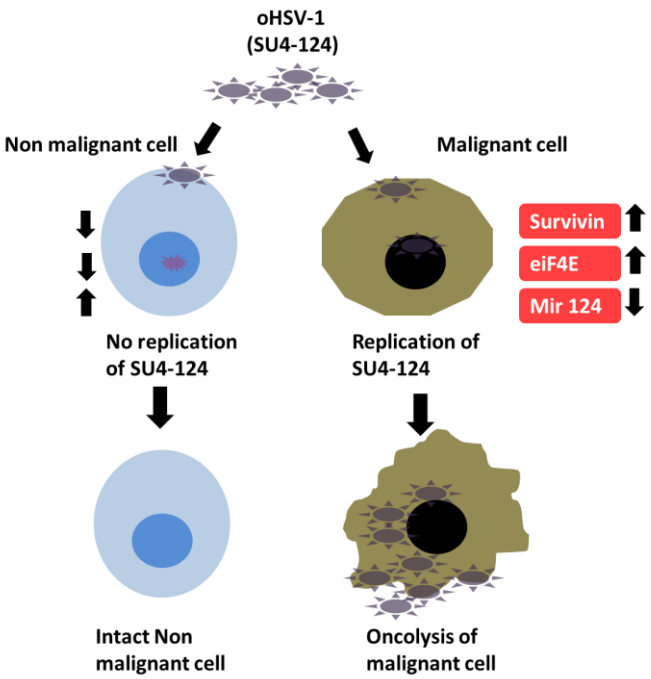


Figure 1.1 Oncolytic virus (OV). OV replicates and kills malignant cells by cell lysis, while unable to replicate in non malignant cells.

1.2.1 General mechanisms of virotherapy

The tumour-destructive mechanism of OVs lies in their capability to replicate through a lytic cycle, which results in lysis of the cancer cell and release of viral progeny to infect neighbouring cells. Moreover, OVs also commence antitumour immune responses, which overcome the immunosuppressive environment of tumours [19]. Excellent safety and promising therapeutic efficacy of oncolytic virotherapy have already shown against various tumours in preclinical animal models and a number of clinical trials [20-27]. Oncolytic virus' tumour-destruction capability may not be reduced or influenced by cellular mechanisms that contribute to tumour's resistance to conventional treatments, such as chemotherapy [28,29]. In fact, many cellular mechanisms responsible for resistance to conventional therapies enhance the replication of OVs. These mechanisms include enhanced oncogene expression (such as *RAS* genes) [30], increased DNA repair activity [31], anti-apoptotic activity [32] and augmented function of survival and proliferation pathways, such as protein kinase B (Akt) [33] and nuclear factor κB (NF-κB) [34]. Crucially, the same mechanisms are also featuring of late-stage cancers, making virotherapy a potentially perfect treatment candidate to target cells that have acquired resistance to conventional therapies.

However, OVs mediated tumour regression is a complex process that involves the activities of several mechanisms. Viral oncolysis was considered to be the most important factor of tumour regression. OVs eradicate malignant cells through lytic replication, which eventually results in lysis of the infected cells. Most viruses penetrate the cells through cell surface receptors and instantly initiate expression of viral genes. Moreover, viruses already carry some viral proteins. The virus takes control of the cellular machinery and halts production of cellular macromolecules to express its own genes for its replication. Some viral proteins are also directly cytotoxic and provoke apoptosis or necrosis of the host cells. In the infected cells, copies of the viral genome are amplified, followed by packaging of the viral progeny and discharge into the extracellular space via cell lysis, or infecting the neighbouring cells through fusion of cell membranes. Propagation of viruses among tumour cells and repetitive replication cycles can result in regression of the tumour.

Moreover, tumour-associated vasculature destruction by virotherapy may also play a major role in tumour destruction. In some cases, tumour-associated cells, such as stromal and endothelial cells within the tumour mass are eliminated by the viruses [35]. This process can hinder the tumour microenvironment, contributing to tumour regression activity. For example, oncolytic vaccinia has been demonstrated to get rid of tumour-supporting vasculature which escalates tumour regression [35].

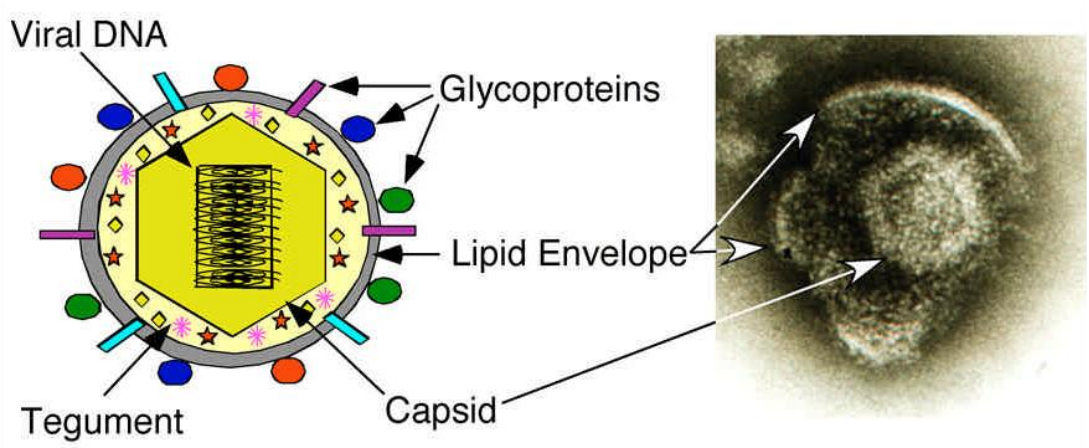
Host immune response has traditionally been considered to obstruct oncolytic effectiveness, as host antiviral immunity usually prevents viral replication. However, virus infection provoked immune responses have now been recognized as a crucial factor contributing to tumour destruction. Despite suppressed immune system in many cancer patients, viral infection activates both innate and adaptive immune responses in the host. Infected tumours can become inflamed and release cytokines to activate immune cells to attack infected cells. Also, virus-mediated cell lysis can discharge cellular proteins into the extracellular space that leads the presentation of specific tumour antigens that are usually intracellular [36,37]. Accordingly, natural killer cells, macrophages, and T cells can attack tumour cells that are usually hidden or not recognised by the immune system.

1.2.2 Herpes Simplex Virus

Herpes simplex virus (HSV) belongs to the subfamily Alphaherpesvirinae in the Herpesviridae family. HSV is a large, double-stranded DNA viruses and it contains two closely related viruses: Herpes simplex virus Type 1 (HSV-1) and Herpes simplex virus Type 2 (HSV-2). HSV-1 is normally associated with oral lesions which are known as cold sores, and HSV-2 mostly causes genital lesions [38,39]. HSV-1 is widely distributed among human population, and around 2/3 of the adult human are seropositive for HSV [40].

1.2.2.1 Herpes simplex virus Type 1 structure

HSV-1 contains the following four structural components: 1) core that contains the double-stranded viral DNA, 2) an icosahedral capsid, 3) a tegument that contains additional viral proteins, and 4) an outer membrane envelope studded with viral glycoprotein spikes [39].



Taylor TJ, et al. Frontiers in bioscience 2002;7:752-64

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Figure 1.2 HSV virion structure. The HSV virion is comprised of (a) the double stranded DNA, (b) icosahedral capsid; (c) tegument layer; and (d) an envelope. EM picture of the virion structural components (right panel).

1.2.2.2 Herpes simplex virus Type 1 life cycle

HSV-1 genome size is 152kb that encodes approximately 84 genes. HSV-1 genome is categorized into three parts: immediate early (IE), early (E) and late (L) genes [39]. HSV-1 enters the cell by receptor-mediated endocytosis process. VP16 tegument protein activates the expression of IE (ICP0, ICP4, ICP22, ICP27 & ICP47) genes, followed by

E genes that encode enzymes and DNA replication protein, and finally, late genes encode structural proteins of progeny virus [39].

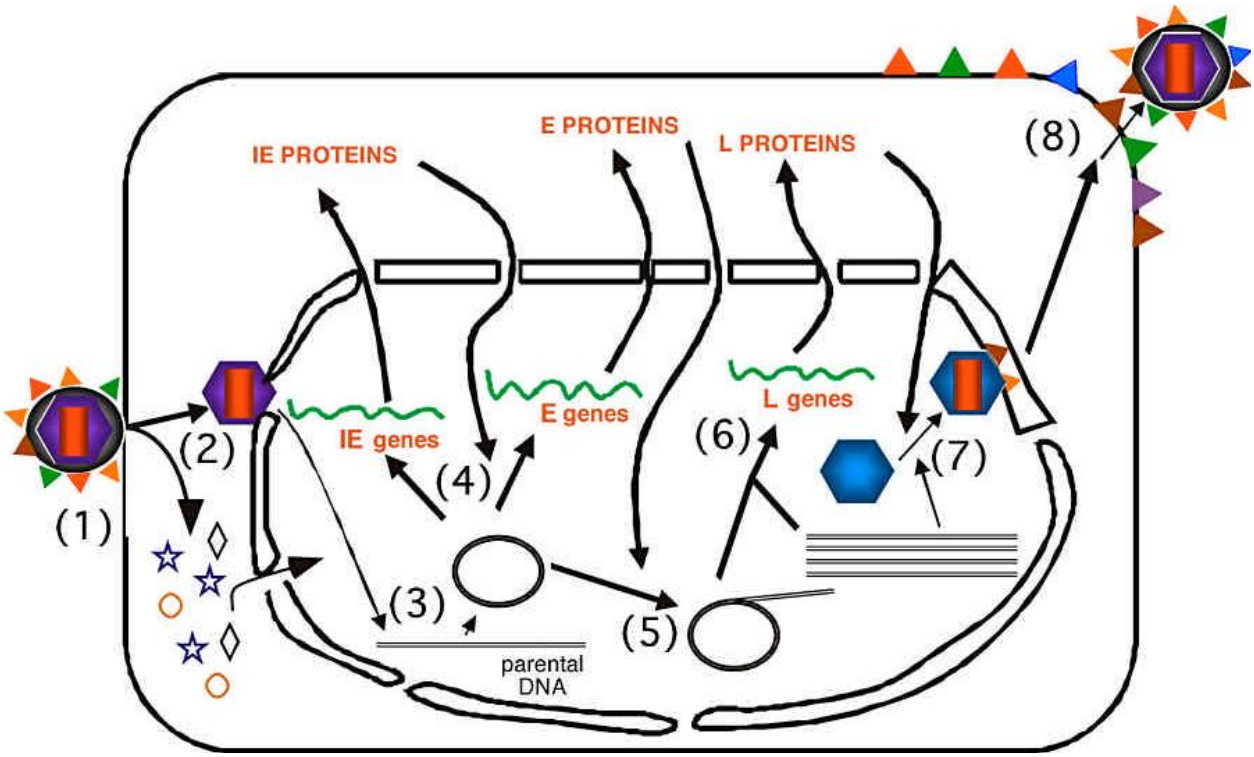

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Figure 1.3 Herpes Simplex Virus replication cycle. The stages of HSV infection and replication are: (1) Binding with receptor and membrane fusion; (2) Viral nucleocapsid and tegument release into the cell cytoplasm and then transport of the nucleocapsid to the nuclear pore; (3) Viral DNA release into the nucleus; (4) Viral immediate early (IE) and early (E) genes transcription and translation; (5) Synthesis of viral DNA; (6) Viral late (L) genes transcription and translation; (7) Assembly of the capsid and DNA packaging; and (8) egress of progeny virions.

1.2.2.3 Oncolytic potential of HSV-1

Genetically engineered HSV-1 vectors are promising therapeutic agents to treat various cancers as they have the capability to replicate specifically in and lyse tumour cells while sparing non malignant cells [41,42]. Oncolytic herpes simplex virus type-1 (oHSV-1) has been investigated for more than a decade in laboratories and clinical trials for the treatment of various types of cancer including glioblastoma [43].

HSV-1 has several advantages for using as an oncolytic virus: 1) HSV-1 is genetically very stable, 2) Its large genome size allows enhanced capacity for insertion of foreign genes, 3) It is easy to attain high titer and highly infectious, 4) clinical virology is well characterized and efficient anti-herpes agents are available such as acyclovir and ganciclovir. Therefore, the virus has been engineered to restrict its replication only in tumour cells and lyse tumour cells without harming non-tumour cells [41,42,44].

1.2.2.4 ICP34.5 and ICP6 deletion mutants of oHSV-1

In the 20th century, wild-type HSV-1 was found to slow down tumour growth. Then 70 years later, mutated viruses were among the first OVs, investigated in pre-clinical studies for their anti-tumour properties [45]. Viral gene deletion or mutation is the most common approaches to making the HSV-1 tumour specific. Potential oncolytic properties of the genetically mutated HSV-1 was first revealed by the thymidine kinase (TK) mutated HSV-1 [46].

HrR3 is an oncolytic HSV-1 [47,48], with a mutation /deletion of ICP6 gene encoding the viral ribonucleotide reductase (RR) [47] and this is one of the early stage oHSV-1s that was investigated in glioblastoma preclinical studies [49]. Ribonucleotide reductase (RR) is essential for the synthesis of deoxyribonucleotides, which is needed for viral DNA synthesis and replication. Increased expression of mammalian RR is found in most of the rapidly dividing cells, and RR deletion mutants (HrR3) efficiently replicate only in the cells that compensate for the loss of ICP6 by expressing the mammalian complement of RR [50].

G207 is a double gene mutated oHSV-1 that lacks neurovirulence γ34.5 and ICP6 gene [51-53]. G207 was among the first to be safely administered to patients with recurrent malignant glioma. HSV-1 infection triggers the activation of protein kinase R (PKR) which is one of the pivotal cellular defenses against viral infection. Activation of PKR leads to phosphorylation of eIF-2α, thereby shutting down protein synthesis in the normal host cell. HSV-1 has the countermeasure against PKR and γ34.5 restores protein synthesis by activating protein phosphatase-1 which thereby dephosphorylates and restores eIF-2α function. Due to some genetic mutations, activation of PKR is less pronounced in malignant cells and deletion of viral 34.5 gene makes the virus non-replicable in non malignant cells [45]. This is the most widely evaluated oHSV-1 in the glioma clinical trials [54,55] and series of clinical studies demonstrate the safety profile of G207, but its oncolytic efficacy needs to be improved [56].

1.3 Challenges of OV

Preclinical in-vitro studies in some laboratories have shown that oHSV-1 could eliminate glioma cells within a short period. This was further supported in immunodeficient glioma xenograft models where intratumoural inoculation of oHSV-1 could eliminate the tumour [57,58]. However, although excellent safety shown by several clinical trials, oHSV-1's therapeutic efficacy requires considerable improvements [59-61]. To be successful in clinics, oHSV-1 needs to overcome some challenges including:

1.3.1 Tumour selectivity without compromising viral replication efficiency

Maximizing oncolytic activity of an oncolytic virus without compromising safety for non malignant cells is a major challenge. High specificity for cancer cells is the central concern when designing OVs. Viral gene deletion to enhance safety and tumour specificity often comes with the price of losing strong oncolytic virulence [62,63]. A common approach to reduce oHSV-1's neurovirulence is to delete the ICP34.5 gene,

which significantly attenuates the viral replication efficiency [62,64]. GBM is highly invasive and infiltrate to surrounding brain parenchyma [65]. So, a highly glioma-specific oncolytic virus is required to eradicate glioma cells successfully without harming surrounding non-tumour cells.

1.3.2 Intratumoural barriers

Often, oncolytic virus treatments are highly effective in animal tumour models but not in clinical trials. In addition to many other differences, established tumours in patients are highly heterogeneous in comparison with those in animal models, containing significant amounts of non-tumour cells, such as stromal cells, infiltrating immune cells and endothelial cells in blood vessels. Viruses are large and often heavily charged particles that do not diffuse as easily in tissues as small molecules. Hence, biological dissemination, in contrast to purely physical diffusion, is necessary for the virus to spread throughout the entire tumour. As OVs by definition can only replicate in cancerous cells, tumour-associated non malignant cells are physical barriers within the tumour against dissemination of the progenitor virus or virus progeny released from infected tumour cells[66]. Glioma tumour mass also consists of numerous non-cancerous cells including various types of immune cells [67] that block the virus spread [68]. Clinical observations support this notion that replicating adenovirus resulted in better overall response rates than a non-replicating adenovirus, although both viruses expressed immune-system-stimulating factors [69,70].

1.3.3 Anti-viral immune response

Immune responses have been noted in numerous studies both in animal models and patients. Virus-targeted antibodies were readily detectable in patients receiving OVs [69,71-73] which might contribute to the attenuation of subsequently administrated virus doses [69,72]. The capability of the host's immune response to inhibit the effectiveness of OVs has been recognised for a long time. For OVs that are common in normal human populations, pre-existing antibodies and adaptive immune responses in addition to

innate immune response can effectively block virus activity. This might be particularly true if the viruses are strongly immunogenic and are delivered systemically since the viruses may encounter strong neutralising antibodies in the blood.

However, the effect of the immune response on virus dissemination is time-dependent and dose-dependent. Initial innate immune responses carried by macrophages [68] and NK cells [74] start almost instantly after virus inoculation [75], either by intratumoural injection or by systemic delivery, and this is followed by adaptive responses including induction of neutralizing antibodies and cytotoxic T-cells [76]. The anti-viral immune response may take some time to reach a full stop on viral dissemination. However, the antiviral immune response is also an important mechanism underlying oncolytic-virus-induced antitumour activity. All patients showed dose-related increases in IL-6 and IL-10 blood levels when treated with adenovirus CG7870 [77]. Some patients had increased serum levels of IFN-γ and IL-8 after treatment with coxsackievirus CVA21[78]. Several experiments with various OVs including HSV-1, adenovirus, parvovirus H-1, NDV, and VSV have shown that intratumoural virus injection induced increased tumour infiltration of various immune cells. It includes antigen-presenting dendritic cells, macrophages and tumour-specific CD4+ and CD8+ T cells [79-81]. Interestingly, tumour-specific lymphocyte infiltration also occurred in distant tumours that were not infected by the therapeutic viruses such as HSV-1, NDV and adenovirus [36,82,83]. In patients with prostate cancer, histological analysis of biopsy samples prepared upon prostatectomy following intratumoural reovirus injections demonstrated a substantial CD8+ T cell infiltration within the reovirus-injected areas [84]. In addition, in the CG0070 adenovirus trial, no correlation between administered virus dose and clinical efficacy was observed, suggesting that virus-mediated oncolysis was not a dominant cause for the observed tumour regressions in this case [69]. The virally induced strong immune response may help to kill cancer cells. However, the immune response can also eliminate the virus to attenuate its antitumour cell lysis. A balanced approach with carefully designed dosing schedule may help to obtain the optimised clinical outcome.

1.4 Strategies to increase the efficacy of oncolytic virotherapy

Strategies to overcome the obstacles of successful oncolytic virotherapy include increasing effectiveness as well as reducing adverse and off-target effects. For example regulating and restricting viral gene transcription and translation to target tissues, enhancement of the oncolytic activity of the viruses themselves, a combination of gene therapy with oncolysis, a mix of virotherapy with chemotherapy or radiotherapy, combining or arming OVs with immune-modulator factors and removing the intratumoural barriers.

1.4.1 Increasing effectiveness and reducing off-target effects by enhancing tumour specificity

Different strategies have been utilized to obtain the tumour selectivity of the OVs [85]. Viral gene attenuation or deletion is a common approach to reduce viral replication in non-tumour cells to reduce toxicity [62,64] . Tumour specific promoter driven viral gene expression is a useful transcriptional regulatory method to restrict virus replication in tumour cells, but this approach still results in basal-level expression of the controlled gene in non-target tissue [86]. Restricting expression on a translational level, in addition to transcriptional regulation, is another strategy to improve tissue specificity [87,88]. Attenuating viral tropism is another strategy to achieve tumour specificity by altering virus entry mediator gene(s).

1.4.1.1 Transcriptional regulation

Tumour cell specific promoter driven expression of an essential viral gene is a very common strategy [89]. This approach has been proven as a good strategy to target a particular cell type and use of a cell-specific promoter can considerably restrict viral gene expression to the desired cell [90]. Most viruses depend on expression of certain essential viral genes for their replication; thus, manipulating the transcriptional control of these genes can be another strategy to ensure tumour specificity. For example, for

HSV-1, putting *ICP4*, which is essential for virus replication, under the control of a tumour specific promoter can restrict lytic replication of HSV-1 to tumour cells [91].

1.4.1.2 Translational regulations

Tumour specific translational regulation of an oncolytic virus has been proven as another useful strategy to enhance tumour specificity and safety without compromising viral oncolytic virulence [87,88,92,93].

1.4.1.2.1 5'UTR mediated translational targeting

Eukaryotic translation initiation factor 4E (eIF4E) binds at 5-terminal, 7-methylguanosine-containing cap of an mRNA, thereby deliver that mRNA to the eIF4F, which is a translation initiation complex that composed of eIF4E, eIF4G (scaffolding protein), and eIF4A (RNA helicase). In normal cellular conditions, eIF-4E is bound by the inhibitory 4EBPs and 4EBPs sequester eIF-4E from interaction with eIF-4G [94]. Upon mitogenic stimulation, the 4EBPs become phosphorylated and dislodged from eIF-4E, thereby liberating eIF-4E to enable eIF-4E to deliver mRNAs to the eIF-4F complex. The eIF4F complex then scans through the 5' untranslated region (UTR) of the mRNA and expose the translation initiation codon by unwinding mRNA secondary structure to and trigger ribosome engagement to facilitate the translation of that mRNA [94,95]. The eif4e level is low in most non malignant cells, but high in almost all cancer cells [42,96-99]. The differential expression pattern of eif4e in tumour and non malignant cells providing an opportunity for cell-specific control [87]. Translation of many growth factors, such as FGF-2, is widely dependent on cellular levels of eIF-4E, owing to the complex structure of their 5' untranslated region (5'UTR). FGF-2 5'UTR insertion in front of the coding area of UL54, restrict its corresponding protein ICP27 expression only in tumour cells, which have high levels of eIF 4E [87].

1.4.1.2.2 MicroRNA mediated translational targeting

A further approach to regulating viral gene expression involves the use of microRNAs (miRNAs) [92,93,100]. miRNA is a class of small noncoding RNA with 21 - 22 nucleotides in length. miRNA is involved in the silencing of protein translation through complementary interaction with 3' untranslated region (3'UTR) of messenger RNA (mRNA) [101,102]. It plays a vital role to maintain a proper balance of various processes, including proliferation, differentiation, and cell death. They have been found to be involved in different diseases, including cancer. Several miRNAs are expressed differentially in various cell types, and some occur in lower levels in cancer cells than in non malignant cells [103]. Loss of tumour suppressive miRNAs increases translation of target oncogenes. On the other hand, upregulation of oncogenic miRNAs repress the translation of target tumour suppressor genes [104,105]. Several miRNAs, such as miR124 [106], miR128 [107], Let7 [108] are known to be upregulated in neuronal cells. Among them, miR124 is one of the most well known overexpressing micro-RNA in the CNS [109,110] and it has been found to be downregulated in glioma cells [103,106,111].

1.4.1.3 Targeting virus tropism for tumour specificity

Antibodies against the cell surface markers can be incorporated into their envelope or capsid to alter the tropism of the virus [112]. A previous study reported that trastuzumab (a monoclonal antibody that binds with HER2) coated lentivirus accumulated in the HER2-overexpressing solid and circulating tumours[112,113]. Tumour-specific oHSV-1 infection can also be achieved by engineering virus glycoproteins such as gD and gB, through recognition of tumour-specific receptor which is mostly expressed in tumour cells[100,114].

1.4.2 Enhance the efficacy of OV by combining with chemotherapy or immune modulator compounds

Recent clinical trials have shown that combining OVs with chemotherapy or radiation therapy improve the anticancer efficacy [115-120]. Similarly, several animal studies had also demonstrated enhanced anti-tumour efficacy or synergistic effects when oncolytic virus

treatment was combined with radiation, chemotherapeutic agents or immune inhibitory chemical compounds [121-124]. The combination of ICP34.5 encoding gene mutated HSV-1 with the alkylating agent temozolomide, the microtubule stabilizer paclitaxel or radiotherapy showed increased [125,126] or strongly synergistic [127,128] anti-tumour effects in various tumour models. DNA repair and DNA damage-inducible protein GADD34 is the cellular homologue of ICP34.5 and chemotherapy-mediated DNA damage inducible GADD34 can complement ICP34.5 deletion mutants for enhanced viral replication [129,130]. Not only oHSV-1, but similar combinational effect were also tested for many other OVs in various types of cancers [119,131-135]. Reovirus, which is another emerging oncolytic virus, also provides synergistic anti-tumour effect with docetaxel, probably through accelerated apoptosis triggered by prolonged mitotic arrest [120,136]. Mechanisms underlying the improved combinational antitumour efficacy can be varied. In some occasions, viral infection or replication enhances the anti-tumour effectiveness of the chemotherapy [129,130] and sometimes, chemotherapy or chemical compound increases viral replication [137]. Adenovirus and doxorubicin combination increase viral replication in soft-tissue sarcomas [137]. Cyclophosphamide which is an anti-cancer chemotherapeutic, as well as an immune suppressor, has been found to increase antitumour efficacy in combination with various OVs [68,138-147]. The chemotherapeutic role of cyclophosphamide enhances tumour cell killing, while immune suppressive effect help to reduce anti-viral immune response against the oncolytic virus [148]. Moreover, peritumoural inflammation caused by the chemotherapy or radiotherapy-mediated necrotic or apoptotic cancer cells might change the peritumoural microenvironment of immunity that may further improve anti-tumour effect of virally induced tumour vaccination [149]. Thus, a carefully scheduled combination of chemotherapy and virotherapy may generate better anti-tumour efficacy [150]

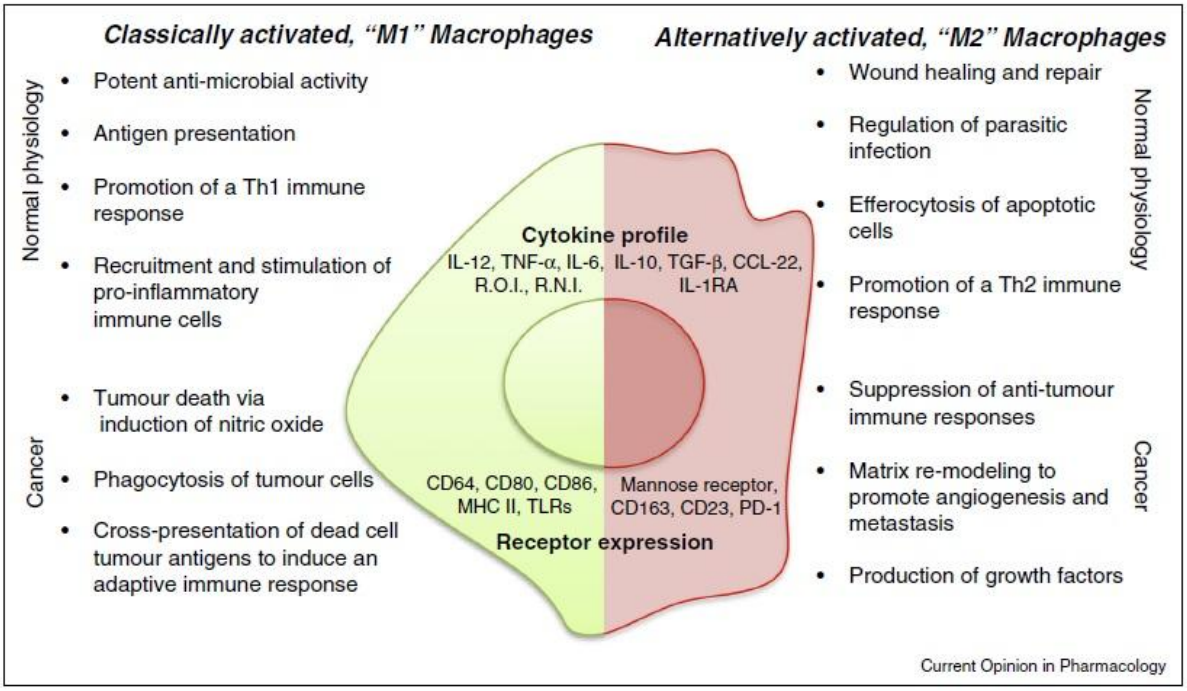
1.4.3 Overcome microglia/macrophage-mediated intratumoural barrier

Microglia is the tissue-resident macrophages of the brain, which is a type of mononuclear phagocytes that include peripheral tissue macrophages, CNS-associated

macrophages, dendritic cells and monocyte-derived cells [151,152]. They are innate immune cells and have diverse functions including chemotaxis, phagocytosis, antigen presentation, cytokine production and neurobiological function that consists of neural development and the maintenance of neuronal cell operating in both healthy and pathological contexts. Microglia contributes to both CNS damage and repair. Roles and phenotypes of microglia depend on the microenvironmental conditions, which can selectively determine the role of microglia [152]. Some studies suggest that microglia plays a profound role in the glioma growth and invasiveness [67,153,154]. Moreover, in the OV context, evidence suggest that activity of macrophages significantly affect the outcome of oncolytic virotherapy [155]. On the other side, oncolytic adenovirus and HSV have been found to enhance the infiltration of macrophages in glioma models [156,157]

1.4.3.1 Microglia/macrophage polarization

There are two functional categories of microglia/ macrophage: M1 (classically activated macrophages) and M2 (alternatively activated macrophages) [158]. Functional states of these two types of macrophage differ regarding activating signals, cytokine/chemokine production, receptor expression, and biological effects. Activated M1 polarized macrophages produce high levels of pro-inflammatory cytokines, such as IL-12, and oxidative metabolites, such as nitric oxide (NO) [159] and at the same time display elevated expression levels of MHC class II and co-stimulatory molecules CD80 and CD86, which have been used as a marker to detect M1 polarized macrophages [160].



Jakeman PG, et al. Current opinion in pharmacology. 2015; 24:23-29

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Figure 1.4 Functions and phenotypes of 'M1' and 'M2' polarisation states of macrophages[155].

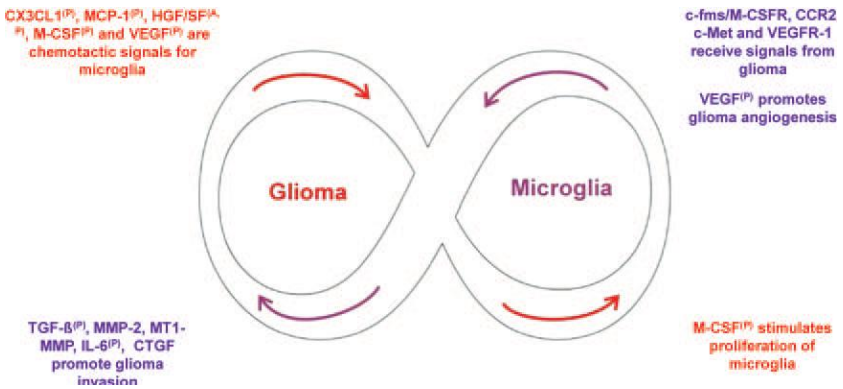
On the other hand, M2 macrophages induce tissue remodeling, angiogenesis, and repair and produce anti-inflammatory cytokines, such as IL-10, and decreased the level of NO production to suppress immune reactions [161]. CD163, CD204, and CD206 are the common markers to distinguish M2 phenotype of the macrophage[162].

1.4.3.2 Microglia/macrophage in glioma

The presence of high numbers of microglia /macrophages has been extensively documented in GBMs and other tumours[153]. Microglia/macrophages are the most common non-tumour cellular infiltrates in GBM [153,154,163-166] whereas mean microglia/macrophages content is around 30-40% (range:5-78%) of total tumour

mass[167,168]. The abundance of microglia /macrophages also depends on the malignancy stage of the brain tumour [165,167]. Moreover, the source of microglia/macrophages were further verified in rodent GBM model, microglia/macrophages contribute up to 46% of the tumour's cellular mass in rodent GBM, where up to 34% originated from microglia (CD45-/CD11b+ cells) that exist in the brain since embryonic development. The remaining 12% originated from blood-derived macrophages (identified as CD45+/CD11b+ cells) [169].

Regardless of the origin of tumour-associated macrophages (TAMs) in the GBM microenvironment, a large body of work suggested that tumour cells can communicate with TAMs [163]. The initial communication is triggered by glioma cells, which release a wave of chemoattractants to attract the robust infiltration of TAMs into the tumour mass. The potent TAM chemoattractants include the monocyte chemotactic protein (MCP)-1, MCP-3, cytokine-stimulating factor (CSF), granulocyte-colony stimulating factor (G-CSF), and many others. Importantly, the chemoattractants are shown to act both ways, where they are released not only by glioma cells to drive TAM infiltration, but also by TAMs to promote glioma growth and metastasis [163,164]. Adding to this complexity, it is shown that glioma cells release cytokines that suppress the proinflammatory nature of TAMs. Among these cytokines include anti-inflammatory cytokines IL-10, IL-4, TGF-beta and prostaglandin E2 [170-172]. In response, TAMs acquire a distinct inactive phenotype similar to inactivated "M2" or myeloid-derived suppressor cells (MDSCs); a phenotype thought to favor the tumour's evasion from the host immune system [173,174].



Li W et al. Neuro-oncology. 2012;14(8):958-78

Figure 1.5 Glioma-microglia crosstalk. Glioma and microglial interaction skewed for the glioma growth and invasiveness. The immunosuppressive microenvironment created by glioma-secreted molecules such asTGF-b, FasL, and IL-10 polarizes glioma-infiltrating microglia toward the M2 phenotype. Glioma (red) generates chemotactic factors, such as MCP-1, that stimulate the recruitment and proliferation of microglia. In turn, microglia (purple) enhance glioma angiogenesis and invasion [158].

1.5 Objective and aims

For more than a decade, oHSV-1 has been tested in clinical trials for patients with various types of cancer including glioblastoma [54,55,115,116,175-178]. Clinical trials of oHSV-1 have confirmed the safety of oHSV-1 administration to the human brain [55], but, the therapeutic efficacy of oHSV-1 to treat one of the most therapeutic refractory tumours like glioblastoma remains unsatisfactory. Factors that could obstruct the anti-glioblastoma efficacy of oHSV-1 includes: (a) reduced potency of oHSV-1 due to deletion or attenuation of viral genes for achieving tumour specificity, (b) suboptimal anti-tumour efficacy due to cellular responses in infected tumour cells and (c) tumour infiltrated non-tumour or immune cell responses which could hinder oHSV-1 replication and promote its clearance. The main hypothesis is that a tissue-specific oHSV-1 can enhance the anti-tumour efficacy of the oHSV-1 therapy, which can be further improved

by sensitizing the oHSV-1 mediated cytotoxicity and by blocking the response of tumour associated microglia/macrophages with a cytotoxic or immune modulator compound. We focused on two aspects of oncolytic virotherapy to overcome the obstacles and enhance the anti-tumour therapeutic efficacy. (I) Viral aspect: improving viral oncolysis potential; (II) Cellular aspect: a) sensitizing tumour cells to oncolytic virus cytotoxicity and b) overcoming tumour infiltrated non-tumour cell barrier.

Specific aim 1, construct a highly tumour specific replication efficient oHSV-1 amplicon system by utilizing transcriptional and translational regulator without deleting any viral gene.

Specific aim 2, sensitize the tumour cells by a potential chemical mediator or chemotherapeutic drug to stimulate oHSV-1 mediated cytotoxicity.

Specific aim 3, overcome the tumour-associated cellular barrier of oHSV-1 in tumour mass.

For specific aim 1, we demonstrated that the tumour specificity can be achieved by regulating an essential viral gene transcriptionally and translationally in an amplicon HSV-1 viral system without deleting any viral genes. For specific aim 2, we showed that a clinically available antibiotic drug used for a decade, nifuroxazide, sensitizes viral cytotoxicity in tumour mass by up-regulating apoptotic genes to boost the antitumour effect of oHSV-1 synergistically. For specific aim 3, we showed that microglia/macrophages are potential barriers to oHSV-1 dissemination in gliomas. We further revealed that an oxindole/imidazole derivative; C16 is capable of overcoming this obstacle to allowing viral replication in those noncancerous cells, which significantly enhances oHSV-1 therapeutic efficacy in glioblastomas in-vivo.

2 Tumour-specific triple-regulated oncolytic herpes virus to target glioma*

2.1 Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumour [65,179,180]. With the best possible treatment, GBM patients survive for only 12 to 15 months [179]. The current treatment for GBM is limited to surgical resection of the tumour, followed by radiation and chemotherapy [180]. Oncolytic virus (OV) therapy has recently emerged as a promising antitumour therapeutic mainly because its tumour specificity can selectively replicate in tumour cells while sparing normal cells [18]. Among the different OVs, oncolytic herpes simplex virus type 1 (oHSV-1) has emerged as one of the most promising OV candidates due to its well-known pathology in humans, extensively researched virology, well-characterized viral genome and its 150kb genome allowing ample space to integrate different transgenes and permitting of specific antiviral therapy as a safety measure [181,182]. The efficacy and safety of oHSV-1 has been widely investigated and tested in preclinical and clinical glioma models [181].

Viral oncolysis is an important feature of OV since it allows the virus to disseminate inside the tumour mass and to release tumour antigens associated with lytic destruction of tumour cells [183]. This mechanism provides OV therapy with the unique ability to provoke an anti-tumour immune response compared to other tumour vaccines [184]. However, acquiring viruses with a high level of lytic activity while maintaining tumour specificity has always been a challenge. Viral oncolysis is an important feature of OV since it allows the virus to disseminate inside the tumour mass and to release tumour antigens associated with lytic destruction of tumour cells [183].

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*A version of this chapter has been published. Zahid M. Delwar, Guoyu Liu, Yvonne Kuo, Cleo Lee, Luke Bu , Paul S. Rennie, William W. Jia. Tumour-specific triple-regulated oncolytic herpes virus to target glioma. Oncotarget. 2016 May 10;7(19):28658-69.

This mechanism provides OV therapy with the unique ability to provoke an anti-tumour immune response compared to other tumour vaccines [184]. However, acquiring viruses with a high level of lytic activity while maintaining tumour specificity has always been a challenge. For most oncolytic DNA viruses, the price of tumour specificity is often attenuated viral activity [63]. For instance, in the case of oHSV-1, a common approach to reduce its neurovirulence is to delete the ICP34.5 gene, which significantly attenuates the viral replication efficiency [62,64]

HSV-1 virus replication starts with the expression of immediate early genes, which initiate a cascade of viral gene expression ending in completion of the viral life cycle. Among the 5 essential genes, ICP4 and ICP27 are absolutely required for viral replication and disabling either one or the other results in a non-replicable virus. Previously, It has been reported that either ICP4 or ICP27 can be transcriptionally and/or translationally regulated without deleting any other viral genes allowing it to retain tumour specificity in a prostate cancer model [185,186]. In the present study, we further tested an amplicon/oHSV-completed system in which the ICP4 gene is controlled by glioma-specific transcriptional, post-transcriptional and translational triple-regulation in a glioma model.

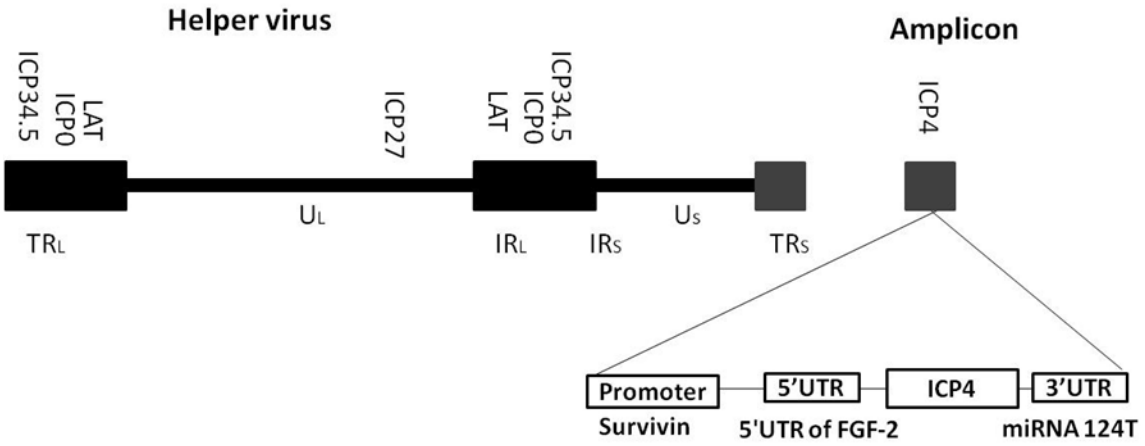

Figure 2.1 Schematic diagram of cancer-specific triple-regulated HSV-1 amplicon virus (SU4-124 HSV-1). The ICP4 promoter was replaced with the survivin promoter. The 5'UTR of rat fibroblast growth factor -2, and 5 copies of the microRNA 124 target sequence 3'UTR were introduced into the ICP4 gene

For this virus, the ICP4 gene is transcriptionally controlled by a survivin promoter, which is an anti apoptotic protein and vastly present in malignant cells [187], it is an ideal tumour-specific promoter for transcriptionally target tumour cells [188-191]. Survivin expression is up-regulated in most malignancies, and it is also played a major role in the oncogenesis and progression of malignant brain tumours [192]. It prevents caspase 9 and obstructs apoptotic pathway [193,194]. Survivin favours cancer survival by multiple functions including cytoprotection, inhibition of cell death, and cell-cycle regulation [195]. In addition, an FGF 5' UTR region and a 3'UTR containing the target sequence for miR124 are added to regulate expression of ICP4 for tumour-specific translation (Figure 2.1). Our results showed that this oHSV has enhanced tumour specificity in glioma model.

2.2 Materials and methods

2.2.1 Cell culture

Human glioma cell lines (U87, U373, SF188, SF126, SF210), a rat glioma cell line (9L), human embryonic kidney cells (293FT) and a monkey kidney cell line (vero) and 7B (ICP4 and ICP27 expressing cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and a 1% antibiotic mixture (penicillin and streptomycin). The human cortical neuron cell line (HCN-2) used was purchased from ATCC (Manassas, USA) and maintained in DMEM medium containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate (ATCC, USA), supplemented with 10% fetal bovine serum. Primary rat neurons were cultured in neurobasal medium supplemented with B27 and 5mM glutamax (Invitrogen, Canada). Human fibroblast (HFF) cells were maintained in MEM (Invitrogen, Canada) medium supplemented with 10% fatal bovine serum. All cultures were maintained at 37^{o}C in 5% CO_2.

2.2.2 Plasmid constructs

CMV-ICP4 HSV-1 amplicon plasmid construction was described by Cleo et al. [90] . CMV-124T HSV-1 plasmid was constructed by inserting five tandem copies of miRNA 124 complementary sequences (5'-CTCGAGCGGTTAATTAACGGGCATTCACCGCG TGCCTTACGATGGCATTCACCGCGTGCCTTAGATCGGCATTCACCGCGTGCCTTA GTCAGGCATTCACCGCGTGCCTTAGTCAGGCATTCACCGCGTGCCTTAGCTATCG ATGCAGT-3') into the 3' end of the ICP4 gene before the poly A signal in CMV-ICP4 HSV-1 plasmid . SU4-124 HSV-1 plasmid was constructed by replacing the CMV promoter of CMV-124T HSV-1 plasmid with a 268 bp survivin promoter [196], upstream to a rat FGF 5'UTR (Figure 2.1) [185] . All plasmids were constructed using a previously described protocol [90,185,186] .

2.2.3 Amplicon virus preparation

To prepare the amplicon viruses, corresponding plasmids were transfected into Vero or U87 cells by using Lipofectamine 2000 transfection reagent (Invitrogen, Canada) according to the manufacturer's instructions. After 48 hours, transfected cells were superinfected with helper (3galΔ3) virus at MOI-2. At 4 to 5 days post superinfection, amplicon viruses were harvested and amplified on SF188 glioma cells. Helper virus was amplified on 7B cells. Viruses were then subjected to titration on Vero and 7B cells to measure the concentration of amplicon and helper respectively, as described previously [90,186].

2.2.4 Virus replication assay

Cells plated on a 24-well plate were infected with the viruses at a multiplicity of infection (MOI) of 0.1 or 1. Virus infection and treatment were carried out in DMEM supplemented with 10% FBS and 1% antibiotics. At 2-3 days post-infection, viruses were harvested and freeze thawed three times and then titrated in triplicate on Vero cells by a standard plaque assay on 12-well plates.

2.2.5 Precursor miRNA transfection

293FT cells were co-transfected with amplicon plasmid (CMV-ICP4 HSV-1 or CMV-124T HSV-1) at a concentration of 200 ng and precursor miRNA (control pre- miRNA or pre-miR 124) at a concentration of 20, 50 or 200 nmol using Lipofectamine 2000 transfection reagent (Invitrogen, Canada) according to the manufacturer's instructions.

2.2.6 Luciferase reporter assay

293FT cells were co-transfected with survivin luciferin reporter plasmid (1.2 µg) and CMV-LacZ reporter plasmid (0.3 µg) using Lipofectamine 2000 transfection reagent (Invitrogen, Canada) according to the manufacturer's instructions. Total protein was

extracted at 24 hours post transfection and subjected to luciferase and β-galactosidase detection using a luciferase assay kit (Promega, Canada) and a β-galactosidase enzyme assay kit (Promega, Canada) respectively, according to the manufacturer's instructions.

2.2.7 Real time PCR

Total genomic DNA from tumours and various organs were extracted using an EZNA tissue DNA kit (Omega bio-tek). The viral ICP27 copy number was obtained by quantitative real time PCR (Quantstudio 6 Flex qPCR apparatus, Applied Biosystems) using 5'-GTCTGGCGGACATTAAGGACA-3' (forward) and 5'-TGGCCAGAATGACAAACACG-3' (reverse) primers. β-actin was used as an endogenous control. 10 ng of DNA were added to a 25 µl master mix of SYBR green (Invitrogen, Canada) supplemented with forward and reverse primers. miRNA 124 in HCN-2, HFF and glioma cells was detected with a miScript PCR Starter kit and an miR124 miScript Primer Assay, following the manufacturer's protocol (Qiagen, Canada).

2.2.8 Cytotoxicity assay

Cells were seeded in a 96-well plate at a density of 1 X 10^4 and allowed to settle overnight. They were then treated with vehicle only or viruses with different MOIs. Cell viability was measured after 2 or 3 days of treatment by means of an MTT assay (Sigma, Canada) according to the manufacturer's instructions. In brief, cells were incubated with MTT solution for 3 hours at 37^{o}C and then incubated with lysis buffer overnight. The next day, cell viability was measured at 595 nm by using a plate reader (Envision 2103 Multilabel Reader, Perkin Elmer).

2.2.9 Western blots

Total protein was extracted from cultured cells by using sample buffer (125 mM Tris-HCL, 50% glycerol, 4% bromophenol blue and 5% 2-mercaptoethanol). Proteins were

boiled in a heat block for 5 minutes, subjected to SDS-PAGE (8% gel), transferred to nitrocellulose membranes and then blocked in 5% nonfat milk (Bio-Rad) in TBS-Tween 20 (TBS-T) for 1 hour at room temperature. The membranes were then incubated overnight at 4^{o}C with either anti-eIF4E antibody (1:1000; Cell Signalling, Danvers, MA), anti-ICP4 antibody (1:750; Abcam, Cambridge, MA), anti-ICP27 antibody (1:1000; Abcam, Cambridge, MA) or anti-β-actin antibody (1:1000; Cell Signaling, Danvers, MA). Then next day, the membranes were washed with TBS-T three times and incubated with the corresponding secondary antibodies (1:3000; Perkim Elmer, Boston, MA) for 1 hour at room temperature. After washing three times with TBS-T, membranes were visualized using ECL reagent (Perkim Elmer, Boston, MA) and a VersaDoc imaging system (Bio-Rad) and band densities were then measured using ImageJ software (NIH, Bethesda, MD).

2.2.10 Immunohistochemistry

Harvested tissues were fixed with 4% paraformaldehyde for 24 hours and then incubated with 30% sucrose for 72 hours. Tissues were then embedded in OCT (Sakura Tissue-Tek), sectioned (20 um) using a cryostat (Leica CM 3050 S), prepared on Fisherbrand™ Superfrost™ Plus microscope slides (Fisher Scientific, Canada) and stored at -80C. During immunostaining, frozen sections were washed with PBS, and incubated with 3% albumin bovine serum (ABS) dissolved in PBS containing 0.1% Triton X-100 for one hour to block unspecific binding. Cells were then incubated overnight with either anti-ICP4 antibody (1:200; Abcam, Cambridge, MA) or anti-ICP27 antibody (1:200; Abcam, Cambridge, MA) diluted in PBS containing 0.1% Triton X-100 solution at 4ºC. The next day after washing 3 times with PBS, sections were incubated with goat anti-mouse IgG Alexa Fluor 488 secondary antibody (1:500; Invitrogen, Canada) for one hour at room temperature. After incubation with secondary antibody, sections were washed 3 times with PBS and mounted with Dapi Fluoromount G (Electron Microscopy Sciences). Sections were then visualized and imaged using a confocal microscope (Olympus, Canada).

2.2.11 Intracranial virus toxicity assay

Female C57BL6 mice were purchased from Harlan Laboratories. CMV-ICP4 HSV-1 or SU4-124 HSV-1 viruses were injected intracranially using a stereotactic frame. Animals were monitored on a daily basis to record any sign of toxicity. At 15 days after the virus injection, the animals were euthanized by CO2 asphyxiation. Then the virus-treated brain tissues were harvested and subjected to cryostat sectioning.

2.2.12 β-galactosidase staining

Frozen-fixed tissue sections were washed twice with PBS and then incubated with 1mg/ml X-Gal solution (Sigma, Canada) diluted with X-Gal staining solution (5mM K_3Fe, 5mM K_4Fe and 2mM $MgCl_2$) at 37^oC overnight. Cells were then counterstained with 1% eosin. Stained cells were visualized and imaged by using a light microscope.

2.2.13 U87 xenograft mouse model

Female athymic nude mice were purchased from Harlan laboratories. 2.5X10^6 human glioma U87 cells in 100 ul of PBS containing 0.75 mg Basement Membrane Extract (VWR, Canada) were subcutaneously implanted into the left flank. When the tumour size reached ~100 mm3, mice were administered a single dose of 3galΔ3, or CMV-ICP4 HSV-1 or SU4-124 HSV-1 virus by intratumoural injection. Tumour volume was measured at different time points using calipers (Height X Length X Wide/2). At the end of the experiment, mice were euthanized by CO2 asphyxiation and tumours as well as several organs (brain, stomach, intestine, liver) were harvested. All in-vivo experimental procedures were approved by the UBC Animal Care Committee and performed according to the guidelines of the Canadian Council on Animal Care.

2.2.14 Statistical analysis

Statistical analysis was performed by using Microsoft Excel and a significance $P<0.001$, $P<0.01$, or $P<0.05$ was determined using a 2 tailed Student's t-test. All data are expressed as means ± SD or ± SE.

2.3 Results

2.3.1 Survivin and eIF4E are overexpressed in glioma cells, but downregulated in normal neuronal cells

Survivin been reported to be upregulated in many cancers, including gliomas [197-203]. A survivin luciferase reporter assay in rat neurons and various glioma cells confirmed overexpression of the gene in glioma cells (Figure 2.2A). Upregulation of survivin transcription in glioma cells was also observed by qRT-PCR (data not shown). Since the ICP4 gene was also translationally regulated by the FGF gene 5' UTR, which requires a high level of eukaryotic translation initiation factor 4 (eIF4E) for successful translation, we performed western blot analysis to measure the eIF4E level in normal neurons and various glioma cells. Our results demonstrated the significantly increased eIF4E expression in glioma cells (Figure 2.2B), which is consistent with previous results by many other laboratories which showed that eIF4E is overexpressed in many cancers as well as in astrocytic tumours [94,97,204].

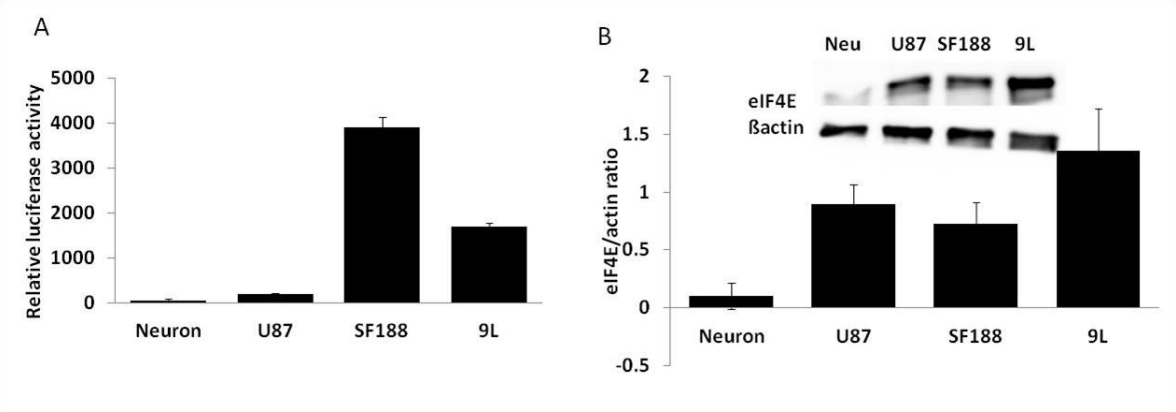

Figure 2.2 Survivin and eIF4E expression in different cells. (A) The indicated cells were transfected with survivin luciferase and CMV-LacZ reporter plasmids. Total protein was extracted 24 hours post-transfection and subjected to a luciferase and lacZ assay. (B) Total protein was extracted from the indicated cells and subjected to western blot analysis to measure eIF4E and β-actin expression. Data are presented as means ± SD

2.3.2 Micro RNA 124 expression is low in glioma cells

To select an effective micro RNA target for our glioma-specific oncolytic virus, we studied miR124 expression profiles in a different glioma and normal cells. PCR analysis demonstrated that the mature miR124 level was significantly high in human cortical neurons (HCN-2) compared with human glioma U87 and SF188 cells (Figure 2.3).

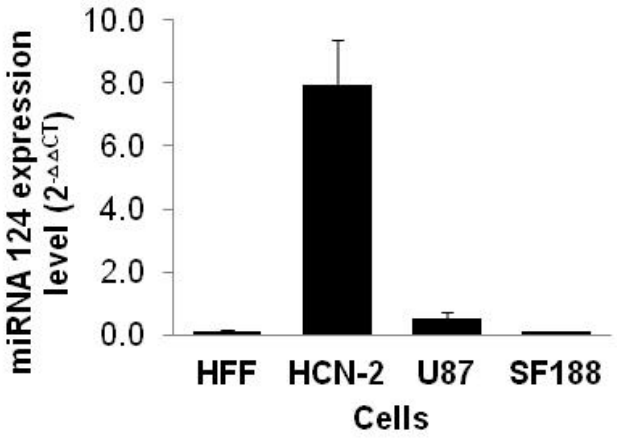

Figure 2.3 miRNA 124 expression levels in different cells. miR124 expression levels in the indicated gliomas and non malignant cells were detected by qRT-PCR. Data are presented as means ± S.D.

2.3.3 miR124 prevented the replication of miRNA regulated virus

To evaluate the specificity of the miR124-regulated ICP4 expression, 293FT cells were co-transfected with different concentrations (20 ng, 50 ng and 200 ng) of miR124 precursor and CMV-124T plasmid. Significantly downregulated ICP4 expression was observed in the presence of miR124 precursor (Figure 2.4A). Moreover, replication of CMV-124T HSV-1 in which the ICP4 gene is controlled by a 3'UTR region with an miR124 target, drastically decreased in miR124 precursor-transfected cells (Figure 2.4B). Furthermore, infection with CMV-124T HSV-1 showed that the ICP4 expression was significantly higher in glioma cells (U87, SF188 and 9L) than in primary cultured neurons. (Figure 2.4C).

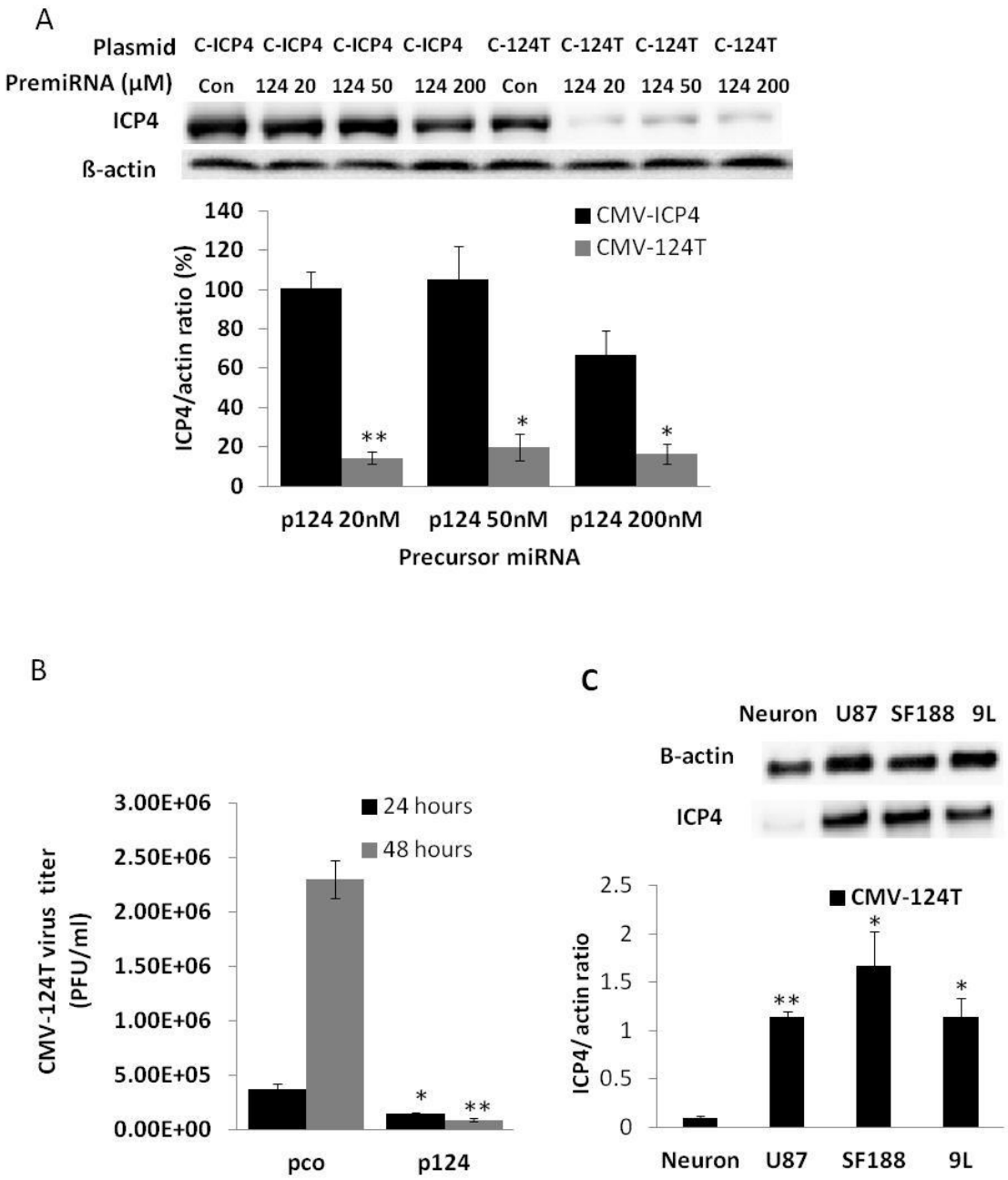

Figure 2.4 miRNA 124 prevents CMV-124T HSV-1 virus replication. (A) 293FT cells were co-transfected with amplicon plasmid (CMV-ICP4 HSV-1 or CMV-124T HSV-1) and control precursor miRNA or pre-miR 124 at a concentration of 20, 50 or 200 nmol. Total protein was extracted at 48 hours post-transfection. βactin and ICP4 protein levels were measured by western blot analysis. (B) 293FT cells were transfected with pre-con or pre-miRNA 124. Cells were superinfected with CMV-124T virus at MOI -1 after 48 hours of transfection. Viruses were harvested at 24 and 48 hours post-infection and titrated in Vero cells. Data are presented as means ± S.D. (* P<0.05, ** P<0.01 vs the

corresponding control or virus from CMV-ICP4 HSV-1 treated cells) (C) 124 micro RNA targeted ICP4 protein expression in the indicated cells. Cells were infected with CMV-124 virus at an MOI of 1. Total protein was extracted after 24 hours of treatment. ICP4 expression was measured by western blotting (* P<0.05, ** P<0.01 vs neurons).

2.3.4 miRNA regulation does not hinder HSV-1 antitumour efficacy

To determine whether incorporation of the miRNA 124 target sequence in the 3'UTR region of the ICP4 gene would hamper HSV-1 oncolytic activity, we evaluated the replication and cytotoxicity of the CMV-124T virus using a panel of glioma cell lines. A single-step virus growth assay (Figure 2.5A) and MTT cell proliferation assay (Figure 2.5B) demonstrated that all glioma cell lines are sensitive to CMV-124T virus oncolysis.

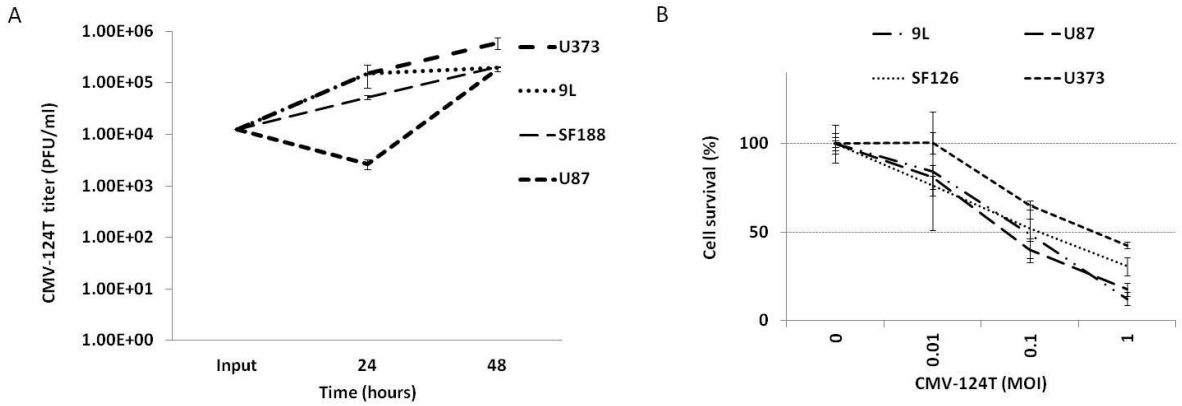

Figure 2.5 Replication & cytotoxicity of miRNA124 targeted amplicon virus. (A) The indicated cells were infected with CMV-124T HSV-1 at MOI- 0.1. Viruses were harvested at 24 and 48 hours post-infection and titrated in Vero cells. Data are presented on a logarithmic scale (B) The indicated cells were infected with CMV-124T HSV-1 at the indicated MOIs. After 48 hours of infection, cytotoxicity was measured by an MTT assay.

2.3.5 Survivin promoter, miRNA 124 and 5'UTR triple-regulated amplicon virus (SU4-124 HSV-1) have a strong antitumour effect on glioma cells

Since a high level of miR124 may not only be restricted to neural cells [109,205], additional measurement of tumour-specific controls is required to prevent virulence toward non-neural cells. To that end, we further tested an HSV-1 vector, SU4-124 HSV-1, of which the ICP4 gene is controlled by the survivin promoter and FGF 5'UTR in addition to the miR124 target in the 3' regions. To evaluate the antitumour effect, different glioma cells were treated with of the SU4-124 HSV-1 virus, different glioma cells were treated with a non-tumour-specific HSV-1 in which the ICP4 gene was driven by a generic CMV promoter (CMV-ICP4 HSV-1), (Figure 2.6A) or with SU4-124 HSV-1 for 48 hours (Figure 2.6B). Inhibitory concentration 50% (IC_{50}) values of SU4-124 HSV-1 against 9L, SF188 and U87 glioma cells were around MOI- 0.1, MOI- 0.1 and MOI- 0.05 respectively, while those of CMV-ICP4 HSV-1 against the above cells were approximately MOI-0.02, MOI- 0.1 and MOI- 0.05, respectively.

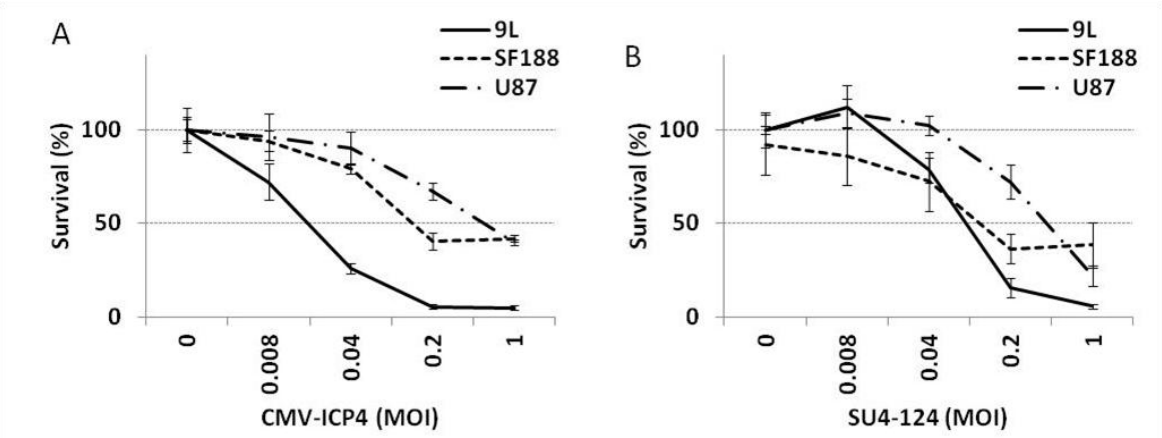

Figure 2.6 Antiproliferative effect of SU4-124 HSV-1. The indicated cells were infected with either CMV-ICP4 HSV-1 (A) or SU4-124 HSV-1 (B) at the indicated MOIs. Cytotoxicity was measured at 48 hours post-infection by an MTT assay.

2.3.6 Triple-tumour-specific regulation significantly increases tumour specificity

Since the mechanism involved in the glioma specificity of SU4-124 HSV-1 virus depends on glioma-specific expression of the essential viral gene ICP4, we investigated the ICP4 expression level of SU4-124 HSV-1 in normal neurons and glioma cells, as compared with the control CMV-ICP4 HSV-1 virus by western blotting. The ratio of ICP4 expression of glioma cells vs. normal neurons was significantly higher for SU4-124 HSV-1 than that for CMV-ICP4 HSV-1 (Figure 2.7A). Furthermore, to verify the tumour-specific growth of SU4-124 HSV-1, we evaluated the growth of the SU4-124 HSV-1 and CMV-ICP4 HSV-1 viruses in neurons and various glioma cells. For SU4-124 HSV-1, the ratios of tumour to neurons in viral replication were 16.7, 51.4 and 157 fold higher than those of CMV-ICP4 HSV-1 virus for U87, SF188 and 9L, respectively (Figure 2.7B).

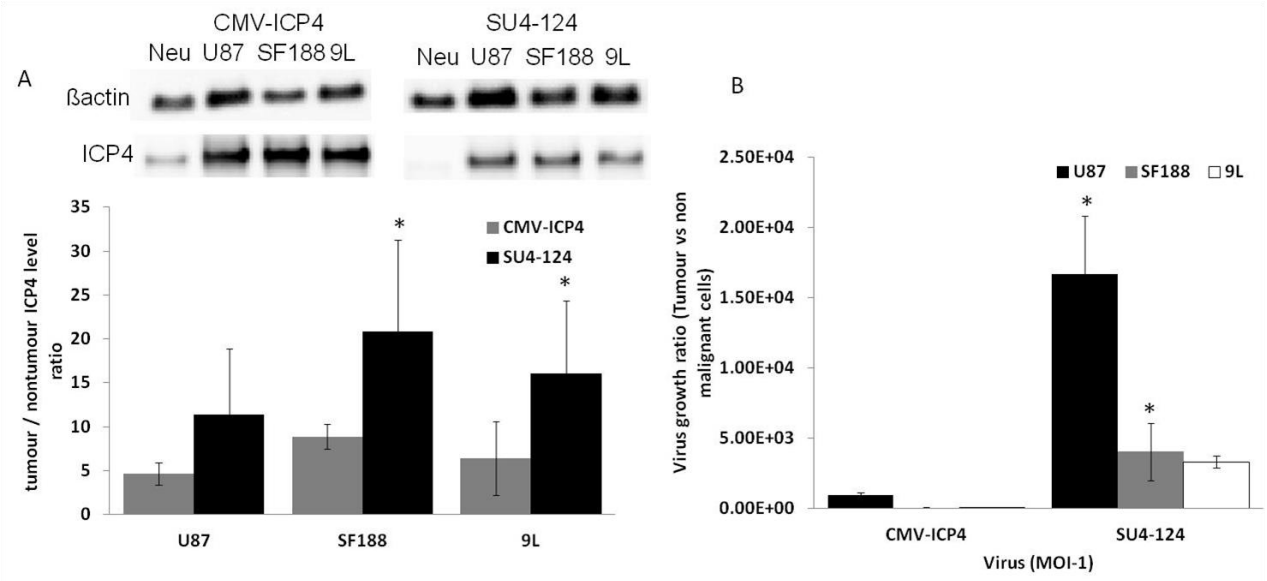

Figure 2.7 Triple regulation significantly augments tumour specificity. (A) Neurons and different glioma cells were infected with CMV-ICP4 HSV-1 or SU4-124 HSV-1 viruses at an MOI of 1. Total protein was collected 6 hours post infection. ICP4 expression was detected by western blotting. (B) Neurons and different glioma cells were infected with either CMV-ICP4 HSV-1 or SU4-124 HSV-1 at an MOI of 1. Viruses

were harvested at 48 hours post-infection and titrated on Vero cells. Data are presented as means ± S.D., * P<0.05 vs corresponding CMV-ICP4 treatment.

2.3.7 SU4-124 HSV-1 virus reduces neuronal toxicity in vivo

To study the neuronal toxicity of the SU4-124 HSV-1 virus, CMV-ICP4 HSV-1 or SU4-124 HSV-1 (2.8X10^5 PFU) were intracranially injected into the brain of C57BL6 mice. Immunostaining for immediate-early viral proteins and virally expressed reporter gene LacZ in the brain showed reduced viral activity in SU4-124 HSV-1-injected mouse brains compared with CMV-ICP4 HSV-1-injected brains (Figure 2.8A and B).

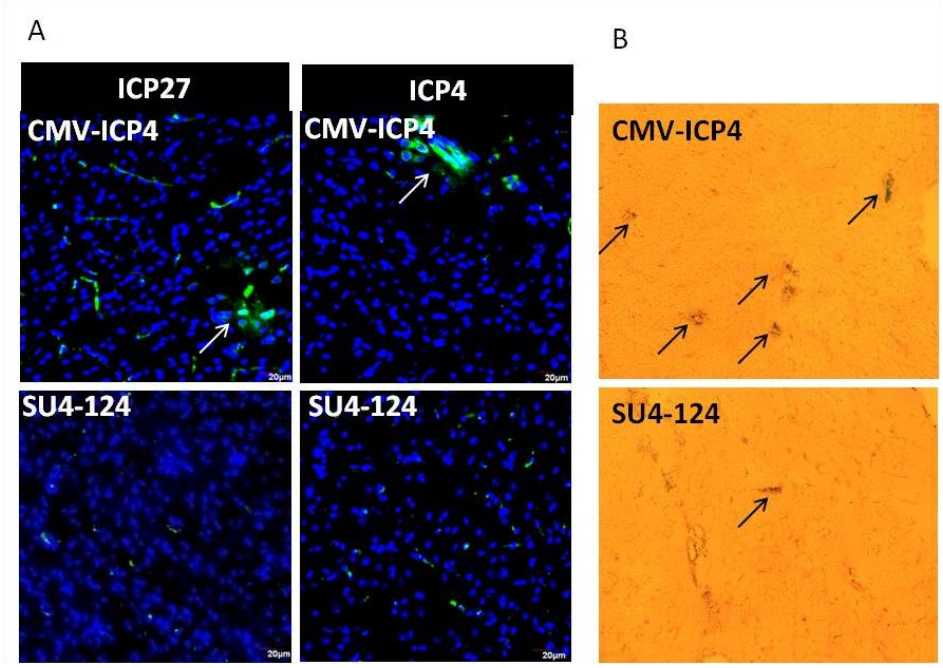

Figure 2.8 Intracranially injected virus toxicity in vivo. 2.8X10^5 PFU/ml CMV-ICP4 HSV-1 or SU4-124 HSV-1 amplicon viruses were injected intracranially into C57BL6 mice. Brain tissue was collected at day 15 post-virus injection. (A) Viral ICP4 and ICP27 expressions were determined by immunohistochemistry of the indicated virus injected brain tissue embedded in OCT and cut into 20-µm sections. (B) Frozen tissue sections were subjected to X-Gal solutions overnight to detect the helper virus.

2.3.8 Tumour-specific triple-regulation enhances the antitumour effect without affecting normal organs in vivo

To evaluate the antitumour efficacy of the SU4-124 HSV-1 virus, subcutaneously implanted U87 tumours were treated with either SU4-124 HSV-1 (2.4X10^7 PFU helper : 6X10^6 PFU amplicon) or CMV-ICP4 HSV-1 (2.4X10^7 PFU helper : 6X10^6 PFU amplicon), or with ICP4- helper virus (2.4X10^7 PFU helper) only by intratumoural injection. Both CMV-ICP4 HSV-1 and SU4-124 HSV-1 caused significant tumour regression at 8 days post injection (Figure 2.9A). Animals were euthanized at day 8 post virus injection (day 17 post tumour implantation) to harvest the tumour and to determine copy number of viral genome by qPCR in the tumour to confirm lytic viral replication inside of the tumours (Figure 2.9B). Meanwhile, SU4-124 HSV-1 injected tumours showed 2.25 fold greater regression than CMV-ICP4 HSV-1 injected mice at day 8 post virus injection (Figure 2.9A). In addition, a 12.1-fold increase in viral genome copies was seen in SU4-124 HSV-1-injected tumours compared to those injected with CMV-ICP4 HSV-1 (Figure 2.9B).

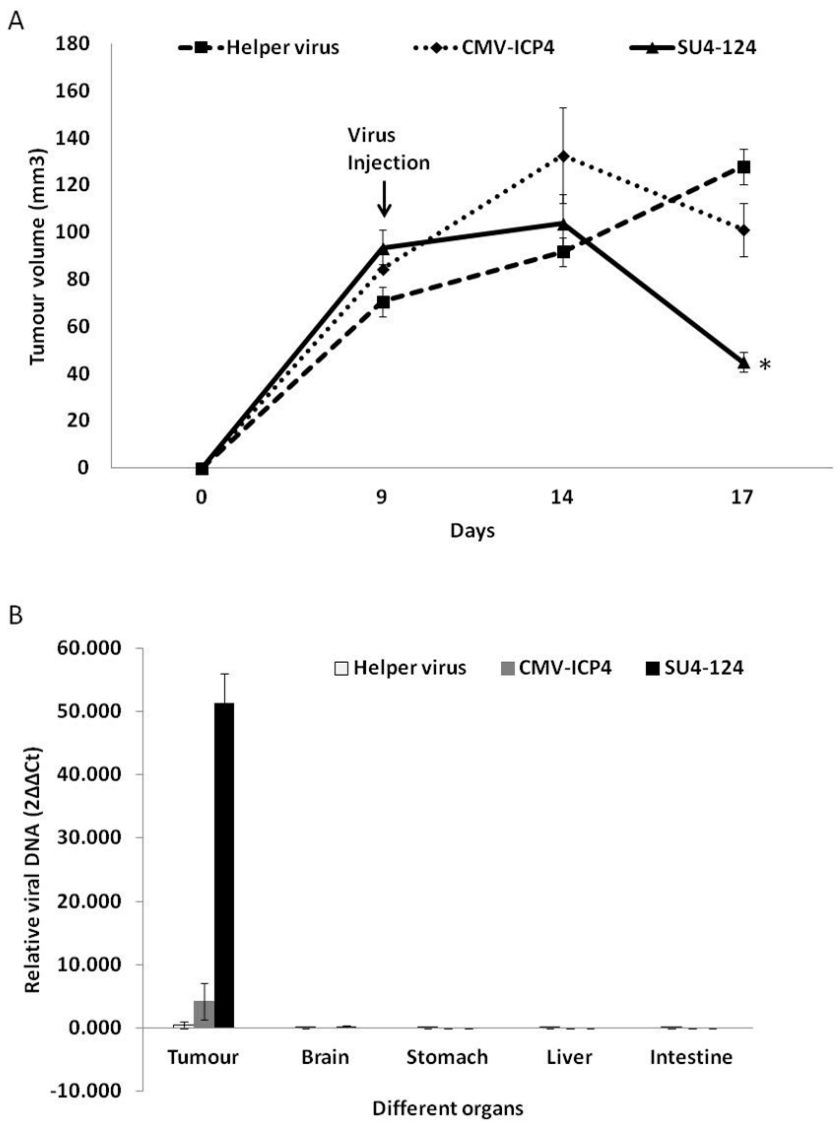

Figure 2.9 SU4-124 HSV-1 significantly augments the antitumour effect without increasing viral spread to other organs. Mice subcutaneously bearing the U87 tumour were intratumourally injected with tumour nonspecific CMV-ICP4 HSV-1 (n=5; 2.4X10^7 PFU helper : 6X10^6 PFU amplicon) or tumour-specific SU4-124 HSV-1 (n=5; 2.4X10^7 PFU helper : 6X10^6 PFU amplicon) or ICP4^{--} 3galΔ3 (n=5; 2.4X10^7 PFU helper only) at a 4:1 helper : amplicon ratio. (A) The tumour volumes were measured using calipers [(height X length X Width)/2)]. Data are presented as means ± S.E, * P<0.001, SU4124 vs helper virus treatment. (B) Total genomic DNA was extracted from the different harvested organs of the indicated mice treated with virus (n=2). Viral genomic DNA (ICP27) was detected by qPCR and normalized to β-actin.

2.3.9 Effect of the amplicon and helper ratio on tumour-specific growth

Since amplicon virus is a combination of helper virus and the amplicon, we evaluated the replication of different amplicon and helper ratio combinations in neurons and U87 cells. We observed a 54, 14.3, 18.2 and 10.7 fold decrease in the titer of neurons compared to U87 cells at an amplicon : helper ratio of 1:4, 1:10, 1:25 and 1:50, respectively.

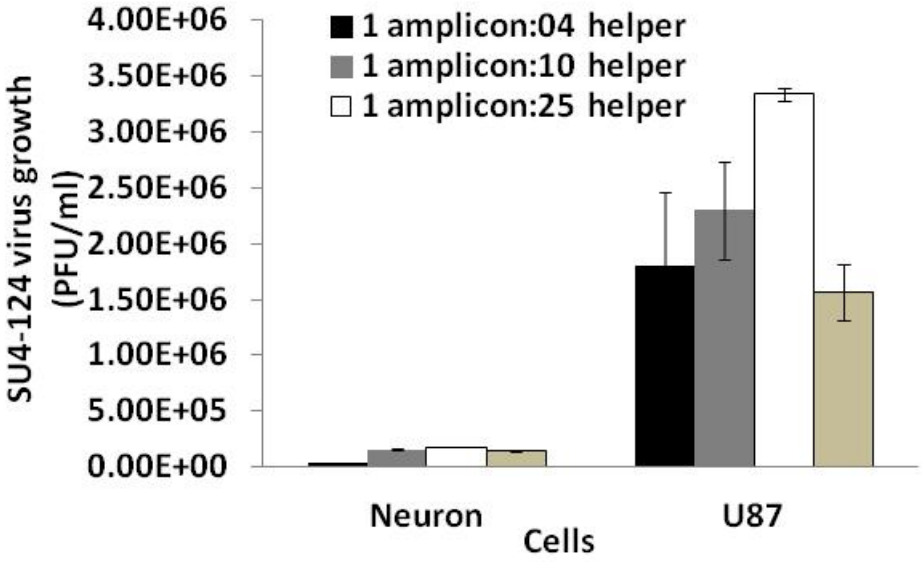

Figure 2.10 Amplicon and helper ratio combination. Neurons and U87 cells were infected using the indicated amplicon and helper (MOI-1) ratio of SU4-124 HSV-1, were harvested at 72 hours post-infection and were then titered on Vero cells.

2.4 Discussion

In this study, we developed a triple-regulated oncolytic amplicon viral system that generates tumour-specific oncolysis with a replication-defective helper virus. Reduced toxicity and the enhanced oncolytic potency compared to the wild type virus were also observed.

Certain microRNAs are differentially expressed in tumour and nontumour cells [206]. We previously showed that inclusion of miRNA 143 and 145 target sequences into the 3'UTR of a HSV-1 essential gene restricted viral replication in prostate cancer cells [186]. In the present study, we confirmed that the miRNA 124 level is very high in normal brain cells compared to glioma cells as reported in several previous studies [106,207,208]. Therefore, miRNA 124 may be a better choice for targeting GBM than miRNA 143 & 145. Here, we also observed that miRNA 124-targeted oHSV-1 (CMV-124T HSV-1) expresses ICP4 in various glioma cells but not in normal neuronal cells. In addition, CMV-124T HSV-1 virus-replication-mediated oncolysis was observed in a panel of glioma cells. However, the virus replication and ICP4 protein expression was completely abolished in the presence of precursor miRNA 124.

Since enhanced tumour specificity often comes at a price, namely reduced viral oncolytic activity [62,63], we attempted to circumvent this disadvantage by using a tumour specific promoter that is highly expressed in glioma cells. Interestingly, the survivin promoter gave rise to transcriptional activity that was higher than CMV and even higher than the native HSV-1 ICP4 promoter. Our results showed that combined with 5'UTR and 3'UTR miR124 translational regulators, survivin promoter-driven ICP4 expression was higher in tumour cells.

Oncolytic virotherapy is mainly used to specifically deplete tumour cells with replication capable viruses through oncolysis: a cell killing mechanism characterized by the lysis of tumour cells through the course of virus replication [18]. However, it has been accepted that there are probably two major mechanisms for the anti-tumour effects by OVs. One is by virally induced cell killing through cell lysis [18] and apoptosis[209]; the other is virally

induced host immune response [210]. In the present study, athymic nude mice were used to develop human glioma mouse model [211], which lacks T-lymphocytes but the innate immune system is partially functional [212]. However, SU4-124 HSV-1 caused tumour regression is likely due to viral lysis and other virally induced direct cell death as the nonreplicable helper virus, which may stimulate similar innate immune response equally effective as the replicable HSV-1 [213], did not show the same efficacy in tumour regression. Agreeable to this notion, it seems that the tumour regression was related to the efficiency of intratumoural viral replication as the CMV-ICP4 HSV-1 virus that had less viral replication rate in the tumour also showed less efficacy in tumour regression compared to that of SU4-124 HSV-1.

Another feature of this study was that we used an amplicon expressing the essential viral gene to supplement a defective helper HSV-1 for oncolytic viral replication. Considering that HSV-1 may establish latency in the brain after treating the glioma, an amplicon dependent oncolytic virus system may be safer clinically than conventional oncolytic HSV-1 since the amplicons are plasmid constructs that are unlikely to persist in cells, which renders replication and reactivation of the helper virus virtually impossible. A potential concern in using an amplicon-supplemented oHSV system is the need to maintain a consistent ratio of helper to amplicon. This was solved in our study by using an essential viral gene carried by the amplicon to supplement the helper virus. Our study showed that, with this design, the ratio of amplicon to helper is always kept below 1 (Figure 2.10), which means that the pure helper virus can always be used to dilute the mixture and maintain a consistent ratio. Thus, this study has shown for the first time that a triple-regulated ICP4 gene expressed from an amplicon can be used to supplement a replication-defective HSV-1 to reduce toxicity and enhance oncolysis to destroy glioma.

3 An antibiotic drug, nifuroxazide synergistically enhances anti-tumour effect of oncolytic herpes simplex virus*

3.1 Introduction

Genetically engineered OVs (OVs) can selectively destroy tumour cells while sparing non malignant cells [52,214,215]. Oncolytic viral therapy has recently emerged as a promising new class of antitumour therapeutics[216]. A herpes virus-based oncolytic virus (T-Vec) has successfully completed a first phase III clinical trial in North America and has been approved by the FDA for the treatment of melanoma. [16,17]. Recently, however, combinations of OVs and cytotoxic compounds has proven very effective in enhancing the anti-tumour therapeutic efficacy [123,217]. Finding new uses for existing drugs can bypass the expensive and time consuming drug discovery process[218]. In search for an effective compound to enhance the efficacy of oHSV-1, we tested here the combinational anti-tumour efficacy of oHSV-1 and a nitrofuran antibiotic, nifuroxazide (NF), a drug which has been used in clinics for decades to treat diarrhoea[219,220]. NF has recently been found to exert anti-tumour activity against melanomas and breast cancers by directly inhibiting the signal transducer and activator of transcription 3 (STAT-3) [221-223]. STAT3 is widely known for its oncogenic function [224] which can be activated by various stimuli including Janus family kinases (JAK), interleukin-6 receptor (IL-6R/gp130), growth factor receptors including EGFR and platelet-derived growth factor receptors (PDGFR), Abl family kinases, and Src family kinases[226].

STAT3 activation increases tumour cell proliferation, survival and invasion, and at the same time, also suppresses anti-tumour immunity[224]. NF inhibits activation of STAT3 by downregulating upstream Janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2) [221].

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*A version of this chapter has been submitted for publication. Zahid M. Delwar, Justin Lardizabal, Yvonne Kuo, Erin Kwa, Gabriel Fung, Honglin Luo, William W. Jia. An antibiotic drug, nifuroxazide synergistically enhances anti-tumour effect of oncolytic herpes simplex virus.

Along with STAT3, NF also inhibits STAT1 and STAT5 [221]. STAT1 is a key regulator of interferon (IFN) signalling and has anti mycobacterial and antiviral activity [224,227]. Type I or type II IFN initially activates Janus kinases (Jak1, Jak2, and Tyk2) that in turn, phosphorylate STAT1 at Tyr701. Upon activation by IFN gamma, phosphorylated STAT1 forms homodimers and initiates the transcription of IFN stimulated genes (ISG) by binding to the gamma-IFN–activated sequence (GAS) [227]. However, STAT1 forms heterodimers with STAT2 and IRF9 and binds to IFN-stimulated response elements (ISRE) in promoters of ISGs[227]. STAT1 signaling is usually considered to be a pro-death and tumour-suppressive pathway. However, recent evidence has suggested that STAT1 activation is associated with a resistance of various tumour cells to chemotherapeutic drugs [227-231]. Since both STAT1 and STAT3 activation is associated with tumour growth and STAT1 plays an important role in the cellular antiviral effect, we hypothesized that NF and oHSV-1 combination could be jointly utilized to enhance viral oncolysis by inhibiting STAT1 and to enhance the antitumour effect by inhibiting STAT 1/3. We studied the effect of NF on oHSV-1 anti-tumour efficacy, and we report here that nifuroxazide synergistically enhanced oHSV-1 anti-tumour efficacy both in-vitro and in-vivo. We also demonstrated that the synergistic antitumour effect of this novel combination is the result of NF mediated STAT inhibition which leads to sensitizing to apoptosis in oHSV-1 infected cells.

3.2 Materials and methods

3.2.1 Cells, viruses and reagents

Human glioma cells (U87), mouse breast cancer cells (4T1), mouse colon cancer cells (CT26), and African green monkey kidney cells (Vero) were obtained from the American Type Cell Collection. All cells were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and a 1% antibiotic mixture

(penicillin and streptomycin). Culture conditions were maintained at 37^{o}C in 5% CO_2. Nifuroxazide was obtained from Millipore, Canada. Ribonucleotide reductase (RR) deficient oHSV-1, HrR3 [41,47-49,232,233], which contains the LacZ gene incorporated into the RR large subunit (ICP6) was a gift of Dr. S Weller, Department of Microbiology, University of Connecticut Health Center, CT, USA. A γ34.5 gene deleted HSV-1, VG34.5-12TR was kindly provided by Virogin Biotech, BC, Canada.

3.2.2 Western blots

Total protein was extracted from cultured cells or harvested tissues by using sample buffer (125 mM Tris-HCL, 50% glycerol, 4% bromophenol blue and 5% 2-mercaptoethanol). Proteins were heated at 100ºC for 5 minutes, subjected to SDS-PAGE (8% - 10% gel), then transferred to nitrocellulose membranes and blocked with 5% nonfat milk (Bio-Rad) in TBS-Tween 20 (TBS-T) for 1 hour at room temperature. The membranes were then incubated overnight at 4^{o}C with anti phospho pSTAT1 (Tyr701) 1:1000; anti phospho pSTAT3 (Tyr705) 1:1000; anti STAT1 1:1000 ; anti STAT3 1:1000 ; or anti-β-actin 1:1000; all from Cell Signaling, Danvers, MA. After overnight incubation with the primary antibody, the membranes were washed three times with TBS-T and incubated with the following secondary antibodies (1:3000; Perkin Elmer, Boston, MA) for 1 hour at room temperature. After washing three times, membranes were visualized using ECL reagent (Perkin Elmer, Boston, MA) and a VersaDoc imaging system (Bio-Rad), and band densities were measured using ImageJ software (NIH, Bethesda, MD).

3.2.3 Cytotoxicity assay

Cells were plated in a 96-well plate at a density of 1 X 10^4. The next day, they were treated with vehicle only, with viruses with different MOIs, with different doses of NF or a combination of NF and viruses for 48 or 72 hours. Cell viability was then measured by an MTT assay (Sigma, Canada) according to the manufacturer's directions. In brief, cells were incubated with MTT solution at 37^{o}C for 3 hours, and they were then

incubated with lysis buffer overnight. Cell viability was measured at 595 nm using a plate reader (Envision 2103 Multilabel Reader, Perkin Elmer).

3.2.4 qPCR array

U87 cells were treated with 25 µM NF or 3.12 MOI HrR3 virus alone or in combination for 24 hours. Total RNA was extracted by using a miRNeasy Mini Kit (Qiagen, Canada) and gene expression profiling was determined by using a RT² Profiler™ PCR Array human IL6/STAT3 Signaling Pathway Plus kit (Qiagen, Canada), following the manufacturer‘s protocol.

3.2.5 U87 xenograft mouse model

Female athymic nude mice were obtained from Harlan Laboratories, Canada. Human glioma U87 (2.5X10^6) cells were subcutaneously implanted into the left flank. Mice began receiving intraperitoneal injection of vehicle or different NF doses when the tumour size reached ~100 mm3. At 48 hours after the initial NF administration, mice were administered an intratumoural injection of a single dose of HrR3 (1X10^6 PFU) virus or vehicle. The HrR3 virus or vehicle injected mice were then intraperitoneally (IP) administered vehicle or different NF doses. An intraperitoneal injection of vehicle or NF was administered twice a week. At day 10 post treatment, mice were euthanized by CO2 asphyxiation and tumours were harvested.

3.2.6 CT26 xenograft mouse model

Mouse colon CT26 (2X10^6) cells were subcutaneously implanted into the flank of female BALB/c mice (Harlan Laboratories, Canada). Mice began receiving intraperitoneal injection of vehicle or 50mg/kg NF when the tumour size reached ~100 mm3, These injections were continued on a daily basis. Three days after initial the NF treatment, tumours were intratumourally injected with a single dose of HrR3 virus (n=5; 2X10^7 PFU) or vehicle with peritoneal daily injections of 50mg/kg NF alone or in

combination with HrR3 virus. Tumours and specific organs were harvested at 23 days after tumour implantation. Tumour volumes were measured using a caliper (height X length X width/2). All in-vivo experimental procedures were approved by the UBC Animal Care Committee and carreid out according to the guidelines of the Canadian Council on Animal Care.

3.2.7 Tissue RNA extraction and real time quantitative PCR

Total RNA from the harvested tumours were isolated with an miRNeasy Mini Kit (Qiagen, Canada). 1µg RNA was then subjected to genomic DNA elimination and reverse transcription using an RT² First Strand kit (Qiagen, Canada). cDNAs were then amplified using RT² SYBR® Green qPCR Master Mix following the manufacturer's protocol. Amplification of cDNA was performed with the following primers. β-actin:5'-ACGAGGCCCAGAGCAAGAG-3'(forward) and 5'-TCTCCATGTCGTCCCAGTTG-3' (reverse), Fas: 5'-ATGCACACTCTGCGATGAAG-3'(forward) and 5'-CAGTGTTCACAGCCAGGAGA-3'(reverse), BCL2:5'-CTCGTCGCTACCGTCGTGACTTCG-3' (forward) and 5'-CAGATGCCGGTTCAGG TACTCAGTC-3' (reverse), BAX-2 5'-AAGCTGAGCGAGTGTCTCCGGCG-3' (forward) and 5'-GCCACAAAGATGGTCACTGTCTGCC-3' (reverse). Results were expressed as the fold change over the untreated control ($2^{-\Delta\Delta CT}$) and β-actin was used as a loading control.

3.2.8 Tissue DNA extraction and quantitative PCR

Using an EZNA tissue DNA kit (Omega Bioteck) total DNA was extracted from harvested tumours and different organs of a CT26 xenograft. Extracted DNAs were subjected to qPCR analysis using Syber Green Master Mix (Invitrogen, Canada) supplemented with ICP27 primers: 5'-GTCTGGCGGACATTAAGGACA-3' (forward) and 5'-TGGCCAGAATGACAAACACG-3' (reverse); β-actin primers: 5'-ACGAGGCCCAGAGCAAGAG-3' (forward) and 5'-TCTCCATGTCGTCCCAGTTG-3' (reverse). Amplification was carried out using a QuantStudieo 6 Flex qPCR system

(Applied Biosystems, Canada). Results were expressed as $2^{-\Delta\Delta CT}$ and β-actin was used as a loading control.

3.2.9 Statistical analysis

Statistical analysis was conducted by using Microsoft Excel and significance $P<0.001$, or $P<0.01$, or $P<0.05$ was determined using Student's t-test. All data were expressed as means ± SD or ± SE.

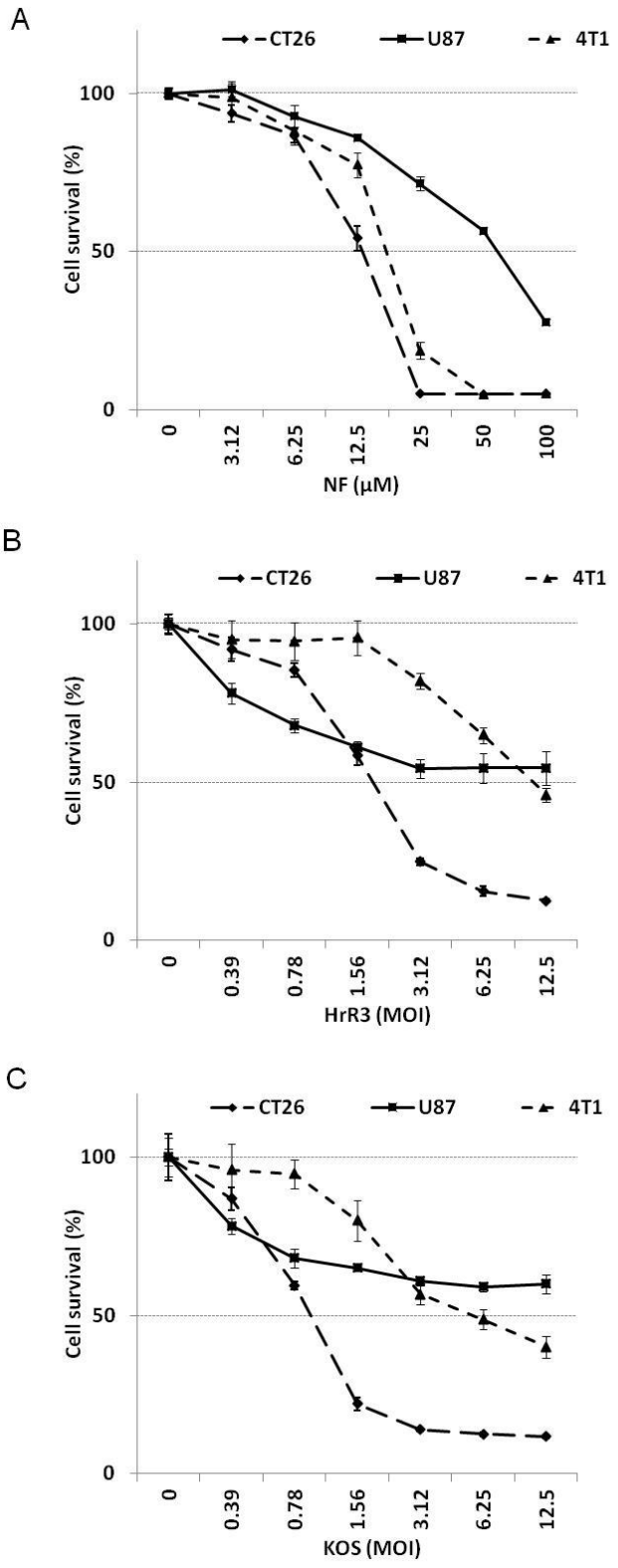
3.3 Results

3.3.1 NF significantly enhances the anti-proliferative effect of oHSV-1 in an synergistic fashion

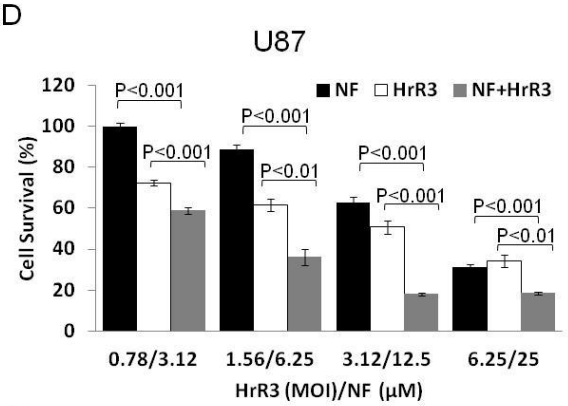
We first evaluated the cytotoxic effect of the each agent on different types of tumour cells. An MTT cell proliferation assay revealed that inhibitory concentration 50% (IC_{50}) values of NF after 48 hours of treatment directed against glioma (U87), colon cancer (CT26) and breast cancer (4T1) cells were around 75 µM, 12.5 µM and 20 µM, respectively (Figure 3.1A), while those of HrR3 viruses were approximately MOI-12.5, MOI-2 and MOI-10 (Figure 3.1B). A similar anti-proliferative effect was obtained with wild-type HSV-1 (KOS) when used to target the above cells (Figure 3.1C).

To evaluate the combinational anti-tumour effect of the NF and oHSV-1, U87, CT26 and 4T1 tumour cells were treated with NF or oHSV-1 (HrR3) alone or in combination. The MTT cell proliferation assay revealed that 6.25 µM NF alone only killed approximately 10% of human glioma U87 cells, and HrR3 alone at an MOI of 1.56 killed approximately 30 % of cells, whereas the combination of NF and HrR3 depleted 65% of the cells within 72 hours (Figure 3.1D). Moreover, a combination of 12.5 µM NF and 3.12 MOI HrR3 increased the anti-proliferative effect 2.6 fold and 2.25 fold compared to NF or HrR3 alone in U87 cells, respectively (Figure 3.1D). A similar enhanced anti-proliferative

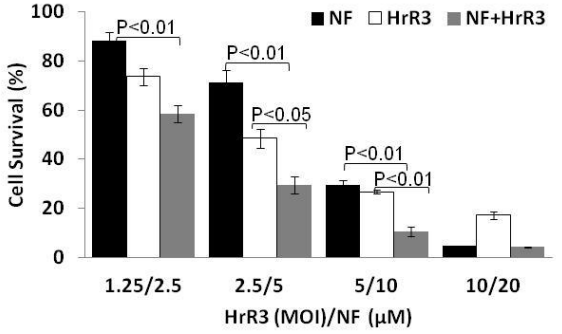
effect of the combinational treatment was observed in mouse colon cancer CT26 cells (Figure 3.1E), mouse breast cancer 4T1 cells (Figure 3.1F) and mouse lung cancer LL2 cells (data not shown) at 48 hours post-treatment. To determine whether the increased cytotoxicity with the NF and HrR3 combination was due to synergy, we analyzed the data with CompuSyn software (Ting-Chao Chou, New York, U.S.A.) and found that 10% to 80%, 10% to 90% and 10% to 90% of the fractions affected by this combination demonstrated synergy in U87 cells, CT26 cells and 4T1 cells, respectively (Figure 3.1G). To determine whether the cytotoxic effect of NF with HSV-1 is virus strain specific, we measured the anti-proliferative effect of NF using the wild-type HSV-1 (KOS) on different types of cancer cells. A similar synergistic anti-tumour effect was observed with the NF-KOS combination in U87, CT26 and 4T1 cells (Figure 3.1H). An ICP34.5- mutated oHSV-1 (VG34.5-12TR) that has the same viral backbone as T-Vec and is sensitive to cellular PKR activity [234,235], also showed a synergistic anti-tumour effect in combination with NF (Figure 3.1I). Therefore, the effect of combining NF and HSV-1 was not strain specific. Finally, we calculated the dose reduction index (DRI) of the NF and HrR3 combination in various affected fractions and found it to be highest mostly with HrR3 in U87, CT26 and 4T1 cells (Figure 3.1J).


A
CT26
U87
4T1
Cell survival (%)
100
50
0
0
3.12
6.25
12.5
25
50
100
NF (μM)
B
CT26
U87
4T1
Cell survival (%)
100
50
0
0
0.39
0.78
1.56
3.12
6.25
12.5
HrR3 (MOI)
C
CT26
U87
4T1
Cell survival (%)
100
50
0
0
0.39
0.78
1.56
3.12
6.25
12.5
KOS (MOI)

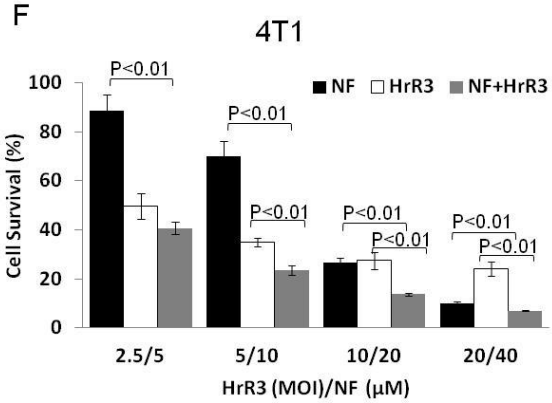
D

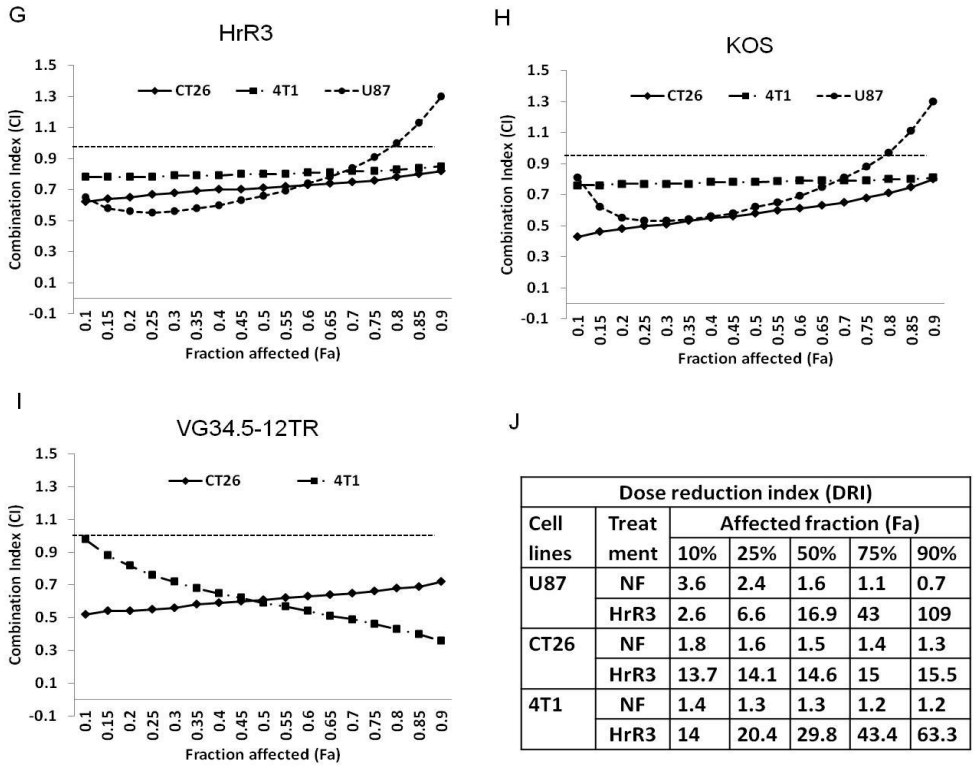

U87
NF
HrR3
NF+HrR3
Cell Survival (%)
120
100
80
60
40
20
0
P<0.001
P<0.001
P<0.001
P<0.01
P<0.001
P<0.001
P<0.001
P<0.01
0.78/3.12
1.56/6.25
3.12/12.5
6.25/25
HrR3 (MOI)/NF (μM)

E


CT26
NF
HrR3
NF+HrR3
Cell Survival (%)
100
80
60
40
20
0
P<0.01
P<0.01
P<0.05
P<0.01
P<0.01
1.25/2.5
2.5/5
5/10
10/20
HrR3 (MOI)/NF (μM)

F


4T1
NF
HrR3
NF+HrR3
Cell Survival (%)
100
80
60
40
20
0
P<0.01
P<0.01
P<0.01
P<0.01
P<0.01
P<0.01
P<0.01
2.5/5
5/10
10/20
20/40
HrR3 (MOI)/NF (μM)



Dose reduction index (DRI)						
Cell lines	Treatment	Affected fraction (Fa)				
		10%	25%	50%	75%	90%
U87	NF	3.6	2.4	1.6	1.1	0.7
	HrR3	2.6	6.6	16.9	43	109
CT26	NF	1.8	1.6	1.5	1.4	1.3
	HrR3	13.7	14.1	14.6	15	15.5
4T1	NF	1.4	1.3	1.3	1.2	1.2
	HrR3	14	20.4	29.8	43.4	63.3

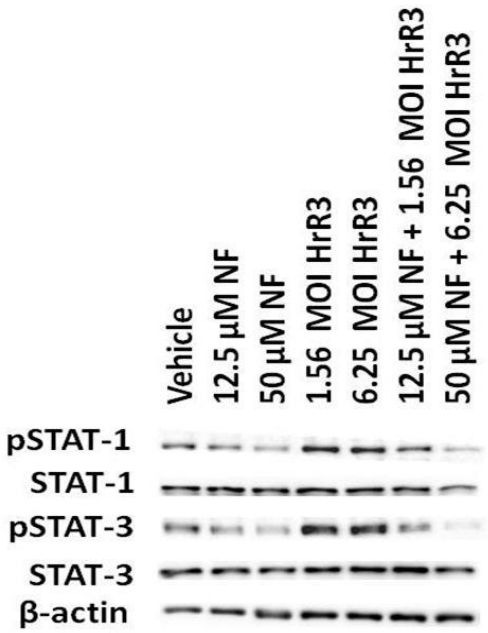
Figure 3.1 NF significantly enhances the cytotoxicity of oHSV-1 and provides an synergistic anti-tumour effect in combination. A-C, Cancer cells were treated with the indicated doses of NF or two different HSV-1 strains, HrR3 or wild-type (KOS), for 48 hours. Samples were run in quadruplicate and the results are expressed as mean ±SD . Cell proliferation was measured by an MTT assay. D-F, U87 (D), CT26 (E) and 4T1 (F) cells were treated with either the indicated doses of NF or HrR3 (oHSV-1) or a combination. NF was applied first, the indicated viral doses were then added and cells were incubated for 72 hours (D) or 48 hours (E and F). Cytotoxicity was measured by means of an MTT assay. Experiments were performed in quadruplicate and results are expressed as mean ± S.D. Statistically significant differences are indicated by $P<0.05$, $P<0.01$ or $P<0.001$. G-I, cells were treated with varying doses of NF or the indicated viruses (HrR3 or KOS or VG34.5-12TR) alone or in combination with NF for 48 hours. Cell viability was measured by an MTT assay and samples were run in quadruplicate and data are means ± S.D. Combination index (CI) values were calculated using Chou-Talalay analysis and plotted against the affected fraction (Fa). CI of < 1, CI = 1 and CI > 1 represent synergistic, additive and antagonistic effect, respectively. The dose reduction index (DRI) was calculated for the NF and HrR3 virus combination in the indicated affected fractions (J).

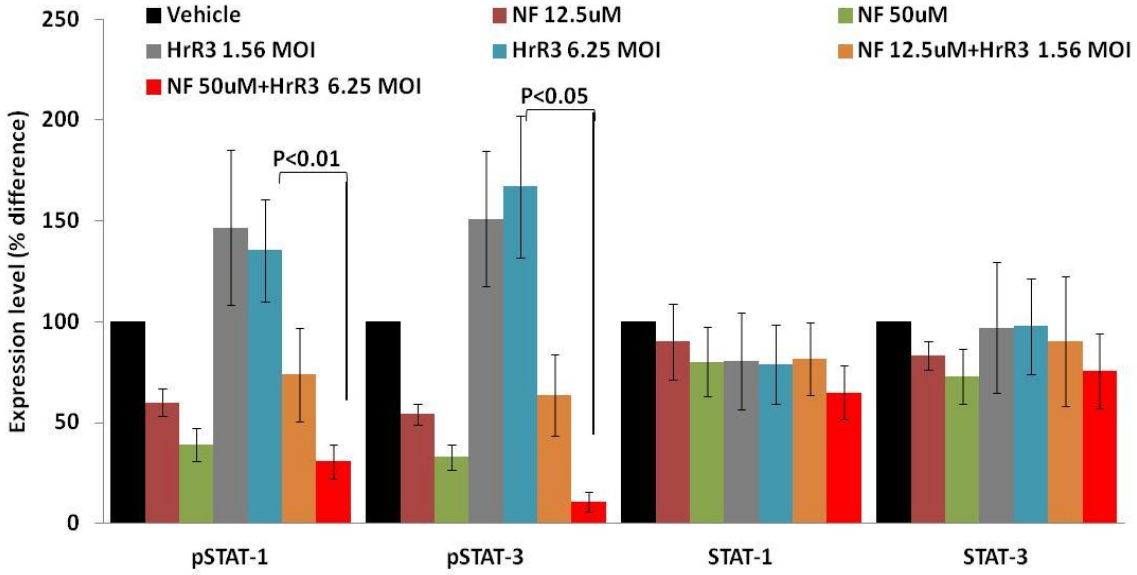
3.3.2 The NF and oHSV-1 combination inhibits STAT activity both *in vitro* and *in vivo*

NF is known to be an inhibitor of STAT1 and STAT3 [221]. Therefore, to evaluate the underlying molecular mechanism of the anti-tumour effect of the NF and oHSV-1 combination, we first examined the STAT1 and STAT3 status of U87 (Figure 3.2A) and CT26 (Figure 3.2B) cells after an overnight treatment with NF and OVs. Interestingly, levels of both STAT1 and STAT3 phosphorylation in these cells were significantly increased after HrR3 virus infection. On the other hand, NF caused a dose dependent inhibition of phosphorylation of STAT1/3. More interestingly, NF effectively suppressed HrR3 induced upregulation of STAT1/3 phosphorylation in both U87 (Figure 3.2A) and CT26 (Figure 3.2B) cells. To our surprise, despite the fact that HrR3 infection elevated STAT1/3 phosphorylation in U87 cells, a combination of high doses of NF and HrR3 (NF 50μM and HrR3 6.25 MOI) reduced STAT1/3 phosphorylation to a level even below that seen with NF alone (Figure 3.2A).

To confirm further that NF is able to inhibit STAT activation *in vivo*, subcutaneously implanted U87 tumours were treated with either HrR3 alone or in combination with 50mg/kg NF or 100mg/kg NF. Expression levels of phosphorylated STAT1/3 in harvested tumour tissues were measured by western blotting. Although we were not able to detect pSTAT1 in the tumour tissue, it was evident that STAT3 phosphorylation was increased in the tumour mass treated with HrR3 alone. Again, the virally induced upregulation of pSTAT3 was dose dependently inhibited by the combination of the virus with i.p. injected NF (Figure 3.2C).

A


Vehicle
12.5 µM NF
50 µM NF
1.56 MOI HrR3
6.25 MOI HrR3
12.5 µM NF + 1.56 MOI HrR3
50 µM NF + 6.25 MOI HrR3
pSTAT-1
STAT-1
pSTAT-3
STAT-3
β-actin


Vehicle
NF 12.5uM
NF 50uM
HrR3 1.56 MOI
HrR3 6.25 MOI
NF 12.5uM+HrR3 1.56 MOI
NF 50uM+HrR3 6.25 MOI
Expression level (% difference)
250
200
150
100
50
0
P<0.01
P<0.05
pSTAT-1
pSTAT-3
STAT-1
STAT-3

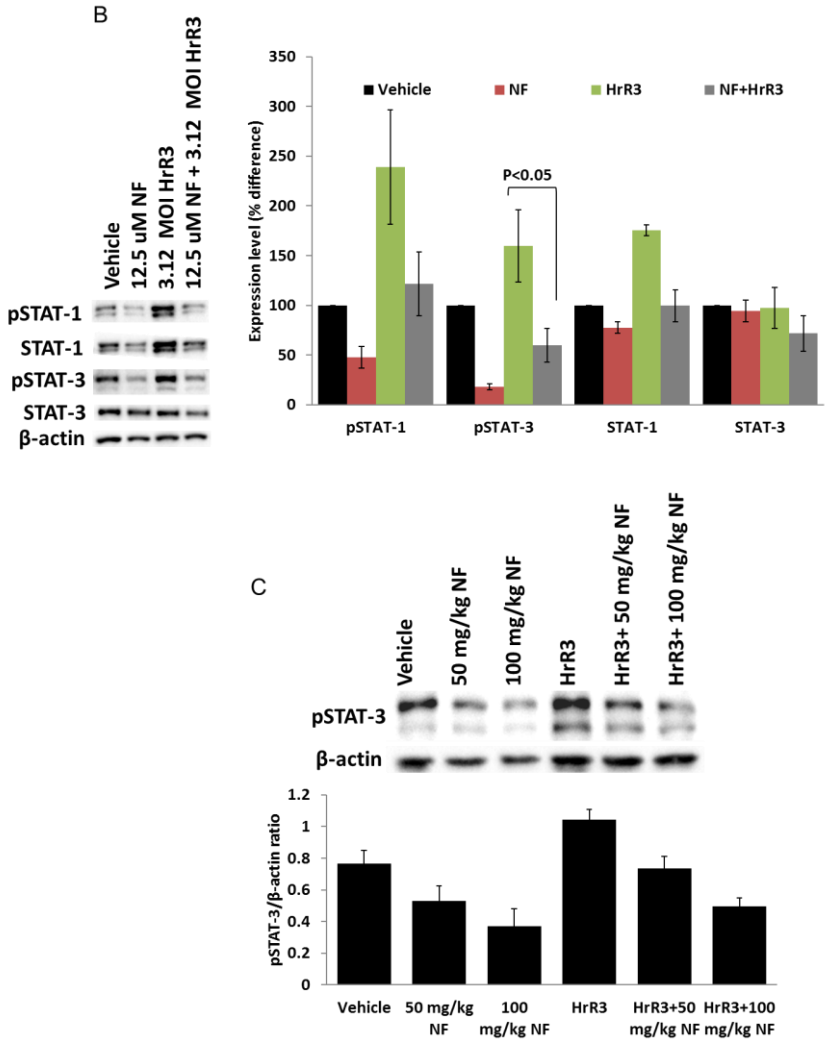


Figure 3.2 STAT inhibition by the NF and oHSV-1 combination. A and B, U87 cells (A) or CT26 cells (B) were treated with the indicated concentrations of HrR3 or NF or a combination. Total protein was extracted after 24 hours and subjected to western blot analysis. Band intensity was measured using Image J software and normalized to β-actin. Samples were run in triplicate and data are as means ± S.E. Statistically significant differences are indicated by P<0.05 or P<0.01. C, Mice subcutaneously bearing U87 tumours were intratumourally injected with a single dose of HrR3 virus (1X10^6 PFU) and/or peritoneally injected doses of NF as indicated. Tumours were harvested on day 10 post treatment and total protein was extracted from a mouse of each treatment group and subjected to western blot analysis in triplicate. Results are reported as means ± S.E.

3.3.3 The NF effect on HSV-1 immediate early gene expression

We then asked whether STAT1/3 inhibition by NF has any effect on viral immediate early gene expression. NF mediated blocking of viral ICP27 expression was evident in tumour cells, whereas ICP4 level was unchanged. Interestingly, similar to 24 hours of treatment, phosphorylation inhibition of STAT-3 was also observed after six hours of NF treatment, but phosphorylation level of STAT1 was unchanged (Figure 3.3).

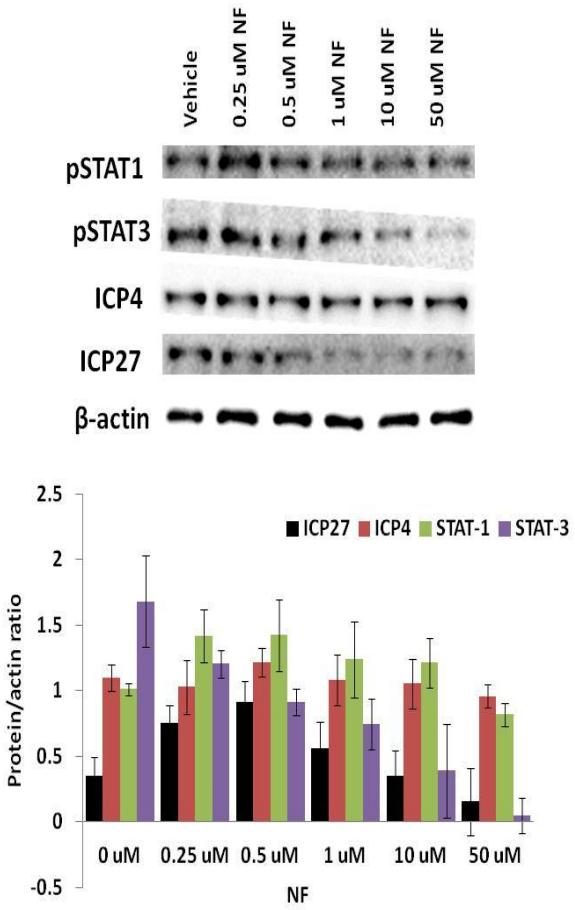


Figure 3.3 NF effect on ICP27 and ICP4 gene expression. U87 cells were treated with HrR3 viruses with an MOI of 1 alone or in combination with indicated concentrations of NF for six hours. Total protein was extracted and subjected to western blot analysis Samples were run in triplicate and data are the means ± S.D.

3.3.4 NF overcomes the anti-apoptotic effect of oHSV-1

Next, we performed a qPCR array analysis of cells treated with either HrR3 or NF alone or a combination of the two. Our data indicate that most cellular death inducing genes such as CDKN1A, MAPK14, CASP4, FAS, BAX etc are suppressed by the oHSV-1 and their expression is upregulated by the addition of NF (Figure 3.4).

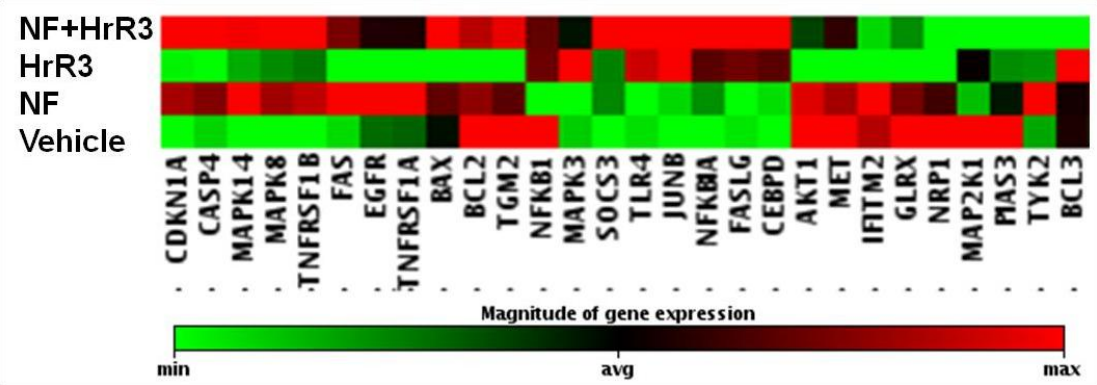


Figure 3.4 PCR array of the NF and oHSV-1 combination in tumour cells. U87 cells were treated with 3.12 MOI of HrR3 or 25 uM of NF alone or a combination for 24 hours. Extracted RNA was then subjected to a qRT-PCR array. The clustergram shows the gene expression levels.

3.3.5 NF enhances both the anti-tumour effect and the safety of oHSV-1 *in vivo*

To evaluate the *in vivo* anti-tumour effect of the oHSV-1/NF combination, subcutaneously implanted CT26 tumours were treated with either 2X10^7 PFU HrR3 alone, 50mg/kg NF alone or both. Significantly increased tumour regression was seen with the combination compared to treatment with NF or HrR3 alone. At day 23 after

tumour implantation, 2.2 fold and 1.9 fold greater tumour regression was seen in the NF plus oHSV-1 treated mice compared to mice treated with oHSV-1 alone or NF alone, respectively (Figure 3.5A).

We then asked whether combination with NF would compromise the safety of oHSV-1. We first isolated the total DNA from the tumour, brain, liver and gastrointestinal tract (GT) and subjected it to qPCR analysis to measure the amount of viral DNA. As expected, similarly high levels of HSV-1 DNA were detected in the HrR3 treated tumours with or without NF. Interestingly, while levels of the viral DNA were almost undetectable in the liver and GT with or without NF, a remarkable level was detected in the brains of animals treated with HrR3 alone but the level was nearly 10 fold less when the virus was combined with NF (Figure 3.5B). Among 5 mice treated with HrR3 (2X10^7 PFU) alone, one mouse was euthanized due to virus related toxicity. No toxicity was observed in mice treated with the NF and HrR3 combination. Furthermore, when two mice were injected intratumourally with a high dose of HrR3 (1X10^8 PFU) in a preliminary experiment, both mice showed signs of severe HSV-1 toxicity (paralysis of the hind leg or immobility) and were terminated immediately. On the other hand, HSV-1 had no toxic effect on mice treated with HrR3 at the same high dose when given in combination with NF (data not shown).

To confirm that NF upregulates pro-apoptotic genes in vivo, we measured the expression ratios of BAX to BCL2 in the tumours by means of qRT-PCR. A decreased BAX\BCL2 ratio was observed in tumours treated with HrR3 alone compared to untreated tumours. The ratio was significantly increased when the virus was combined with NF (Figure 3.5C). In addition, Fas was also downregulated by oHSV-1 but increased in amount by combining with NF (Figure 3.5D).. These data indicated that NF promoted apoptosis in oHSV-1 infected tumours.

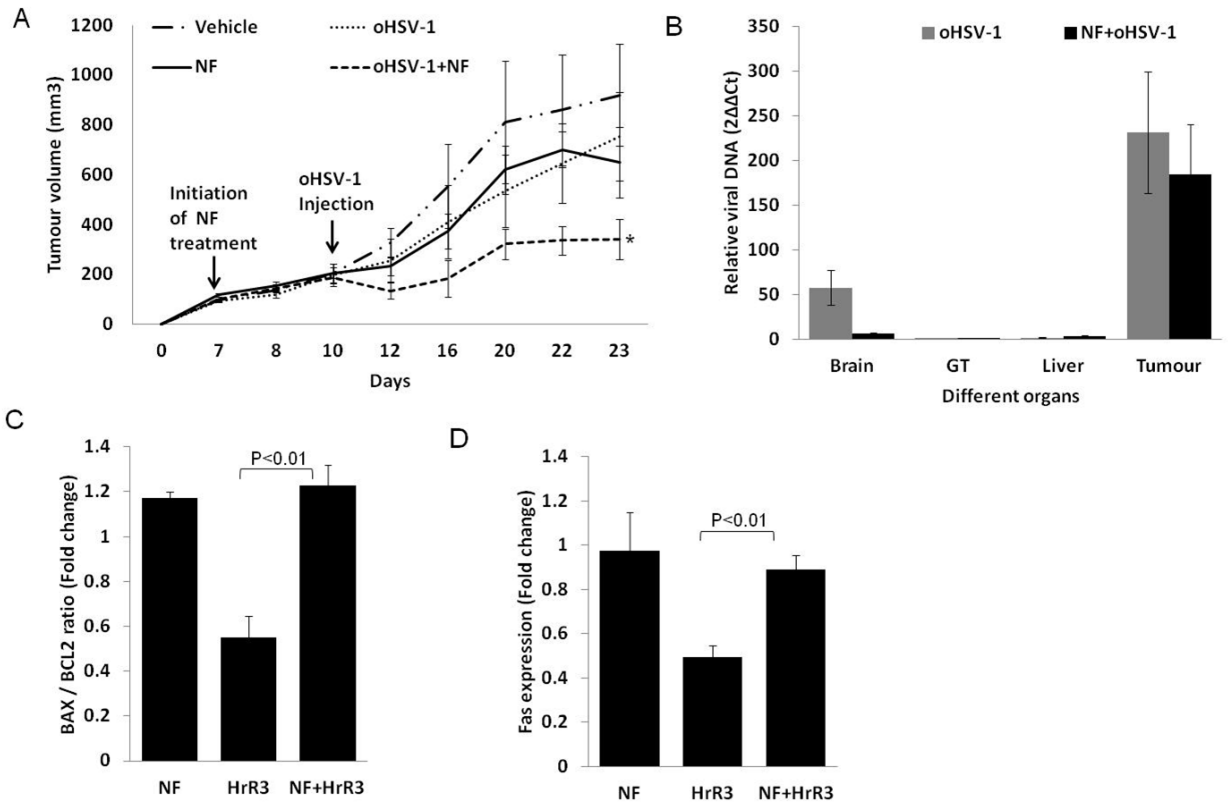


Figure 3.5 Enhanced anti-tumour effect of the NF and oHSV-1 combination in-vivo. A, BALB/C mice subcutaneously bearing a CT26 (colon cancer) tumour were intratumourally injected with a single dose of HrR3 virus (2X10^7 PFU) or vehicle and given daily peritoneal injections of 50mg/kg NF alone or in combination with HrR3 virus (5 mice in each group). Tumours and indicated organs were harvested at 23 days after tumour implantation. Tumours size were measured using calipers (length X height X width /2). Data are the means ± S.E. and statistically significance differences between treatment with the NF and oHSV-1 combination, NF alone, and oHSV-1 alone are indicated by the p value, * P<0.05. B, total genomic DNA was extracted from the harvested tumours and other organs (n=2). Viral DNA (ICP27) was detected by qPCR and normalized to β-actin . Samples were run in quadruplicate and data are the averages of 2 mice ±S.E. C-D, total genomic RNA was extracted from the harvested tumours and subjected to qRT-PCR analysis. Samples were run in quadruplicate and normalized to β-actin. Data are the means (±S.E.) of the fold change difference over the untreated control (2ΔΔCT) of two mice from each treatment group.

3.4 Discussion

Oncolytic virus (OV) in combination with chemotherapeutics or immune modulators has been found to be effective in enhancing therapeutic potency [217,236-241]. In addition, recent clinical trials also demonstrated that OV exerts an enhanced therapeutic effect when used in combination with chemotherapy [242]. Therefore, combinational therapy is believed to be a promising approach for enhancing the therapeutic efficacy of OV. In this report, we confirmed the cytotoxicity of NF in gliomas and in colon and breast cancer cells, a finding that is in agreement with previously reported results using melanoma and breast cancer models [221,222,243,244]. Furthermore, we demonstrated a strong synergistic anti-tumour effect with the NF and oHSV-1 combination in various tumour models both *in vitro* and *in vivo*.

The synergy observed was probably independent of an ICP6 deficiency in HrR3 since wild-type HSV-1 (KOS) combined with NF also demonstrated synergistic cytotoxicity. Similar synergy was also seen in a γ35.5 deleted oHSV-1 (VG34.5-12TR) which is similar to T-Vec, the first OV drug approved by the FDA[16,17]. Since NF has been clinically used as a prescription drug for decades[220,245], our findings are easily translatable to clinical application and may augment the therapeutic efficacy of T-Vec.

Our data indicated the reduced expression of pro-apoptotic genes in oHSV-1 infected tumour cells. The apoptotic pathway is frequently activated as an inherent defense against viral infections[246,247] and viruses are known to be able to counteract the cellular apoptotic mechanism to elongate the life of infected cells to support the viral replication[248-250]. HSV-1 induces apoptosis at an early stage of infection, but the progression of infection leads to the production of anti-apoptotic viral proteins by early and late herpes virus genes, which thereby block apoptosis[246]. The anti-apoptotic function of oHSV-1 was evident from the reduced expression of BAX, CDKN1A, MAPK14, CASP4 and FAS in oHSV-1 infected tumour cells. BAX is widely known for its apoptosis inducing role[251]. CDKN1A is a CDK interacting protein which inhibits cell cycle progression [252]. MAPK14 is a stress activated protein kinase that suppresses cell proliferation[253]. Caspase 4 induces apoptosis by stimulating ER stress[254]. Cell death

surface receptor Fas activated by the Fas ligand results in the induction of apoptosis[255]. Similar to our finding, Elise et al. also observed a blockade of Fas mediated apoptosis by HSV-1[256]. Strikingly, expression of these genes was rescued by NF in oHSV-1 infected tumour cells. It is not clear whether the pro-apoptotic effect of NF is related to its inhibition of STAT1/3 activity. However, this may explain the reduced viral toxicity and viral proliferation in normal tissues such as the brain as systemically delivered NF may strengthen the anti-viral defense of normal organs by promoting the death of infected cells.

The mechanism responsible for the effect of NF on oHSV-1 infected tumour cells may be more complicated. The anti-cancer synergy might be the "net" effect of various factors involved in anti-viral and anti-cancer mechanisms. NF was reported to be a potent inhibitor of STAT1 and STAT3 phosphorylation, which may be associated with its anti-tumour activity[221]. Interestingly, considerable upregulation of STAT1/STAT3 phosphorylation was observed after infection with oHSV-1. Since STAT1 is widely known as an immune stimulatory factor[257] which promotes cellular resistance to bacterial and viral infections [224,227], elevation of STAT activation after oHSV-1 infection therefore might indicate a cellular defence reaction to the viral infection. Activation of STAT1 after viral infection may be mediated by interferons (IFNs) that are released following the infection and inhibit HSV-1 replication [258]. On the other hand, STAT signalling is suppressed by a suppressor of cytokine signalling (SOCS) that blocks STAT1 activation[259,260]. We found that NF completely abolished the oHSV-1 upregulated phosphorylation of STAT1 and STAT3 in infected tumour cells. In addition, our data also demonstrated that a combination of NF and HrR3 upregulated SOCS-3 expression in tumour cells. Thus, one may speculate that reduction of phosphorylated STAT-1 may enhance oHSV-1 replication in these cells. However, NF at doses that inhibited STAT phosphorylation could not enhance oHSV-1 replication in tumour cells (data not shown), indicating that the mechanism of this synergistic interaction is independent of viral replication. Failure to enhance oHSV-1 replication by NF might be related to decreased STAT3 activity since activation of STAT3 was previously reported to increase oHSV-1 replication[261]. In addition, STAT3 is known for its oncogenic function

and has been a target for anti-cancer drugs [224]. Inhibition of STAT3 induces apoptosis [262,263] and autophagy mediated cell death [262]. What is surprising is that NF dose-dependently downregulated STAT3 phosphorylation and that this inhibition was even more effective in combination with HrR3 in tumour cells.

Since we did not find significant differences in virus titers in HrR3 infected tumours with or without NF treatment, it appears that the anti-viral and pro-viral effects of NF are nearly balanced, resulting in no net effect on viral replication. However, the anti-tumour effect of NF inducing STAT inhibition and pro-apoptotic activity may contribute to sensitizing the tumour to viral induced cytotoxicity, which may cause the synergistic anti-tumour outcome both in vitro and in vivo. Nevertheless, these results demonstrate that nifuroxazide, a clinically used anti-diarrheal drug, can not only synergistically increase the anti-tumour effect of the oncolytic virotherapy but also can enhance the safety of the virus in normal tissues. Given that NF is a prescription drug already in clinical use and that the synergy occurs between NF and VG34.5-12TR, a T-VEC like oHSV-1 that has been recently approved, our findings may be very translatable to potential application in virotherapy in a clinical setting.

4 Microglia/macrophages mediated inhibition of oncolytic virotherapy is STAT1/3 dependent *

4.1 Introduction

OVs (OVs) have been a therapeutic arsenal to specifically destroy cancer cells through oncolysis: a killing mechanism characterized by cancer cell lysis through the course of lytic virus replication[18]. In addition to the direct cell killing by the virus, it has been demonstrated that virally induced immune response plays a pivotal role in OV therapy[210]. As OVs can kill cancer cells via a mechanism distinct from the killing effects of conventional chemotherapy and radiotherapy, OVs are potentially ideal for treating cancers that are non-responsive to conventional treatment. Among the various OVs, herpes simplex virus type 1 based OVs are the farthest advanced. A herpes virus-based OV (T-Vec) was recently approved by FDA for the treatment of melanoma after a successful completion of the clinical trials in North America.[16,17]

Glioblastoma multiforme (GBM) is a treatment-refractory brain tumour with a poor prognosis [264]. The most investigated oncolytic HSV-1 (oHSV-1) to treat GBM are mutant HSV-1s with deletions in viral gene ICP6 [47-49] or neurovirulent γ34.5 or both [41,51-53]. Despite excellent safety profile, the clinical efficacy of many oHSV-1s has been disappointing [55,265]. This may largely attribute to host anti-viral immune responses that inhibit effective replication and intratumoural dissemination of OVs after delivery [68,266-268]. Clinical GBM tumour bed is highly heterogenetic at the cellular level. Most importantly, a significant portion of cells in the GBM tumour mass are non-tumourous [163], which by definition is resistant to replication of OVs.

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*A version of this chapter will be submitted for publication. Zahid M Delwar, Yvonne Kuo, Yan Hua Wen, William Jia. Microglia/macrophages mediated inhibition of oncolytic virotherapy is STAT1/3 dependent.

Among all types of cellular infiltrates, microglia/macrophages as important innate immune cells are the most common ones in GBM [153,163-165], whereas mean microglia/macrophages content is around 40% (range:5-78%) of total tumour mass [167,168]. Badie and Schartner have further verified the source of microglia/macrophages in rodent GBM model. They showed that microglia/macrophages contribute up to 46% of the tumour's cellular mass in rodent GBM, where up to 34% originated from microglia (CD45-/CD11b+ cells) that exist in the brain since embryonic development. The remaining 12% originated from blood-derived macrophages (identified as CD45+/CD11b+ cells) [169].

GBM associated microglia/macrophages play an important role in tumour progression [153,154,163,164]. There is a positive correlation between the number of microglia /macrophages and malignancy of brain tumours. Malignant gliomas like glioblastomas and anaplastic gliomas showed the largest number of a mixed cell population containing microglia /macrophages and low malignancy glial tumours contain fewer microglial cell [165]. Regardless of the origin of macrophages in the GBM microenvironment, a large body of work suggested that tumour cells communicate with macrophages[163]. Importantly, the communication is shown to act both ways, where glioma cells attract macrophage infiltration, but also macrophages promote glioma growth and metastasis [163,164].

The abundance of the microglia/macrophages in human GBM may be partially responsible for the poor efficacy of OVs. A previous study found that chemical ablation of microglia/ macrophages subsequently enhances the antitumour effect of oHSV-1 in glioma-bearing rodent model [269]. While it has been accepted that the microglia/macrophages are a part of innate anti-viral immune responses, details of the interaction between the macrophage/microglia and OV-infected glioma cells has not been systemically investigated. As the most predominant non-tumour cells in GBM, a better understanding of this interaction is essential for the development of novel strategies for oncolytic virotherapy against GBM. This report addresses the mechanism and effect of microglia/ macrophages in response to oHSV-1 infected human

glioblastoma cells. We hypothesized that, by modifying the cellular activity of glioma associated microglia/macrophages, we might enhance the efficacy of oHSV-1 for the treatment of GBM. Indeed, we found that the presence of microglia/ macrophages is sufficient to hinder oHSV-1 replication in glioma cells. Our findings suggest that microglia impedes the dissemination of oHSV-1 among glioma cells mainly by uptaking oHSV-1. Furthermore, while the uptaken oHSV-1 fails to replicate in microglia/ macrophages, some viral carried genes can still be expressed. We further found that phosphorylation of STAT-1 and 3 are critical for inhibition of viral replication in microglia/macrophages. We then discovered that C16, an oxindole/imidazole derivative [270] selectively inhibits the phosphorylation of STAT-1 and 3 to allow viral replication in tumour-associated macrophages that increased viral dissemination and tumour destruction in a subcutaneous mouse glioma model.

4.2 Materials and methods

4.2.1 Microglia isolation and culture

E18 Sprague-Dawley rat was obtained from Charles River Laboratories (Charles River, Wilmington, MA). Rat primary microglia isolation and culture was conducted according to the standard protocol [271]. In brief, cortices were isolated from day 18 embryonic E18 Sprague Dawley rat brain. After 30 minutes incubation in trypsin/EDTA (Invitrogen, Canada), harvested tissue was washed with culture medium and minced in the presence of Dnase I (Invitrogen, Canada). Then the cell suspension was centrifuged, resuspended in fresh culture medium and plated on 10 cm culture dishes in high confluence. Microglia cells were grown in Dulbecco's modified eagle's medium (Sigma, Canada) supplemented with 10% fatal bovine serum (Invitrogen, Canada), 1% antibiotics (penicillin and streptomycin) and maintained at 37^{o}C in 5% CO_2. Culture medium was changed in every 3-4 days. After 7-10 days, microglias were harvested by gently rocking the plate for a couple of times. Finally, microglias floating in the

supernatant were plated in poly-L-lysine coated plate. Cell purity was routinely tested by immunocytochemistry staining for ITGAM (1:200; ProSci Incorporated, CA) which is a microglia specific integrin protein.

4.2.2 Cell culture

U87 (human GBM) cells and Vero (African green monkey kidney) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). BV2 (Mouse microglia) cells were kindly provided by Dr. Stephanie Booth, Department of Medical Microbiology and Infectious Diseases, University of Manitoba. All cells were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fatal bovine serum and 1% antibiotics (penicillin and streptomycin). All cells were maintained at 37^{o}C in 5% CO_2.

4.2.3 Virus replication assay

G207 virus was obtained from NeuroVir Therapeut Inc, San Diego, CA. U87 (5X10^4) cells alone or with the indicated number of microglia cells in co-culture were incubated overnight with culture medium (DMEM with 10% FBS and 1% antibiotic). Next day, cells were infected with G207 virus at a multiplicity of infection (MOI) of 1. Viruses were harvested after 2-4 days post-infection. After three times freeze-thaw cycles, viruses were titrated on Vero cells by a standard plaque assay on 12-well plates by triplicates.

4.2.4 Drugs and reagents effect on virus replication

U87 ($5X10^4$) alone or U87 + microglia co-culture ($5X10^4$ + $5X10^4$) or BV2 microglia cells ($5X10^4$) alone were seeded into a 24-well plate. After overnight incubation for allowing the cells to attach, cells were pretreated with indicated concentration of NFkB inhibitor, Bay-11 (Santa Cruz, Canada) or indicated concentration of Imidazolo-oxindole derivative C16 (Milipore, Canada) or vehicle for 1-2 hours. Then cells were infected with oHSV-1 (G2O7 or HrR3) virus at an MOI of 1 in the presence of chemical inhibitors for

another two days. Therefore, viruses were harvested and titrated in Vero cells by plaque forming assay in 12-well plates by triplicates.

4.2.5 Western blots

Total protein was harvested with sample buffer (125 mM Tris-HCL, 50% Glycerol, 4% Bromophenol blue and 5% 2-mercaptoethanol) and boiled for 5 minutes. Protein samples were subjected to SDS-PAGE (8% gel), transferred to nitrocellulose membranes and blocked with 5% nonfat milk (Bio Rad) in TBS-Tween 20 (TBS-T) for 1 hour at room temperature. The membranes were then incubated with primary antibody (anti β-actin 1:1000; Cell Signalling, Danvers, MA or anti STAT1 1:1000; Cell Signalling, Danvers, MA or anti STAT3 1:1000; Cell Signalling, Danvers, MA or anti phospho STAT1 (Tyr701) 1:1000; Cell Signalling, Danvers, MA or anti phospho STAT3 (Tyr705) 1:1500; Cell Signalling, Danvers, MA or anti phosphor-eIF2α (ser51) 1:1000; Cell Signalling, Danvers, MA or anti ICP27 1:1000 ; Abcam, Cambridge, MA or anti ICP4 1:750; Abcam, Cambridge, MA) for overnight at 4^{o}C. Next day, membranes were washed with TBS-T for three times and incubated with the corresponding secondary antibody (1:3000; Perkim Elmer, Boston, MA) for an hour at room temperature. Membranes were washed with TBS-T for three times before visualization using ECL reagent (Perkim Elmer, Boston, MA) and VersaDoc imaging system (Bio-Rad). Band density was measured by using ImageJ software (NIH, Bethesda, MD).

4.2.6 RNA extraction and RT-PCR

U87 and BV2 microglia cells were infected with oHSV-1 at an MOI of 1. Total RNA was isolated 24 hours post-infection from BV2 or U87 cells using Triazol reagent (invitrogen, Canada). RT-PCR was performed by using one-step real time PCR using KAPA SYBR® FAST One-Step qRT-PCR Universal (D-MARK Biosciences, Canada) following the manufacturer's protocol. cDNA was amplified with the following primers. ICP4: 5'-GGCCTGCTTCCGGATCTC-3' (forward) and 5'-GGTGATGAAGGAGCTGCTGTT-3' (reverse) ; ICP27: 5'-GTCTGGCGGACATTAAGGACA-3' (forward) and 5'-

TGGCCAGAATGACAAACACG-3' (reverse) ; β-actin: 5'-ACGAGGCCCAGAGCAAGAG-3' (forward) and 5'-TCTCCATGTCGTCCCAGTTG-3' (reverse) ; ICP8: 5'-GCGCCCCATGGTCGTGTT-3' (forward) and 5'-CTCCGCCGCCGAGGTTC-3' (reverse); GC: 5'-GCCGCCGCCTACTACCC-3' (forward) and 5'-GCTGCCGCGACTGTGATG-3' (reverse); VP5: 5' - TGAACCCCAGCCCCAGAAACC 3' (forward) and 5' - CGAGTAAACCATGTTAAGGACC 3' (reverse). Results were expressed as $2^{-\Delta\Delta CT}$.

4.2.7 β-galactosidase staining

Cells plated into a 8-well chamber slides were infected with G207 virus and mock infected cells were considered as control. After 24 hours of post-infection, cells were fixed by 0.5% glutaraldehyde solution. Fixed cells were washed twice with PBS and then incubated with 1mg/ml X-gal solution (Sigma, Canada) diluted with X-gal staining solution (5mM K_3Fe, 5mM K_4Fe and 2mM $MgCl_2$) at 37^oC for one hour. Stained cells were then visualized and imaged by using a light microscope.

4.2.8 iNOS (Inducible Nitric Oxide Synthase) inhibition effect on oHSV-1 growth

U87 ($5X10^4$) alone or U87 + microglia co-culture ($5X10^4$ + $5X10^4$) were seeded into a 24-well plate. After overnight incubation for allowing the cells to attach, cells were pretreated with indicated concentrations of aminoguanidine hydrochloride (Tocris Bioscience, Canada) or vehicle for 24 hours. Then cells were infected with G207 virus at an MOI of 1. One hour postinfection, cells were again treated with aminoguanidine hydrochloride for another three days. After three days post-infection, viruses were harvested and titrated on vero cells by plaque forming assay in 12-well plates by triplicates.

4.2.9 Phagocytosis assay

Phagocytosis assay was performed according to the manufacturer protocol (Phagocytosis assay kit, Cayman Chemical, MI). Briefly, microglia cells were infected with G207 or vehicle at an MOI of 1 and 10% latex beads-rabbit IgG-FITC solution was added to each well of a 24-well plate with the virus treatment or 1-hour post-infection after washing with PBS for three times. After 24 hours of treatment, cells were incubated with trypan blue solution for two minutes to quench non-specific staining. After two times washing, cells were lysed by using 1X lysis reagent (Promega, Madison, WI). Fluorescence intensity was measured in a plate reader (Envision 2103 Multilabel reader, Perkin Elmer) using an excitation of 485 nm and an emission of 535 nm.

Virus and IgG latex beads interaction were detected by co-incubating either vehicle or 1% or 10% or 20% of IgG latex beads and G207 (MOI:1). After 24 hours of co-incubation, cells were incubated overnight with 1mg/ml X-gal solution (Sigma, Canada) for LacZ staining. Cells were counterstained with dapi to quantify the total number of cells in each microscopic field. Stained cells were then visualized and imaged by using a light microscope (magnification 20X). LacZ positive cells and dapi stained cells are manually quantified. Data are represented as the ratio of the number of LacZ positive cells and the total number of cells in each image (n=5).

4.2.10 Cell conditioned medium collection & treatment

For collecting the cell conditioned medium U87 ($5X10^4$) or microglia ($5X10^4$) or U87 + microglia co-culture ($5X10^4$ + $5X10^4$) were incubated in cell culture medium for 24 hours in a 24 well plate. To check the effect of cell conditioned medium on G207 growth, previously collected conditioned medium (50%) were added into U87 ($5X10^4$) cells (seeded on a 24-well plate) and then infected with G207. Viruses were harvested three days post-infection and titrated by plaque assay in vero cells.

4.2.11 Cell proliferation assay

Cells were seeded in a 96-well plate at a density of 1 X 10^4 (U87). After overnight incubation, cells were treated with only vehicle or indicated MOI of viruses or indicated concentration of drugs or reagents. After 2 to 3 days of treatment, cell viability was measured by means of MTT assay (Sigma, Canada) following manufacturer's instruction. In brief, cells were incubated with MTT solution for 3 hours at 37^{o}C and then incubate with lysis buffer for. Cell viability was measured at 595 nm by using a plate reader (Envision 2103 Multilabel reader, Perkin Elmer).

4.2.12 U87 xenograft model

5 to 6 weeks old female athymic nude mice were obtained from Harlon laboratories. Human glioma U87 cells were implanted subcutaneously into the lower flank. When tumour size reached ~75 to 100 mm3, vehicle or C16 5mg/kg was administered intraperitoneally (IP). 2 days (Figure 4.6) or 4 days (Figure 4.7) of initial C16 administration, vehicle or oHSV-1 were injected intratumourally. Tumour volume was measured by using a caliper (Height X Length X Wide/2). At the end of the experiment, mice were euthanized by using CO2 asphyxiation, All in-vivo experimental procedures were approved by the UBC animal care committee and performed according to the guidelines of the Canadian Council on Animal Care.

4.2.13 Tissue DNA extraction & qPCR

DNA were extracted from 4% paraformaldehyde fixed tumour tissues by using an EZNA tissue DNA kit (Omega bioteck). Extracted DNAs were subjected to qPCR analysis using Syber green master mix (Invitrogen, Canada) supplemented with ICP27 primers: 5'-GTCTGGCGGACATTAAGGACA-3' (forward) and 5'-TGGCCAGAATGACAAACACG-3' (reverse); β-actin primenrs: 5'-ACGAGGCCCAGAGCAAGAG-3' (forward) and 5'-TCTCCATGTCGTCCCAGTTG-3' (reverse). Amplification was done using Quantstudieo 6 Flex qPCR machine (Applied biosystems, Canada).

4.2.14 Immunohistochemistry

Harvested tumours were subjected to cryostat sectioning after fixed for 24 hours with 4% paraformaldehyde. Tissues were fixed for 24 hours with 4% paraformaldehyde, followed by 72 hours incubation with 30% sucrose. Tissues were then embedded in OCT (Sakura tissue tek), sectioned (20 um) using a cryostat (Leica CM 3050 S) and placed on Fisherbrand™ Superfrost™ Plus microscope Slides (Fisher Scientific, Canada). Slides were then washed with PBS, permeabilized with 0.125% Triton X-100 for 5 minutes and incubated with 5% goat serum (SantaCruz, Canada) for an hour to block unspecific binding. Cells were then incubated overnight with either anti-HSV-1 antibody (1:50; Abcam, Cambridge, MA) or anti F4/80 antibodies (1:50; Abcam, Cambridge, MA) at 4ºC temperature. Following day, after three washing, sections were incubated with either goat anti-rabbit IgG Alexa Fluor 488 or goat anti-rat IgG Alexa Fluor 568 secondary antibody (1:500; Invitrogen, Canada) for an hour at room temperature. After three times washing, sections were then mounted with Dapi fluromount G (Electron Microscopy Sciences) and visualized and imaged by using a confocal microscope (Olympus, Canada).

4.2.15 Statistical analysis

Statistical analysis was performed by SPSS 18 or Microsoft Excel, and significance ($P<0.05$) was determined by using independent-samples T test or a significance $P<0.001$, $P<0.01$, or $P<0.05$ was determined using a two tailed Student's t-test respectively. Data are expressed as mean ± SD or ± SE.

4.3 Results

4.3.1 Presence of microglia hinders the oncolytic efficacy of oHSV-1 against U87 cells

Efficiency of G207 replication in U87 cells was determined by one step viral growth assay (Figure 4.1A). G207 anti-proliferative effect was evaluated by means of an MTT assay. A dose-dependent antiproliferative effect was observed and the IC_{50} was observed for G207 at MOI=1 after 72 hours of infection (Figure 4.1B). Our results confirmed that G207 can effectively replicate and lyse U87 cells.

We then measured G207 growth in U87 cells in the presence of different numbers of microglia. The addition of microglia cells in U87 culture inhibited G207 replication in a “dose” dependent manner. G207 replication was reduced by 50% and almost 100% by addition of 6.25x10^3 and 1x10^5 microglia cells in U87 (5X10^4) cultures, respectively (Figure 4.1C). To further confirm that microglia-mediated oHSV-1 growth suppression is not strain-specific, we tested different HSV-1 strains, including HrR3 (ICP6 mutated), b17-TK (a TK- mutant) and KOS (wild type) in this experiment. We observed similar viral replication inhibition among all HSV-1 strains tested (Figure 4.1D).

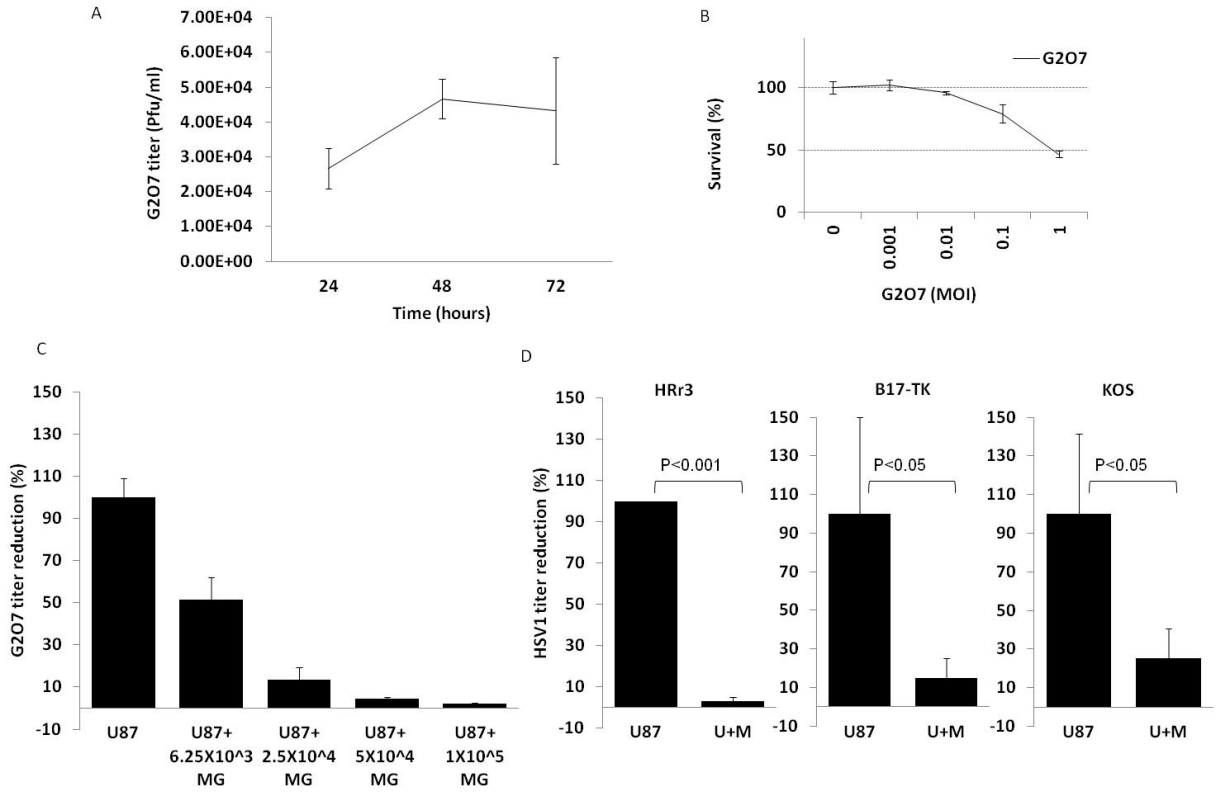

Figure 4.1 Presence of microglia in GBM culture suppresses growth and oncolytic capacity of oHSV-1. The growth of G207 in U87 cells was determined by single step growth assay (A). The cytotoxic effect of G207 was measured by the MTT cell proliferation assay at three days post-infection in U87 cells (B). To study the effect of microglia on G207 growth in U87 (5X10^4) cells alone and also in U87 (5X10^4) in combination with different numbers of primary rat microglia (MG) cells, the cells were infected with G207 at a multiplicity of infection (MOI) of 1 (C). U87 (5X10^4) cells alone and U87 (5X10^4) + MG (5X10^4) co-culture were infected at an MOI of 1 with ICP6 gene-mutated oHSV-1 HrR3, TK-deleted oHSV-1 b17-TK, or wild-type HSV-1 KOS (D). Virus replication efficiency was determined using single step virus growth assay at four days post-infection. Data are reported as means ± S.D.

4.3.2 oHSV-1 triggers M1 polarization of microglia

Since M1 polarized (activated) microglia is characterized by high level of inducible nitric oxide synthase (iNOS) [272], our results demonstrated that G207 treatment significantly upregulated iNOS expression in the microglial cells with or without U87 cells either by co-culturing (Figure 4.2A) or using U87 conditioned medium (Figure 4.2B).

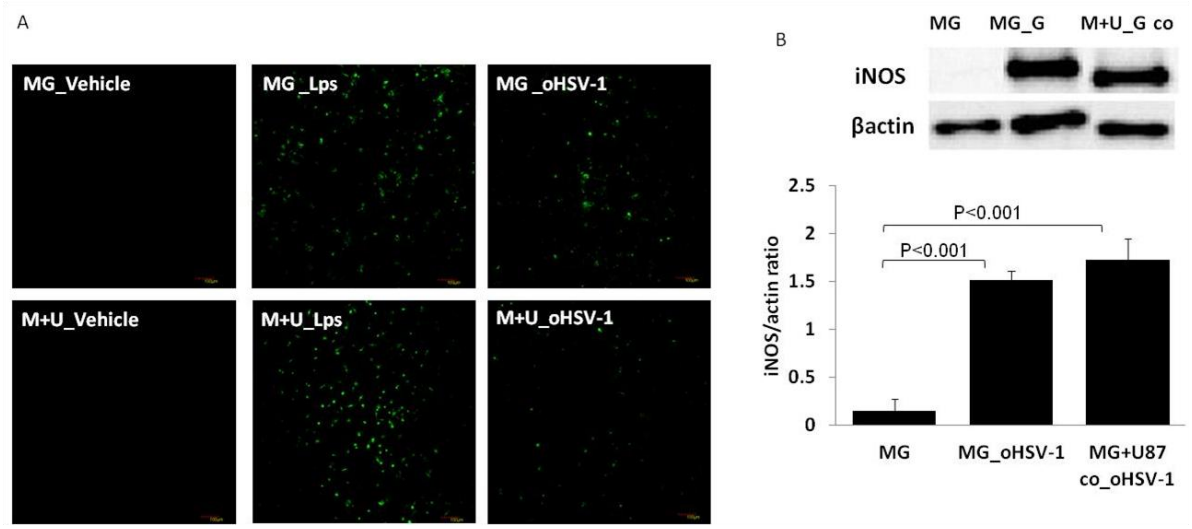

Figure 4.2 oHSV-1 effect on microglia polarization. A, Microglia (MG) or microglia and U87 co-culture (M+U) were immunostained for iNOS after 24 hours treatment with G207 at an MOI of 3 (right panel) or lipopolysaccharide (LPS) (Middle panel) or mock preparation (Left panel). B, Microglia (MG_G207) or microglia incubated with U87 conditioned medium (MG+U87 co_G207) was infected with G207 virus, mock treated microglia (MG) cells were used as a control. Total protein was extracted at 24 hours post-infection. The iNOS and β-actin expression was determined by western blot analysis (B). Band density of iNOS was normalized to β-acting (means ± SD).

4.3.3 Nitric oxide (NO) and other microglia secreted products have no noticeable effect on oHSV-1 replication

Data demonstrated that Deta-No had no antiviral effect against G207 up to 100 uM. Although higher concentrations (such as 500 μM) of Deta-No can suppress G207 replication in U87 cells (Figure 4.3A), it may attribute to the cytotoxicity of the compound, as cell proliferation assay indicated that 500 μM & 1000 μM of Deta-No alone killed 50% and 75% cells, respectively (Figure 4.3B). Moreover, no difference in viral production was found between aminoguanidine-treated and vehicle treated microglia/U87 co-cultures (Figure 4.3C). In addition, no inhibition of oHSV-1 (G2O7) replication in glioma cells were observed by the microglia or microglia+glioma co-culture conditioned medium (Figure 4.3D).

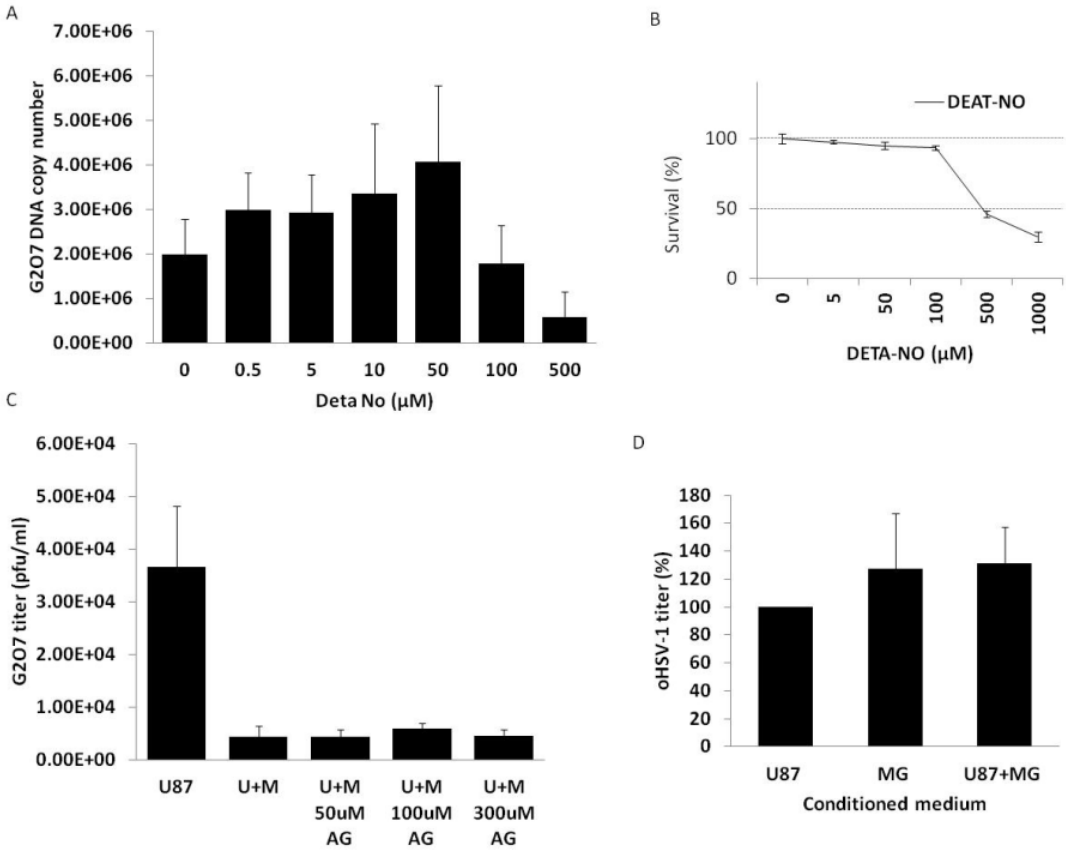

Figure 4.3 Effect of microglia secreted products on oHSV-1 efficacy. A and B, U87 cells were treated with indicated concentrations of Deta-No (an NO donor) in combination with G207 at an MOI of 1 for 48 hours. Harvested virus was then titrated by qPCR to determine viral ICP4 DNA copy number (A). MTT assay determined indicated

concentration of deta-No effect on U87 cell proliferation after 48 hours of treatment (B). C, U87 or U87 + microglia co-culture (U+M) cells were pre-treated with different concentrations of aminoguanidine hydrochloride (AG), an inhibitor of iNOS or vehicle for 24 hours. Then cells were treated with G207 for another three days in the presence of AG or vehicle. Virus titers were determined by plaque assay on vero cells. D, Conditioned medium was collected from U87, Microglia (MG) and co-culture (U+M) cell culture supernatants. U87 cells were infected with G207 at an MOI of 1 with above mentioned conditioned mediums. Viruses were harvested 72 hours post-infection and titrated on vero cells.

4.3.4 Microglia uptake oHSV-1 via phagocytosis

The capacity of primary cultured microglia to engulf the beads by phagocytosis was significantly reduced in the presence of G207 (Figure 4.4A). On the other hand, latex beads reduced number of G207 “infected” primary cultured microglial cells in a concentration-dependent fashion (Figure 4.4B). One possible explanation is that G207 and IgG latex beads may compete for phagocytosis. Thus, it seems that oHSV-1 enter the microglia via the same phagocytotic mechanism as IgG beads. To further verify that microglia and igG latex beads are competing, we performed a similar experiment in BV2 microglia cells. Similarly, co-incubation with G207 caused decreased phagocytotic uptake of IgG latex beads in BV2 microglia cells as well (Figure 4.4C). Interestingly, when BV2 cells were pre-treated with G207, the uptake of IgG latex beads was significantly increased, suggesting that virus may stimulate the phagocytic activity of the BV2 cells (Figure 4.4D).

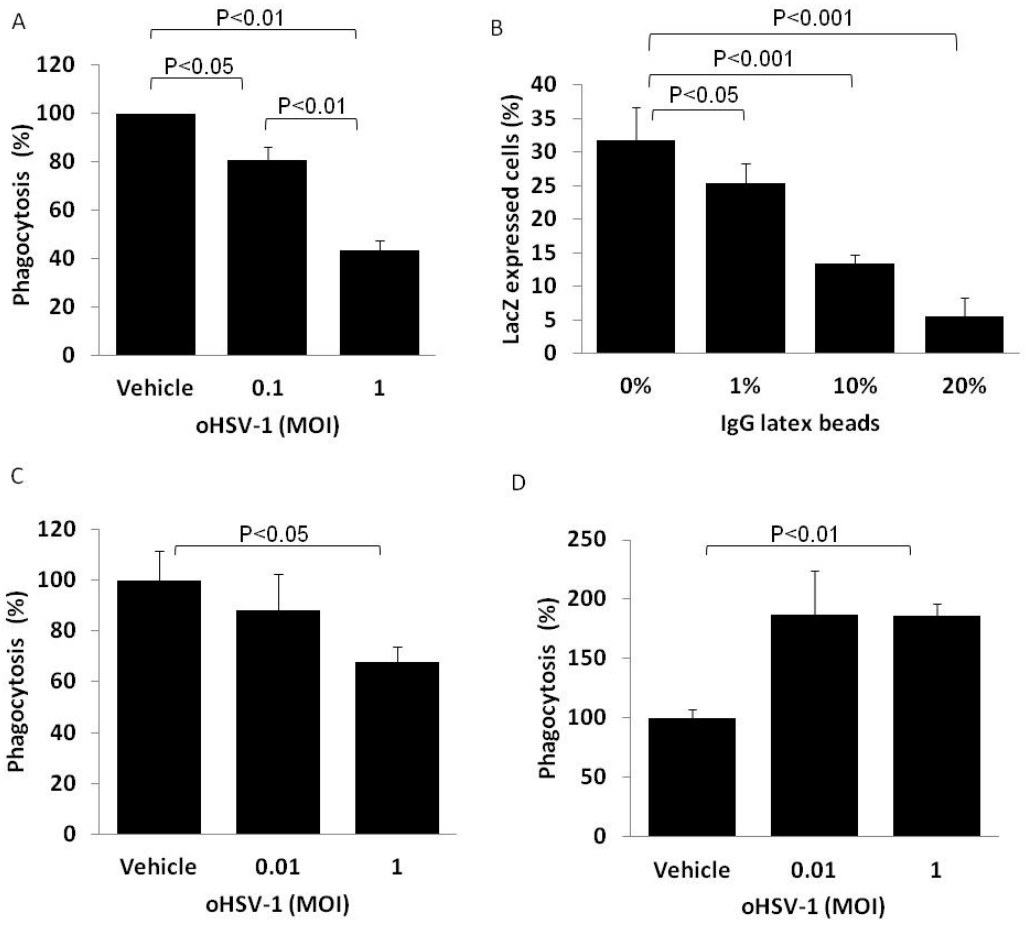

Figure 4.4 oHSV-1 and microglia phagocytosis. A and B, microglia cells were infected with a mock or indicated MOI of oHSV-1 (G207) virus in the presence of 10% latex beads-rabbit IgG-FITC solution. Phagocytosis was measured by fluorescence plate reader after 24 hours of incubation (A). Microglia cells were incubated with G207 at an MOI of 1 with vehicle or indicated concentration of latex beads IgG-FITC solution. Cells were stained with β-galactosidase after 24 hours of treatment to study the virus entry. LacZ positive cells were quantified from 5 randomly selected fields by using a light microscope (means ± SD) (B). C and D, similar experiments were conducted on BV2 microglial cell line. BV2 microglia cells were infected with mock preparation or indicated MOI of G2O7, co-incubated with 10% latex beads-rabbit IgG-FITC solution (C) or 1 hour post-infected cells were washed three times with PBS and then incubated with 10% latex beads-rabbit IgG-FITC solution (D). After 24 hours of treatment, the degree of phagocytosis was measured by fluorescence plate reader.

4.3.5 oHSV-1 infection induces microglia cell death

To determine the fate of the oHSV-1 infected microglia cells, we performed MTT cell prolifeartion assay. Data demonstreated that oHSV-1 inhibits the survival of microglia cells (Figure 4.5).

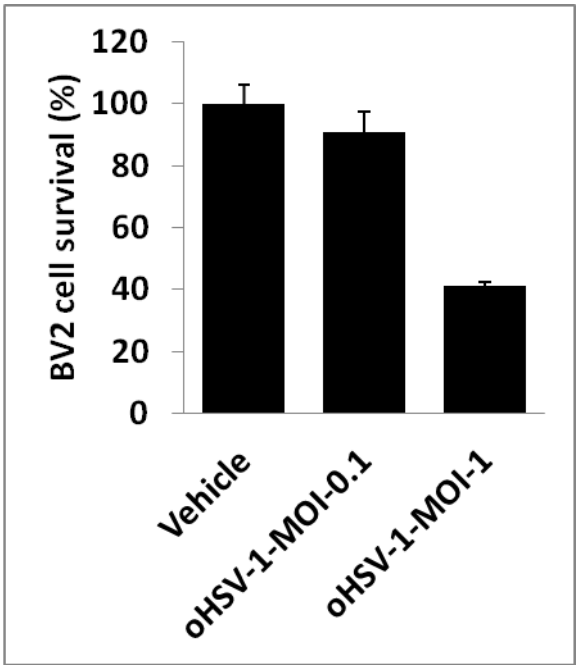

Figure 4.5 oHSV-1 effect on microglia cell proliferation. BV2 microglia cells were seeded in a 96-well plate at a density of 1 X 10^4. The next day, they were treated with vehicle only, with oHSV-1 (G2O7) viruses with different MOIs for 48 hours. Cell viability was then measured using an MTT assay.

4.3.6 Microglia forms a replicative barrier to prevent oHSV-1 dissemination

We next asked whether oHSV-1 can infect and replicate in microglia cells. Both rodent primary cultured microglial cells and BV2 microglia infected with G207 showed LacZ

staining, indicating that the virus can enter the cell and express the reporter gene carried by the virus[273] (Figure 4.6A). Concentration-dependent HSV-1 infection in microglia cells was also observed by GFP expressing UL5354 virus, an ICP27- HSV-1 that is replication deficient but expresses GFP reporter gene as well (data not shown). However, growth assay of G207 in primary cultured rat microglia cells (Figure 4.6B I) and BV2 cells (Figure 4.6B II) demonstrated that G207 failed to produce its progeny in microglia cells (Figure 4.6B). These results suggest that microglia internalized the viruses but did not allow viral replication.

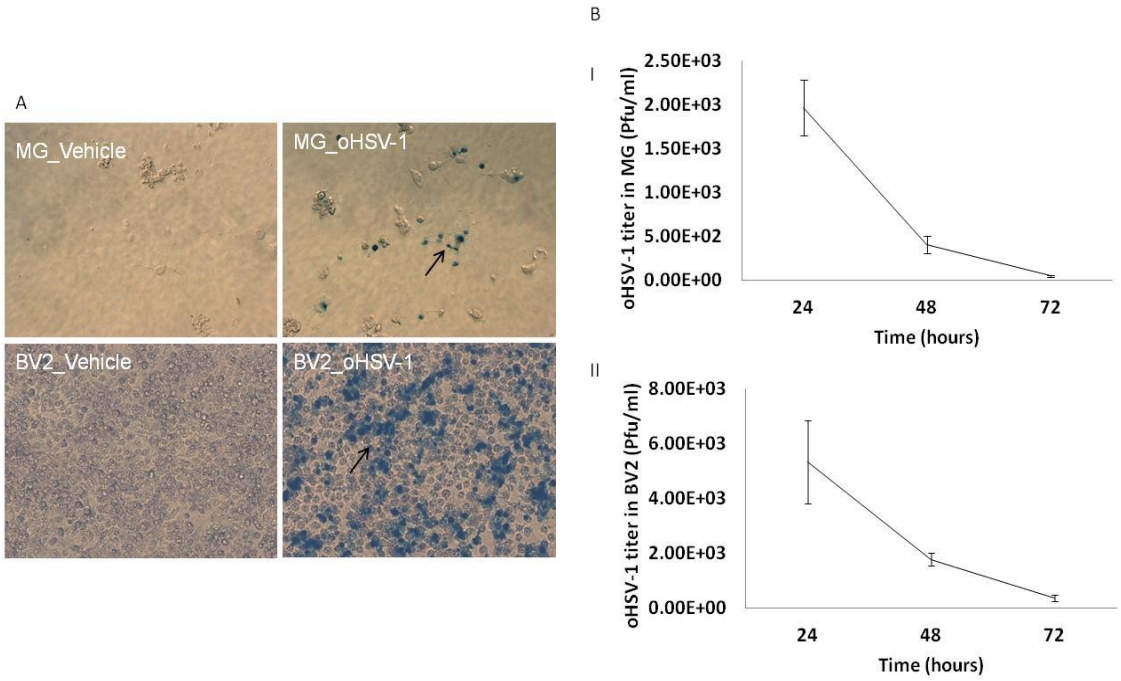

Figure 4.6 oHSV-1s are capable of infecting microglias, but they are unable to replicate. Rat primary microglia (MG) or BV2 microglia cells were infected with G207 virus at an MOI of 3 or mock preparations. To detect viral reporter gene expression, cells were stained for β-galactosidase (arrow) after 24 hours of infection (A). MG or BV2 microglia cells were infected with G207 at an MOI of 1 to study the replication capacity of G207. Viruses were harvested at 24 hours, 48 hours, and 72 hours post-infection and then titrated on Vero cells (B).

4.3.7 oHSV-1 gene expression profiling in BV2 microglia cells

To reveal the mechanism by which the HSV-1 replication is prevented in microglial cells, we measured transcript levels of a panel of viral genes in glioma (U87) and microglia (BV2) cells. The viral genes included ICP4, ICP27, ICP8, VP5 and Glycoprotein C (gC), representing immediate early, early and late genes, respectively. Quantitative RT-PCR results showed that transcription of ICP27, ICP8, VP5 and gC but not ICP4 were significantly suppressed in BV2 cells compared to U87 cells (Figure 4.7).

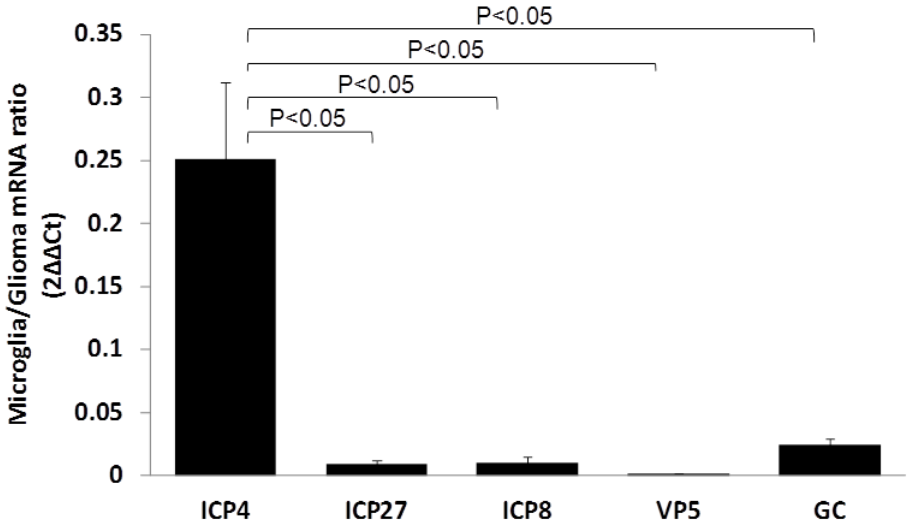

Figure 4.7 oHSV-1 gene expression in microglia cells. The mRNA levels of different genes of the G207 virus (ICP4, ICP27, ICP8, VP5 and GC) were detected by RT-qPCR. Ratios of the relative mRNA level ($2^{-\Delta\Delta CT}$) of G207 infected BV2 and U87 cells (BV2/U87) are presented.

4.3.8 C16 overcomes microglia-mediated oHSV-1 replication barrier

To understand the events of intracellular signaling in G207 stimulated microglia, we have further tested effects of a PKR inhibitor (C16), an NFkappaB inhibitor (Bay11) and an iNOS inhibitor (Aminoguanidine hydrochloride) on G207 replication in BV2 cells. Treatment with 1 & 10 μM C16 significantly enhanced the replication by 9 times and 8 times, respectively (Figure 4.8Ai). However, Bay11 and aminoguanidine hydrochloride had no effect on oHSV-1 replication in BV2 cells (Figure 4.8Aii and iii). In agreement to that, C16 but not Bay11 nor aminoguanidine hydrochloride upregulated expression of ICP4 and ICP27 by 1.8 and 25-fold, respectively (Figure 4.8B). To verify that C16 mediated viral gene transcriptional augmentation is not due to the double deletions in ICP34.5 and ICP6 in G207, we then checked the effect of C16 on wild type (KOS), and ICP6 mutated (HrR3) HSV-1 infected BV2 cells. C16 treatment also upregulated ICP27 expression in KOS and HrR3 infected BV2 cells (Figure 4.8C). Finally, we asked whether C16 can overcome the microglia-mediated suppression of viral replication in glioma cells. As shown in Figure 4.8D, G207 viral replication increased by 33% in the glioma-microglia co-cultures treated with C16.

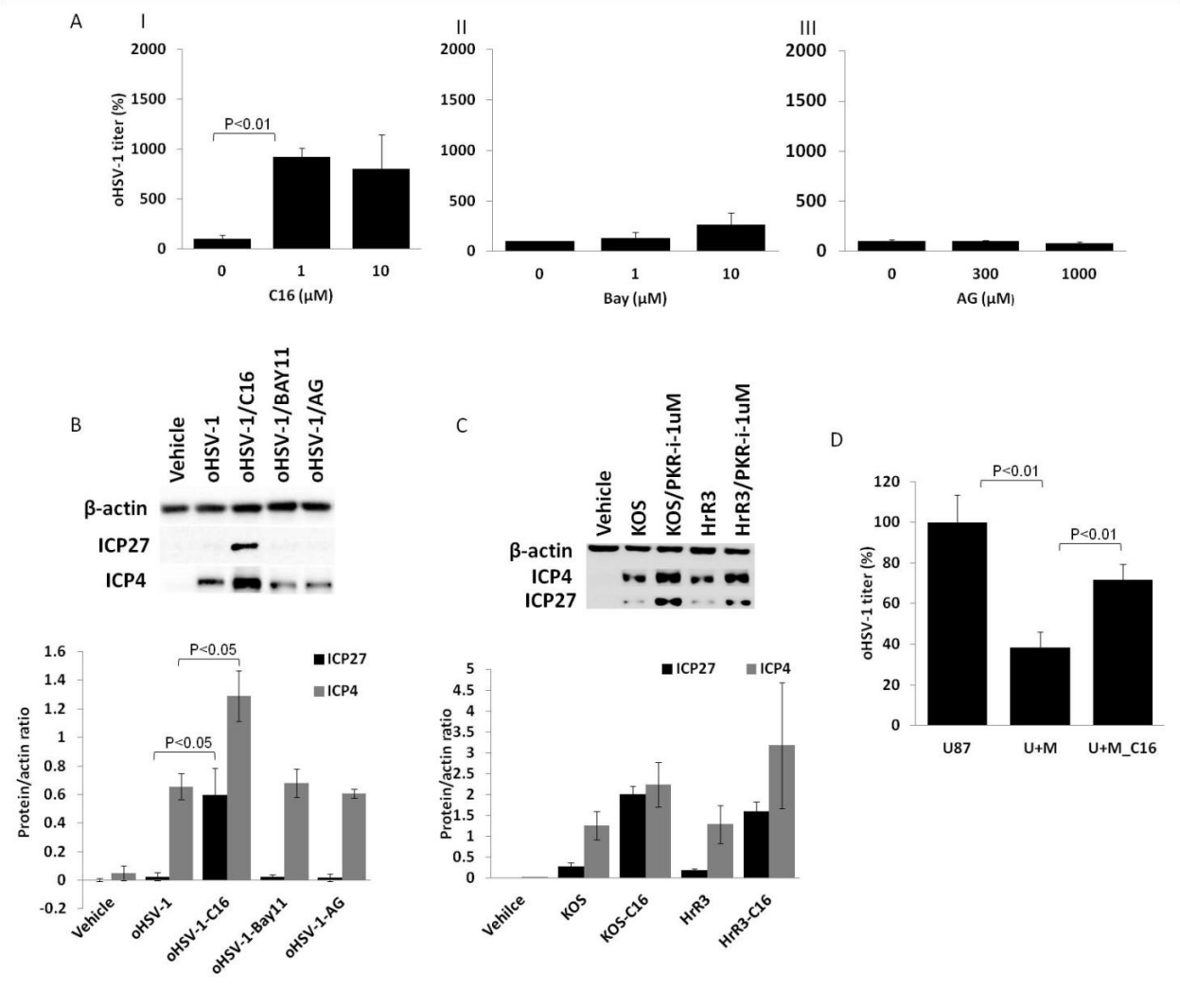

Figure 4.8 Mechanism of oHSV-1 gene expression blockade in microglia cells. BV2 cells were pre-treated with the indicated chemical compounds for 1-2 hours and then infected with oHSV-1 at an MOI of 1 (A-D). The viruses were harvested after 48 hours of oHSV-1 (G207) and indicated chemical inhibitors treatment (A and D). Total proteins were harvested from microglia cells infected with G2O7 (B), HrR3, or KOS (C) at 24 hours post treatment with either 1μM of C16 (B and C) or 1μM of Bay11 (B) or 100μM of AG (B). ICP27, ICP4 & β-actin expression was measured by western blot assay (B and C). U87 and MG co-culture were pretreated with 0.5 μM of C16 for 1 hour. After 48 hours of treatment with oHSV-1 (G207) and C16, viruses were harvested and titrated by plaque-forming assay on vero cells (D).

4.3.9 Cell type-specific C16 effect on oHSV-1 replication

To determine the C16 effect on the replication of oHSV-1 in tumour and normal brain cells, we performed oHSV-1 replication assay in glioma and astrocytes. Virus growth assay revealed that oHSV-1 replication was reduced in astrocytes, while unchanged in U87 glioma cells (Figure 4.9).

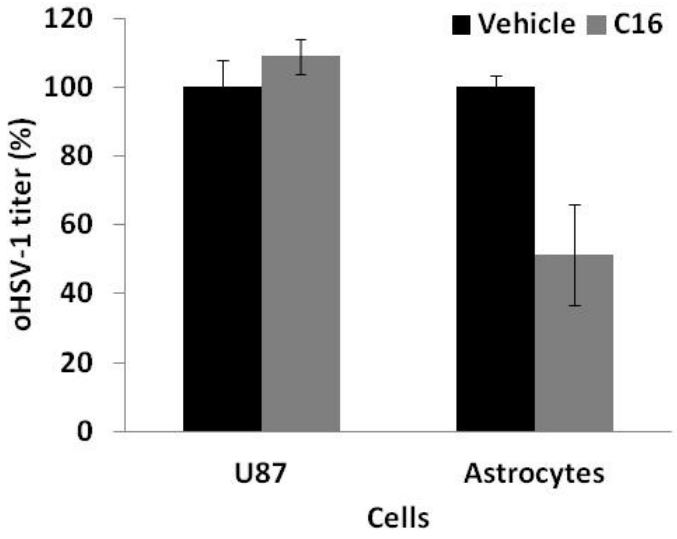

Figure 4.9 Effect of C16 on oHSV-1 replication in tumour and nontumour cell types. The indicated cells were pre-treated with vehicle or 1uM C16 for 2 hours before infecting them with oHSV-1 (G207) at an MOI of 1. Viruses were harvested after 24 hours of infection and titrated on Vero cells.

4.3.10 C16 rescue oHSV-1 in microglial cells by inhibiting STAT 1 and 3 activities

As a part of an effort to understand the mechanism of C16 on facilitating viral replication in microglia/macrophages, we first looked activity of PKR pathway by measuring the level of phosphorylated eIF2α in G207 infected BV2 cells since C16 was reported as a PKR inhibitor. To our surprise, there was no change in phosphorylated eIF2α regardless of the ICP34.5 status of the virus (Figure 4.10). We then examined activities of STAT1 and STAT3 in BV2 cells with and without G207 infection. G207 upregulates phosphorylation of STAT1 (Tyr701) and STAT3 (Tyr705) in BV2 cells (Figure 4.10A), which is significantly suppressed by C16. Interestingly, the overall expression level of STAT1 but not STAT3 was upregulated in G2O7 infected microglial cells, and that was also reduced after C16 treatment. The C16 caused inhibition in STAT1/3 phosphorylation was also confirmed in LPS treated BV2 cells (Figure 4.10B).

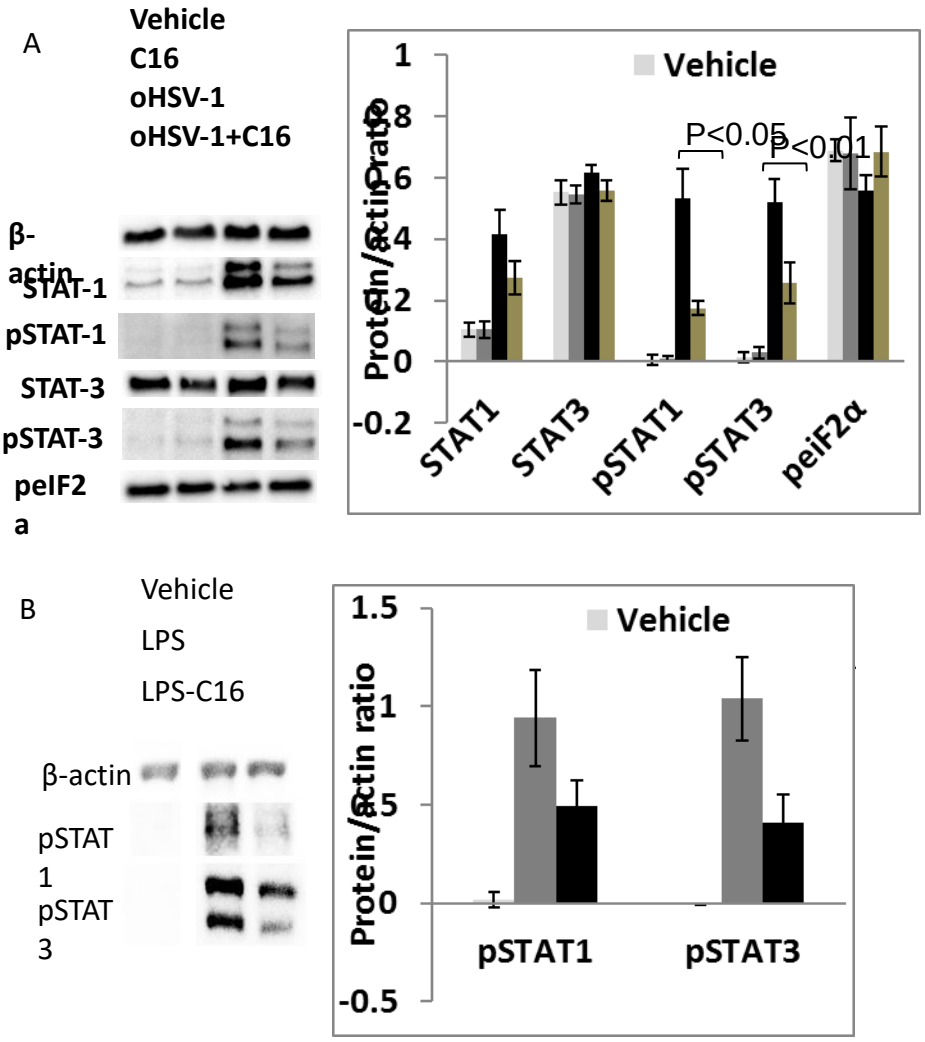

Figure 4.10 C16 inhibits STAT 1 and 3 phosphorylation. BV2 cells were pre-treated with vehicle or C16 (1uM) for 2 hours and then incubated with vehicle or oHSV-1 (G2O7) at an MOI of 1 or with the oHSV-1 and C16 combination for 24 hours (A). BV2 cells were treated with either vehicle or LPS (1µg/ml) alone or a combination of LPS (1µg/ml) and C16 (1µM) for 24 hours (B).Total protein was harvested and subjected to western blot assay. β-actin expression was used for loading control (means ± SE).

4.3.11 Cell type specific eIF2α phosphorylation inhibitory effect of C16

Since C16 is knoiwn for its PKR inhibitory function, therefore we evaluated its effect on eIF2α phosphorylation in oHSV-1 infected cells. We observed that eIF2α phosphorylation was inhibited in oHSV-1 infected astrocytes, but its level was unchanged in U87 and BV2 cells (Figure 4.11).

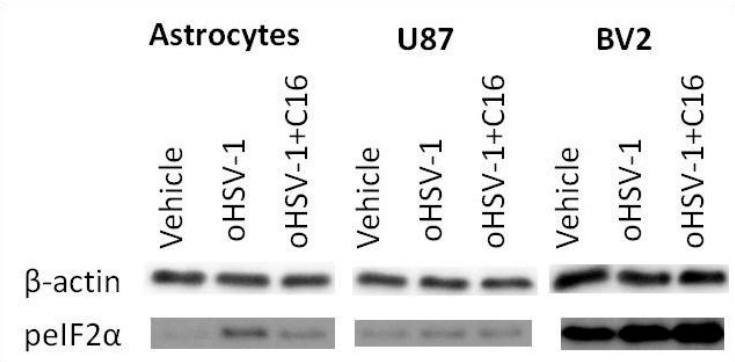

Figure 4.11 eIF2α phosphorylation inhibition effect of C16 in various cell types infected with oHSV-1. The indicated cells were pre-treated with vehicle or 1uM C16 for 2 hours before infecting them with G207 at an MOI of 1. Total protein was extracted at 24 hours post-treatment and subjected to western blot analysis.

4.3.12 C16 selectively facilitates oHSV-1 replication in glioma xenograft by overcoming barriers of tumour-associated macrophage

To demonstrate that the above effect by C16 can be translated into the enhanced efficacy of intratumoural replication of oHSV in vivo, C16 was i.p. injected into the animals bearing subcutaneously implanted U87 tumours that received G207 intratumourally. Administration of C16 significantly enhanced oHSV-1 titer in the tumour

mass (Figure 4.12A). Immunohistochemistry analysis demonstrated increased number of cells harboring replicating HSV-1 in animals co-treated with oHSV-1 and 5mg/kg C16 compared to those treated with oHSV-1 alone. Furthermore, numerous cells with co-localization of HSV-1 and macrophage markers (F4/80) were seen in animals co-treated with oHSV-1 and C16 but not oHSV-1 alone, indicating increased viral replication in the macrophages after C16 treatment (Figure 4.12B).

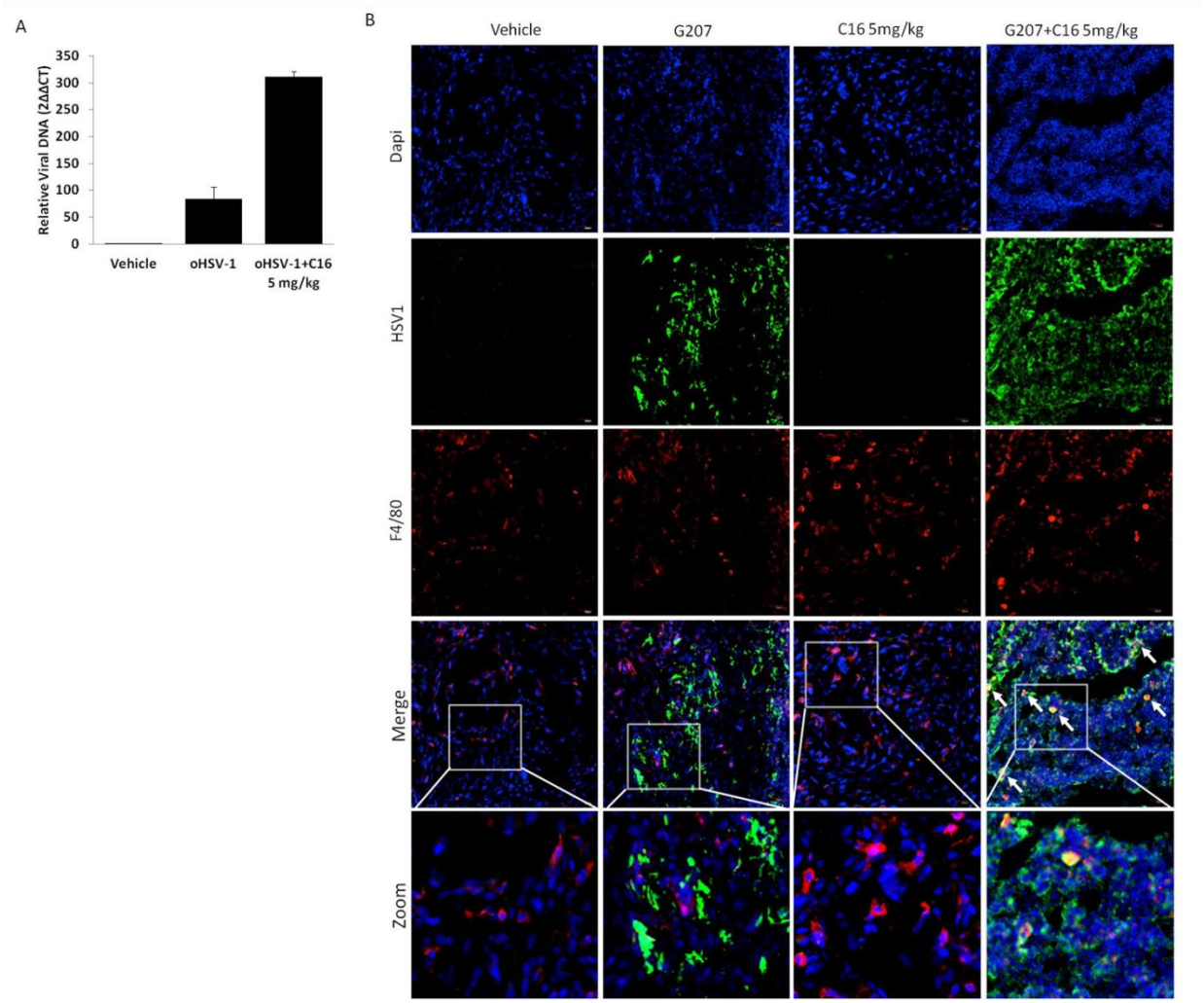

Figure 4.12 C16 triggers oHSV-1 protein expression in microglia/macrophages and enhances virus concentration in glioblastoma in-vivo. U87 xenograft mice were treated with vehicle or 5mg/kg of C16 with or without 6X10^6 PFU oHSV-1 (G207) viruses (n=2). Two doses of G207 or vehicle and 4 doses (in every 3 or 4 days) of C16 were administered in total. Tumours were harvested at day 13 post treatment. Total genomic DNA was extracted from the harvested tumours (n=2), and viral DNA (ICP27) was measured by qPCR (normalized to β-actin). Data shown are regarding relative $2^{-\Delta\Delta CT}$ value ± SE (A). Infiltrated macrophages (F4/80) and replicable viruses were detected in U87 tumours when they were subjected to immunostaining (B).

4.3.13 Anti-tumour effect of C16

As we observed potent STAT3 inhibitory effect by C16, which is well known for its oncogenic function, we then asked if C16 can prevent the survival of tumour cells. Cytotoxic effect of C16 was observed in various types of tumour cells (Figure 4.13).

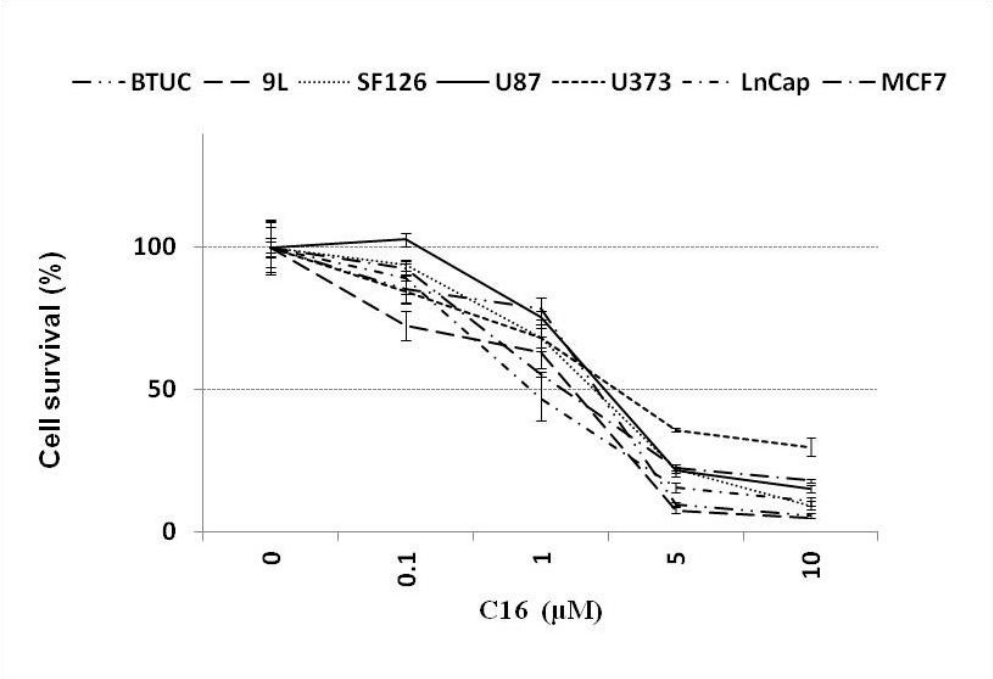

Figure 4.13 Anti-tumour effect of C16 in-vitro. Cells were treated with indicated concentration of C16 for 72 hours. Cytotoxicity was measured by an MTT assay. Data are presented as means ± S.D.

4.3.14 C16 significantly improve human glioma xenograft regression

Finally, we asked whether C16 mediated facilitation in oHSV-1 replication in the tumour is capable of augmenting the anti-tumour oncolysis. Indeed, tumour sizes in animals treated with C16 (5 mg/kg) and the oHSV-1 combination were 9.3 fold, 8.2 fold and six fold smaller than those treated with vehicle, C16 alone and oHSV-1 alone, respectively (Figure 4.14A). Furthermore, virus copy numbers measured by qPCR in the liver, brain and gastrointestinal showed no difference among all groups, demonstrating that viral replication was restricted only in the tumours without spreading to the normal organs (Figure 4.14B). Kaplan-Meier analysis showed the increased survival of the C16 and oHSV-1 combination treatment compared to other groups (Figure 4.14C).

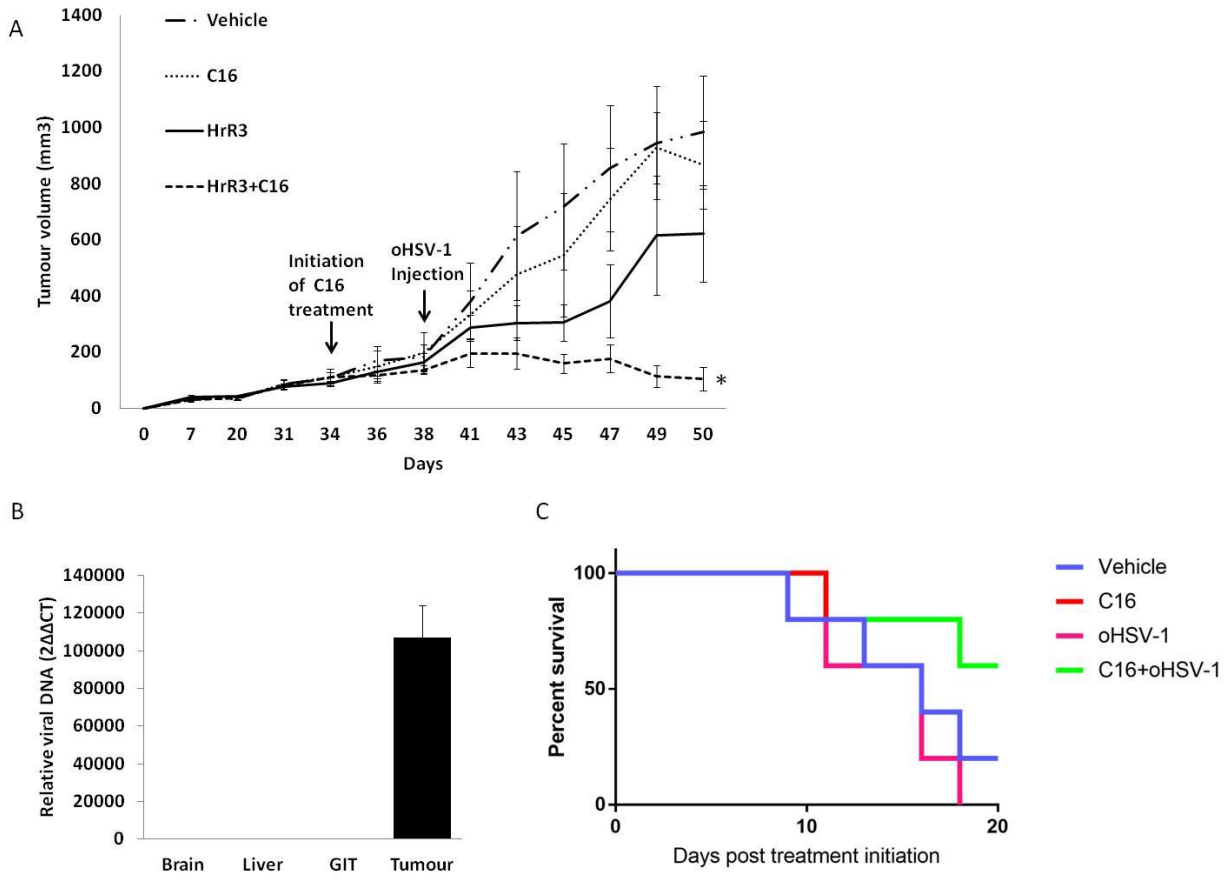

Figure 4.14 C16 increases oncolytic efficacy of oHSV-1 in-vivo. U87 xenograft mice were treated with vehicle or 5mg/kg of C16 with or without a single dose of 1X10^8 PFU oHSV-1 (HrR3) viruses (n=5). Multiple doses of vehicle or C16 were injected intraperitoneally (IP) on alternate days. Tumour volume was measured by using a caliper (Height X Length X Wide/2) (A). In the results, * represents significant (P≤ 0.05) tumour regression by oHSV-1 and C16 combination vs. vehicle or individual C16 or individual oHSV-1 treatment (± SE). Survival of the animals was represented using Kaplan-Meier survival analysis (B). Tumours and other organs were harvested once the animals reached the endpoint, and total genomic DNA was extracted from the harvested organs (n=3). Viral DNA (ICP27) was measured using qPCR (normalized to β-actin). Data shown are relative $2^{-\Delta\Delta CT}$ value ± SE (C).

4.3.15 Schematic diagram of microglia effect on oHSV-1 efficacy

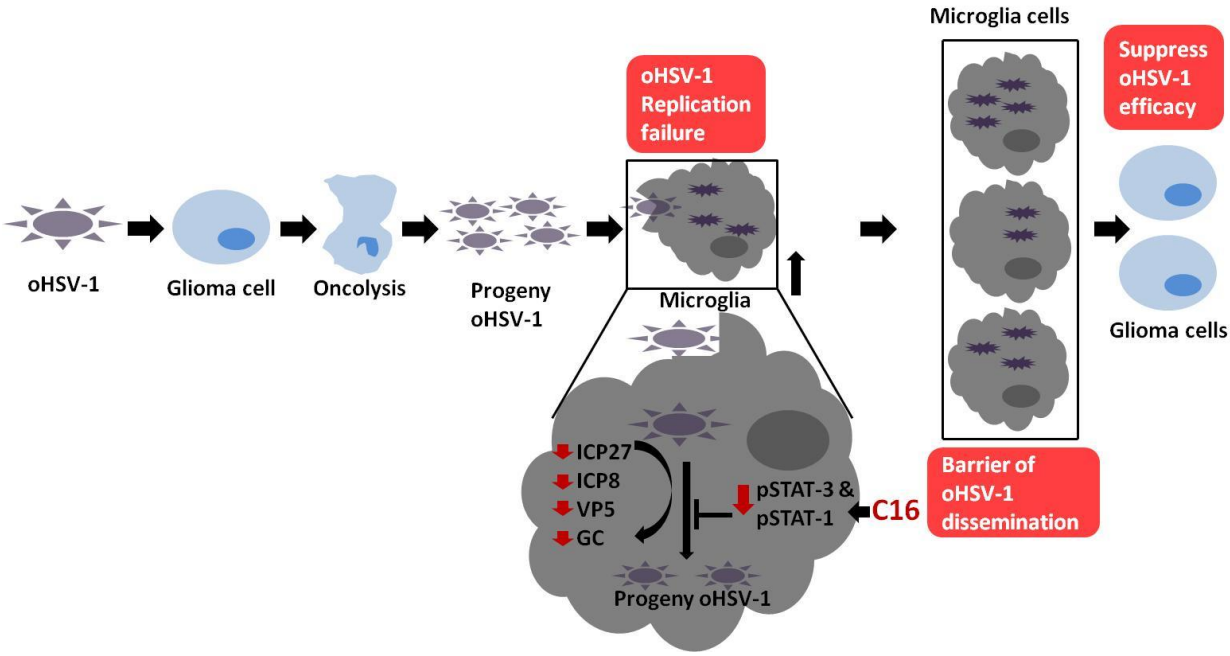

Figure 4.15 Schematic diagram of microglia barrier on oHSV-1 therapy.

4.4 Discussion

In this study, we demonstrated that the presence of microglia/macrophages impedes oHSV-1 oncolytic replication in GBM cells. This finding is consistent with the previous reports that inhibition of innate immune response enhances efficacy[68,266-268,269] of OVs. Another very recent study by Han et al. reported that a single dose of TGFβ helped to overcome the innate immune resistance mediated by microglia/macrophages and NK cells[274] and thereby enhanced the anti-tumour efficacy of oHSV-1. Interestingly, we observed that after oHSV is taken up by microglia/macrophages, some viral genes such as ICP4 and virally carried exogenous genes such as lacZ reporter can still be expressed albeit viral replication would not be permitted. These findings suggest that HSV-1 can potentially target microglia/macrophages to express the therapeutic gene(s) that modify the tumour microenvironment.

Our gene expression profiling in microglia cells infected by oHSV-1 demonstrated that expression of immediate early ICP27 gene, early ICP8 and late VP5 and gC gene belonging to HSV-1 are significantly decreased in microglia cells. Down-regulation of the early and late genes such as ICP8[275] and gC[276] might be the result of the silence of the immediate early gene ICP27 that is required for viral DNA synthesis and late gene expression [275,277]. However, the silence of ICP27 may be a necessary but not sufficient condition to block HSV replication in microglia since restoring ICP27 expression by transfecting an exogenous copy of ICP27 could not rescue the viral replication (Data not shown).

Focusing on the interaction between oHSV-1 and microglia, we then asked how the underlying molecular mechanism of microglia/macrophage-mediated inhibition of viral gene expression operated. It is well known that the interferon-inducible, dsRNA-dependent protein kinase, PKR, plays a key role in the innate immunity response to viral infection [278]. After infection with HSV-1, normal host cells activate PKR thereby shutting down protein synthesis through inactivating eukaryotic initiation factor-2 (eIF-2α) via increased phosphorylation. It has also been known that γ34.5 of HSV-1 counteracts PKR and IFNs-mediated antiviral effect [279] by recruiting the cellular protein phosphatase

1α, which reverses the PKR-mediated phosphorylation of eIF2α [279,280]. Originally, we thought that inhibition of viral protein synthesis might be attributed to eIF2α mediated blockage of protein synthesis, which led to our tests using the oxindole/imidazole derivative C16, a known PKR inhibitor[270,281]. However, subsequent results indicated that activation of PKR is unlikely to be the mechanism behind the blockage of viral replication in microglia/macrophages for the following reasons: 1) the inhibition of viral gene expression occurred at transcriptional level; 2) C16 enhanced viral replication in all viral strains tested regardless of their γ34.5 status[62,64]. Thirdly, in agreement with previous studies, we also observed inhibition of eIF-2α phosphorylation by C16 in primary astrocytes[270,281]; however, no change was seen in microglia and glioma cells, suggesting a possible cell-type specificity of C16 on PKR inhibition. Instead, we found that C16 significantly inhibited phosphorylation of STAT1/3 in oHSV-1-infected microglia/macrophages and thereby rescued the replication of oHSV-1. More interestingly, we found that inhibition of STAT1/3 phosphorylation by C16 was only evident in oHSV-infected cells. Previously, C16 was reported to inhibit three dormant host fucosyltransferase genes (FUT3, FUT5, and FUT6) expression in wild type HSV-1 infected human diploid embryonic lung fibroblasts, which thereby stimulated HSV-1-induced expression of sialyl Lewis X independently to PKR stimulated translation inhibition. Interestingly, they also observed that C16 treatment in HSV-1 infected cell reduced IL6 production which is one of the main cytokine to activate STAT signaling. STAT blockade effect of C16 observed in our study might be the outcome of C16 mediated inhibition of IL6[225].

STATs are well known for their anti-mycobacterial and antiviral effect [224,227,282]. Among different members of the STAT family, STAT1 is the most known for regulating antiviral interferon (IFN) signaling cascade[283,282] and interferon has in turn been reported to inhibit HSV-1[258]. As expected, elevated STAT1 phosphorylation was observed in oHSV-1-infected microglia/macrophages that might be the consequence of cellular defence to the viral infection.

Similar to the action of STAT1, the phosphorylation level of STAT3 was also upregulated in oHSV-1-infected microglia/macrophages. STAT3 is another member of the STAT family which is known for its oncogenic function, tumour cell proliferation, survival and invasion [224]. Inhibition of STAT-3 activation induces cell death mediated by apoptosis [262,263] and autophagy [262]. The role of STAT3 in HSV-1 infection is not clearly understood. A previous study by Okemoto et al. demonstrated a positive correlation between STAT3 and oHSV-1 replication in glioma cells[261]. There have been no studies on for the role of STAT3 in antiviral function of microglia/macrophages. Our data demonstrated enhanced oHSV-1 replication in microglia/macrophages upon C16-mediated inhibition of STAT1 and STAT3. However, C16-mediated virus replication enhancement was only evident in microglia/macrophages and not in astrocytes or glioma cells. In agreement with our findings, functions of both STAT1 and STAT3 were reported to be cell-type specific[284,285]. Shin et al. have demonstrated that HSV-1-mediated countermeasures against IFN are ineffective in monocytes[285]. Apparently, the STAT1 pathway is more functional in monocytes and macrophages. Since ICP27 plays a vital role in countermeasures to cellular IFN response[279,286], silencing ICP27 by STAT1/3 may render HSV-1 ineffective in evading the intracellular antiviral mechanism within microglia/macrophages.

Our *in vivo* data in U87 human glioma xenograft model also confirmed that C16 rescued the oHSV-1 gene expression in tumour-infiltrated macrophages and augmented oHSV-1 viral load in tumours. Moreover, we observed that the effect of C16 on enhanced viral replication in macrophages was restricted to tumours; no viral replication was detectable outside of tumour tissue by sensitive PCR method. It remains to be confirmed whether the selective facilitation of oHSV-1 oncolysis by C16 was due to locally delivered virus or attributable to the difference between normal microglia/macrophages and tumour-associated macrophages/microglia.

Unfortunately, we were not able to distinguish the roles of STAT1 and STAT3 in blocking viral replication in those cells due to lack of a highly selective agent.

Nevertheless, our results suggest that activation of STAT1/3 is sufficient to inhibit viral replication of oHSV-1 even when the PKR-eIF2α axis remains active in microglia/macrophages. Furthermore, since STAT3 has been a target for cancer treatment and our data demonstrates that C16 inhibits the proliferation of various types of cancer cells, inhibition of STAT3 activity by C16 may significantly facilitate oHSV-1-induced tumour regression and enhance viral dissemination in tumour mass.

In summary, our present study suggests that anti-tumour efficacy of oHSV-1 is partially determined by the amount of non-tumourous cells in the tumour mass, especially microglia/macrophages that uptake the viruses but do not permit viral replication (Figure 4.15). This is mainly due to upregulated STAT1/3 phosphorylation that may be independent of PKR activation. However, we found that some viral and exogenous genes carried by oHSV-1 and uptaken by the microglia/macrophages can still be expressed. This suggests that the tumour-associated microglia/macrophages may be utilized for expressing virally carried therapeutic genes. More importantly, we discovered that C16 is a strong phosphorylation inhibitor of STAT1/3 that can selectively restore viral replication in microglia/macrophages to allow enhanced intratumoural viral production and dissemination. Inhibition of STAT3 phosphorylation by C16 may further suppress tumour growth. The combination effects of the above dramatically increase anti-tumour efficacy of the oncolytic virus. Our research has provided strong evidence that C16 is an excellent candidate for clinical application in combination with oncolytic virotherapy on account of its capacity to significantly augment the anti-tumour efficacy of oHSV-1.

5 Conclusions and future directions

Among the various types of viruses, oncolytic herpes simplex type-1 is probably the most advanced oncolytic virus in the clinic as it is the first oncolytic virus approved by the USA FDA. Despite the tremendous recent progress of oHSV-1, the efficacy of the oHSV-1 in treating glioma remains to be improved in the clinics [55,85]. Ensuring the safety of virotherapy is of the highest importance, but the importance of enhancing viral oncolytic antitumour activity is equally critical for OV's success in the clinic [274,287]. Here, we have demonstrated that, to boost the antitumour efficacy of oncolytic herpes virotherapy, three strategies are possible: (1) engineering the virus transcriptionally and translationally for tumour-specific replication to enhance tumour targeting without compromising viral oncolytic activity (Chapter 2), (2) enhancing virus-mediated cytotoxicity by counteracting virally induced anti-apoptotic mechanisms. (Chapter 3), and (3) facilitating intratumoural virus dissemination by overcoming the barriers formed by tumours associated microglia/macrophages (Chapter 4).

5.1 oHSV-1 tumour specificity

Viral gene deletion is a common approach for achieving tumour specificity [63]. Attaining tumour specificity by attenuating or deleting viral gene(s) sometimes compromises the lytic activity of the virus that results significantly reduced therapeutic efficacy [62,64]. To circumvent this disadvantage, various tumour-specific transcriptional targets have been utilized previously to attain tumour specificity [90,288]. Choosing a perfect tumour-specific promoter to transcriptionally regulate OVs is often tricky since the promoter needs to be not only upregulated in tumour cells but also completely silent in normal cells. The survivin promoter [197-203] is a good choice to regulate OVs transcriptionally. However, low-level promoter activity may still result in virus replication in non-tumour cells [90] to cause unwanted toxicity. Single transcriptional regulation by a tumour-specific cellular promoter also may not be sufficient to completely inhibit the replication in normal cells,

as it has been shown that some HSV-1 immediate early gene, such as ICP0, can upregulate the activity of cellular promoters that are supposed to be silent [289].

Thus, multiple tumour-specific regulations might be another approach to overcome off target replication of OVs. The combination of transcriptional and translational targeting strategy has been reported successful to enhance OV's tumour specificity [185]. Previously, Cleo et al., demonstrated that inclusion of miRNA 143 or 145 target sequences into the 3'UTR of an HSV-1 essential gene limit viral replication in prostate cancer cells [186]. We also applied this mechanism in addition to the transcriptional regulation by utilizing the differential expression pattern of eukaryotic translation initiation factor 4 (eIF4E) and micro RNA 124 [94,97,204] in tumour and non-tumour cells. In a very recent study, Zhang et al., of our group, reported that targeting multiple miRNAs significantly improves the tumour specificity of oHSV-1 compared to single miRNA target [290]. However, my study only investigated miRNA 124, the addition of another or multiple miRNAs might further enhance the safety feature of our oHSV-1. Since we only regulated ICP4 [291,292] gene expression, and it has been reported previously by Cleo et al., that low-level expression of ICP4 can still support virus replication in normal cells [90], another replication-essential gene such as ICP27 could ensure extra safety to treat glioblastoma.

5.2 Cytotoxicity of oHSV-1 infection

The interaction between the host and HSV-1 is very complex, and both sides want to take control of the cellular machinery. Since virus replication and life cycle depend on the host cellular machinery, HSV-1 changes the host cellular process for maintaining efficient replication [293]. On the other hand, cellular defense mechanism fight against the viral infection [294]. Among various cellular armors, interferon (IFN) is the first line of defense against viral infection [282,295]. Even though many studies have demonstrated HSV-1 mediated induction of IFN production [296], it is not clearly understood whether IFN is capable of blocking the virus infection, as several studies have reported that HSV-1 is

skilled to countermeasure IFN response [285,297]. However, both classes of IFN, Type 1 (IFNα and IFNβ) and Type 2 (IFNγ) were found essential to defend HSV-1 infection [298]. Type 1 can be produced by most of the cell types, while type 2 is only produced by a few types of immune cells, such as natural killer cells, CD4+T helper 1 (Th1) cell, CD8+T cells [282], and macrophages[299]. In fact, the differential pattern of IFN response in various cell types [296] might explain the cell type-specific HSV-1 countermeasures to IFN response [285]. HSV-1 response to offset type 1 IFN has been reported to be elicited by HSV-1 virion host shut-off protein [300]. On the contrary, HSV-1 response against type 2 IFN seems ineffective, since type 2 IFN producing monocytes/macrophages were nonpermissive to HSV-1 infection [285]. Along with others, we observed that oHSV-1 efficiently replicate and lyse various tumour cells, while it fails to replicate in microglia and macrophages. Further investigations are required to verify the effect of type 2 IFN on HSV-1 for those tumour-associated immune cells.

In the context of the virus-host interaction, inducing apoptosis in the virus-infected cells is another intrinsic cellular defense to limit virus spread[246,247,301], which is mainly provoked by IFN [302]. In response, HSV-1 encodes several genes, primarily driven by immediate early ICP27 and ICP4 [303-305], to prevent the apoptosis as a method to counteract cellular defense [246,248,249,306]. Our study also suggests the critical role of ICP27 in the anti-apoptotic function of HSV-1. First, we found ICP27 and not ICP4 was downregulated in nonpermissive microglia/macrophages (Figure 4.8), and those infected microglia/macrophages died after the infection without producing viral progeny (Figure 4.5). On the other hand, ICP27 is highly expressed in HSV-1 permissive tumour cells and downregulated ICP27 expression in NF-treated tumour cells is also associated with increased apoptosis (Figure 3.3). Again, no change in the level of ICP4 expression was observed in the NF-treated cells. Thus, it seems that the anti-apoptotic mechanism of HSV-1 requires ICP27 in both permissive and nonpermissive cells.

The above findings demonstrated that whether ICP27 is expressed or not, it is determined by the host cells. In tumour cells, ICP27 is highly expressed, and HSV-1 replication is permitted. In microglia/macrophages, where the HSV-1 replication is not

permitted, the ICP27 is silenced although expression of ICP4, another essential viral transcriptional factor is still expressed normally. Moreover, we found that the activity STAT1/3 may determine ICP27 expression in a cell-type specific fashion. In HSV-1 permissive tumour cells, inhibiting phosphorylation of STAT1/3 suppresses the ICP27 expression that facilities apoptosis of the infected cells. In microglia/macrophages, on the other hand, inhibiting phosphorylation of STAT1/3 resulted in the expression of the ICP27 and turned nonpermissive cells into permissive for HSV-1 replication.

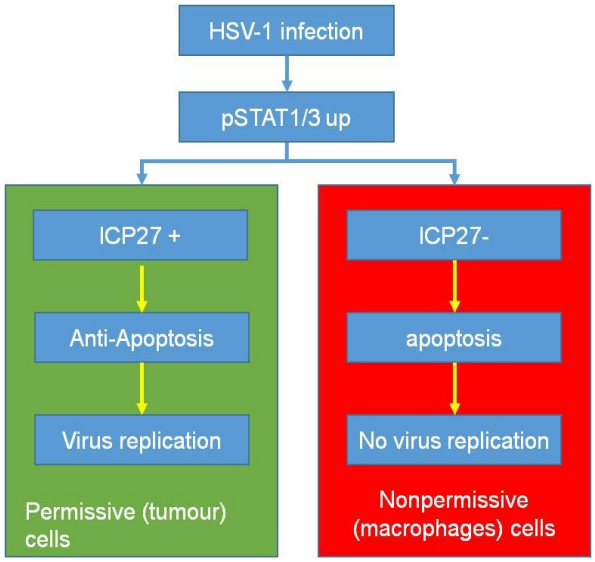

Figure 5.1 Schematic diagram of apoptosis regulation in oHSV-1 permissive and nonpermissive cells.

IFN signaling cascade is primarily regulated by the signal transducer and activator of transcription (STAT) [283]. The STAT family consist of STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 [224]. Among them, STAT1, STAT2, STAT4, and STAT6 have immunoregulatory functions [224]. Our demonstration of significantly enhanced

STAT1/3 activity following oHSV-1 infection in both microglia and glioma cells might be a common consequence of host-virus interactions. STAT1 plays a key role in cellular defense against virus infection [282,283], possibly explaining the increased activation of STAT1 after the oHSV-1 infection. On the other hand, STAT3 has received keen interest in anti-cancer research due to its anti-apoptotic function [224]. Since pSTAT3 has been reported to deactivate innate immune cells [307,308] and plays a major role to prevent apoptosis, enhanced activation of STAT3 observed in the virus-infected cells might be a part of the anti-apoptosis strategy by HSV-1 to block host defense mechanism. Moreover, a recent study demonstrated that oHSV-1 replication is positively correlated with STAT3 activity [261], which supports the notion that activation of STAT3 may facilitate HSV-1 replication. The differential effects of STAT1 and 3 in HSV-1 replication as well as the cell type dependence, remain to be studied in more details. Moreover, an STAT3-specific inhibitor “Stattic” and STAT upstream JAK1 and JAK2 inhibitor “Ruxolitinib” (data not shown) also showed synergistic enhancement with oHSV-1, which confirmed our findings. However, further studies are needed to specify the targets in the JAK-STAT signalling pathway responsible for this synergy.

Other STAT inhibitors may be investigated for their uses in improving the efficacy of oncolytic virotherapy. Also, since STAT is involved in cellular anti-viral mechanism for many viruses, STAT inhibition mediated therapeutic enhancement can be useful in other oncolytic virus settings.

5.3 Tumour microenvironment effect on oHSV-1

Since virus-mediated cell lysis [309] is one of the major mechanisms for the antitumour effects by OVs, numerous oncolytic virus tropism/replication enhancing molecules have been studied to stimulate its therapeutic efficacy [112]. As the oHSV-1 infection stimulates the activation of innate immune cells (Figure 4.2), a significant portion of these molecules are considered as immune inhibitory molecules, which suppress the anti-viral cellular response to allow better viral replication [310-314]. Our data is supported by a few

very recent studies that ablation of microglia/macrophages subsequently enhances the antitumour effect of oHSV-1 in the glioma bearing rodent model [269,274,312]. Among various cells in heterogeneous [309] clinical GBM tumour bed, microglia/macrophages are the most abundant non-tumour cells [168,315], whose number can be further increased by following the oHSV-1 infection[54]. Moreover, they not only play an important role to restrict HSV-1 [316,317], but also stimulate GBM growth and invasiveness [318-320]. Altogether, suppressing microglia/macrophages in GBM may be especially useful for depleting tumour cells by oncolytic virotherapy successfully.

However, the mechanism of microglia/macrophages and HSV-1 interaction is not completely understood. Since microglia/macrophages release a wide array of pro-inflammatory mediators, including nitrogen intermediates, cytokines, and chemokines in response to pathogen stimulation [321], one may expect that those factors released by microglia can be inhibitory to viral replication [322]. Surprisingly, we could not observe any effect of microglia conditioned medium (Figure 4.3) on oHSV-1 replication. One possible explanation is that the concentration of pro-inflammatory mediators in our condition medium may not be high enough to cause direct suppression of the virus as observed by others [312]. Since we do not know the concentrations of those factors released by microglia in vivo, we cannot rule out the possible anti-viral effect of these pro-inflammatory mediators released by the microglia in the tumour mass.

We found that the microglia phagocytosis pathway (Figure 4.4) plays a major role in clearance of oHSV-1 [323,324]. After phagosome-lysosome fusion, it is likely that lysosome constituents are inactivated and eventually degrade virus particles [324]. However, many pathogens such as *Salmonella typhimurium*, *Legionella pneumophila*, and *Mycobacterium tuberculosis* may survive inside of phagosome [325]. Double-stranded DNA acanthamoeba polyphaga mimivirus has also been reported to be internalized by macrophages and subsequently replicated inside the macrophage [326]. Previous studies indicated that HSV-1 gains entry into corneal fibroblasts [327] and ocular cells [328] through phagocytosis-like uptake, involved with plasma membrane protrusions to internalization and rearrangement of the actin cytoskeleton to traffic the virions in large phagosome-

like vesicles [327]. Our finding that microglia/ macrophage generate a physical barrier for the dissemination of oHSV-1 in glioma mass may be useful to understand the mechanism of their interaction.

Our study only investigates the oHVS-1 interaction with microglia/macrophages; further study of oHSV-1 interaction with other GBM-associated cells may shed light on the implication of GBM microenvironment for oHSV-1 antitumour efficacy. Other innate immune cells such as NK and DC also prevent HSV-1 infection [309], and it has been recently reported that TGFβ-mediated inhibition of microglia/macrophages and/or NK cells could enhance oHSV-1 replication in glioblastoma [274]. Regarding adaptive immunity, CD8+T cells play a major role in preventing HSV-1 viral spread[310], and its effect on the oHSV-1 anti tumour efficacy need to be evaluated.

In contrast to inhibiting anti-viral response, several studies were reported to make use of the virally stimulated host immune response [210]. For example, many OVs are armed with releasable factors to stimulate an immune response, two of them are in advanced clinical development stages, the HSV-1 based talimogene laherparepvec [17] and the vaccinia virus based JX-594 [329]; both of them carry a GMCSF transgene to stimulate immunity in tumour microenvironment [112]. Though it is expected that stimulation of immune response could prevent oncolytic virus dissemination in the tumour mass but, stimulated immune response might contribute to depleting tumour cells as well. On the other hand, too much pro-viral effect might cause a safety concern. So, a balance between the pro and anti-viral approaches would be necessary to enhance oncolytic viral therapeutic efficacy without compromising the safety (Figure 5.2).

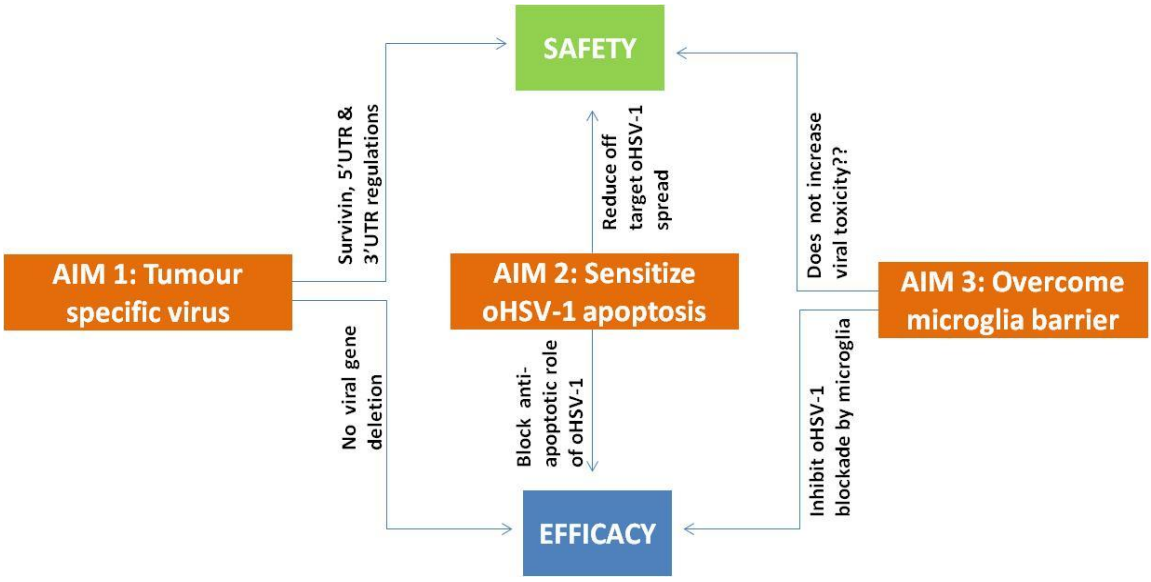

Figure 5.2 Balanced approach of the present study to enhance oHSV-1 efficacy without compromising the safety.

5.4 Conclusions

Even though a herpes-based oncolytic virus is currently available in the USA, pharmacies as a prescription drug to treat melanoma [16,17], its efficacy needs to be greatly improved to successfully treat other types of solid and metastatic tumours in clinics. To this end, this project successfully demonstrated possible ways to overcome the barriers of oncolytic virotherapy. Our data demonstrated the use of a novel transcriptional and translational triple regulated oncolytic amplicon oHSV-1 system without deleting any viral gene that not only increases tumour specificity but also significantly induces antitumour efficacy to eradicate glioblastoma successfully. To further enhance the antitumour property, we identified that a prescription antibiotics, nifuroxazide sensitizes oHSV-1 anti-proliferation effect by overcoming virus-mediated anti-apoptotic effect. Finally, we showed that an oxindole/imidazole derivative, C16 rescues oHSV-1 from microglia/macrophages mediated physical barrier in glioblastoma

by blocking the STAT activity of those nonpermissive immune cells. Eventually, this project provides a guideline for improving the therapeutic efficacy of oncolytic herpes virotherapy to make it more therapeutically effective in clinics without compromising the safety.

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