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Oncolytic HSV1716 in combination with targeted anti cancer agents: identification of synergistic interactions and their mechanisms of synergy

Submitted in fulfilment of the requirements for the degree of PhD College of Medical, Veterinary and Life Science
University of Glasgow

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Unless otherwise specified, all the results reported were obtained by the author's own efforts.

Abstract

Oncolytic viruses are multifunctional cancer agents with huge clinical potential, and recently the first Herpes Simplex Virus (HSV) oncolytic virus has been approved as a licensed cancer treatment. Increasingly, it is becoming apparent that no one cancer treatment is likely to be a 'golden bullet' – a treatment that, on its own is enough to cure all cancers. The answer seems to lie in combination therapies; by combining more than one type of treatment the chances of success, in terms of patient survival, increase.

The aim of the project was to investigate the potential of HSV1716 in combination with other anti-cancer agents. As there is a vast array of current and potential cancer therapies, a high throughput screen using a range of cancer cell lines spanning a number of indications currently of clinical interest to Virttu Biologics was set up. This exploratory screen revealed a number of interesting results – synergies between HSV1716 and other drugs were seen across a number of different classes of drugs. This thesis first describes this 'fishing' exercise, then investigates the mechanism of action by which a subset of those drugs, highlighted as acting either synergistically or enhancing the amount of cell death in combination with HSV1716, are acting.

MTOR inhibitors (targeted agent), Doxorubicin (a chemotherapeutic) and two receptor tyrosine kinases, Sorafenib and Sunitinib, were identified in the screen. Subsequent analysis of these combination revealed that, despite the differences between the classes of drugs, all worked to greatly reduce viral replication, indicating that mechanisms other than viral oncolysis are killing cancer cells.

The mechanism by which these cells were dying was investigated, HSV1716 in combination with mTOR inhibitors increased levels of intrinsic, mitochondrial driven apoptosis.

Much of the observed enhanced cell killing was seen at low level of HSV1716 infection – where only 1 in 10 cells was infected with virus. It was postulated that there is also some form of secreted signal that sensitises non infected cells to apoptosis. If this is the case these cells may be sensitised to the effect of drugs – and hence the levels of cell killing would be increased relative to

the non viral sensitised cells. The experiments detailed in this thesis indicate that this is indeed the case: HSV1716 infected cells secrete a ‘death signal’ that can be exported to non-infected cells. This signal itself increases cell death in non-infected cells but may also sensitise cells to the effect of drugs.

Within the clinic, oncolytic viruses are effective agents at reducing tumour bulk by viral oncolysis and promote an anti-tumour immune response. The work presented in this thesis suggests that the virus may also induce infected cells to secret a factor that sensitises the surrounding cancer cells, generally resistant to apoptosis, to become more sensitive to apoptosis. These sensitised cells are then more susceptible to the effects of other anti-cancer agents.

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Abbreviations

Abbreviation	Full name
AIF	Apoptosis inducing factor
AKT	serine/threonine-protein kinase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
BAX	Bcl-2-associated X protein
BAK	Bcl-2 homologous antagonist/killer
BCL2	B-cell lymphoma 2
BID	BH3 interacting-domain death agonist
BIK	Bcl-2-interacting killer
BIM	Bcl-2-like protein 11
CAR	Coxsackie virus and adenovirus receptor
CDN	cyclic dinucleotide
CI	combination index
cpe	cytopathic effect
CTL	cytotoxic T cell
CXCR3	Chemokine receptor CXCR3
DAMP	danger associated molecular pattern signals
DC	Dendritic cells
DCP	Death cell protease
DDR	DNA damage and repair
DISC	death-inducing signalling complex
DMEM	Dulbecco's modified Eagle Medium
DMSO	Dimethyl sulphate
DNA	Deoxyribonucleic acid
cpe	Cytopathic effect
CEV	cell-associated enveloped virion
E	Early genes
EEV	extracellular enveloped virion
EGFR	Epidermal growth factor receptor
EM	Electron microscopy
ENT-1	Equilibrative nucleoside transporter-1
ER	Endoplasmic reticulum

ERK1/2	Originally: Extracellular signal-regulated kinases MAPK – mitogen-activated protein kinase ERK1=MAPK3 ERK2=MAPK1
ET	Electron tomography
FACS	fluorescence-activated cell sorting
FADD	Fas-associated death domain
Fas	Type-II transmembrane protein
FCS	Fetal calf serum
FKBP12	Binds the immunosuppressant molecule tacrolimus
cGAS	cyclic GMP-AMP synthase
GADD34	growth arrest and DNA damage-inducible protein
GEM	gemcitabine
GFP	Enhanced green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP-AMP	Cyclic guanosine monophosphate–adenosine monophosphate
GTP	Guanosine-5'-triphosphate
H_20	water
HCC	Human hepatocellular carcinoma
HDAC	Histone Deacetylase inhibitors
HDAP	Herpes Simplex Virus-Dependent Apoptosis
HER	Human Epidermal Growth-Factor Receptor, for example HER2, HER3, HER4
HIRED	HSV1716 infection Related Exportable Death
HMBA	hexamethylene bisacetamide
HNSCC	squamous cell carcinoma of the head and neck
hr	hour
HRK	Activator of apoptosis harakiri
Hsp	Heat shock protein
HSV	Herpes simplex virus
HVEM	herpes virus entry mediator
IAP	inhibitor of apoptosis proteins
ICD	Immunogenic cell death
ICP	Infected cell polypeptide
IE	Immediate early
IFN	Interferon
IFNAR	Interferon-α/ß receptor, consisting of subunits IFNAR1 and IFNAR2

IL	Interleukin
IMV	intracellular mature virion
IP-10	Interferon gamma-induced protein 10
IRF9	Interferon response factor
IR_L	Internal long repeat
IR_S	Internal short repeat
ISRE	IFN-stimulated response elements
i.t	Intra tumoural
i.u	Infectious units
JAK	Janus kinase, non-receptor tyrosine kinase
K bp	Kilo base pairs
L	Late genes
LAT	Latency associated transcript
LDH	Lactate dehydrogenase
MAP	Mitogen-activated protein
MAP3K	MAP kinase
MCL-1	Induced myeloid leukaemia cell differentiation protein
MDSC	myeloid derived suppressor cells
MEK	Also MAP2K = Mitogen-activated protein kinase kinase, e.g. MEK1=MAP2K1, MEK2=MAP2K2
MHC	Major histocompatibility
MIG	Monokine induced by gamma interferon
MOI	Multiplicity of infection
MPM	Malignant pleural mesothelioma
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NaCL	Sodium chloride
NaOH	Sodium hydroxide
NDV	Newcastle disease virus
NF-κB	nuclear factor-κB
NK	Natural Killer cells
NSCLC	Non-small-cell lung carcinoma
NTR	nitroreducatase
^oC	Degree centigrade
oHSV	Oncolytic herpes simplex virus
OV	Oncolytic virus
PAGE	Polyacrylamide gel electrophoresis

PAMP	pathogen-associated molecular pattern
PCNA	Proliferating cell nuclear antigen
PDK1	Phosphoinositide-dependent kinase-1
PD-L1	Programmed death-ligand 1
PI3K	Phosphatidyl-Inositol-3-kinase
PKR	Protein kinase R pathway
PNS	Peripheral nervous system
Raf	Serine/threonine specific protein kinase that are related to retroviral oncogenes
RAG2	Recombination activating gene 2
Ras	G protein
RNA	Ribonucleic acid
rpm	Revolutions per minute
RR	Ribonucleotide reductase
RT	Room temperature
SDS	Sodium dodecyl sulphate
Smac	second mitochondria-derived activator of caspase
STAT	Signal Transducer and Activator of Transcription protein
STING	Stimulator of interferon genes
S6K1	Serine/threonine kinase in the PI3 pathway
TAA	tumour associated antigens
TK	Thymidine kinase
TKI	Tyrosine kinase inhibitor
TLR	Toll like receptors
TMZ	temozolomide
TNF	Tumour necrosis factor
TR_L	Terminal repeats
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAIL	TNF-related apoptosis-inducing ligand
T-VEC	Talimogene Laherparepvec
µg	microgram
U_L	unique region – long
µM	Micro molar
U_S	unique region - short
UV	Ultraviolet
VDEPT	Virus directed enzyme prodrug therapy

VEGFR	Vascular endothelial growth factor receptors
VF-CM	virus free conditioned medium
VSV	Vesicular stomatitis virus

Chapter I- Introduction

1.1 Oncolytic virotherapy

Advances in surgery, radiotherapy and chemotherapy have improved the outlook for many cancer patients, and targeted therapies, such as kinase inhibitors and angiogenesis inhibitors, offer the potential to arrest tumour growth and extend survival. More recently, immunotherapeutics, in particular immune checkpoint inhibitors, a new class of cancer treatment that harnesses the innate powers of the immune system to fight have been approved for use in patients with a wide range of cancer indications (Teng et al., 2016, Khanna et al., 2016). These therapies may hold greater potential than current treatment approaches, and even the hope of a cure. This represent a huge step change in cancer treatment – some patients are achieving complete remission from diseases that would have previously have had an extremely poor prognosis. Unfortunately, not all patients respond to these new treatments and there still remains an urgent need for more effective therapies for primary and metastatic disease.

Many alternative cancer treatments are being investigated and one of the most promising is the use of Oncolytic Viruses (OVs). Many virus families are currently being developed as OVs, both naturally occurring and engineered viruses. To date, adenoviruses, poxviruses, HSV, Coxsackie virus, poliovirus, measles virus, Newcastle disease virus, reovirus, and others have all undergone early clinical phase clinical trials (Patel and Kratzke, 2013).Table 1 describes the primary advantages and disadvantages of the most common oncolytic viruses in development both preclinically and in clinical trials.

Using viruses to treat cancer is not a new idea. For more than 100 years there have been clinical observations that cancer patients who contracted viral infections would enter periods of remission. During the 1950s and 60s there was considerable activity using wild-type viruses as anti cancer treatments but many of these trials were limited by the toxicity of the wild-type virus, for a historical perspective see (Kelly and Russell, 2007). Progress has only recently been possible as advances in virology and molecular biology have

allowed either the identification of naturally occurring viruses with intrinsic tumour selectivity or by genetically engineering oncolytic viruses.

An oncolytic virus is a virus that preferentially infects and kills cancer cells. As the infected cancer cells are destroyed by oncolysis, they release new infectious virus particles or virions to help destroy the remaining tumour. Oncolytic viruses not only to cause direct destruction of the tumour cells, but also to stimulate host anti-tumour immune responses.

Of all the oncolytic viruses currently being studied, oncolytic herpes simplex viruses (oHSV) are the only ones that have successfully completed clinical trials and become an approved, licensed treatment for cancer in the US and Europe. Herpes viruses have a number of features that lend themselves to success, both now and in the future as improved oncolytic agents. These features are summarised in Table 2.

OHSV replicates and kills cancer cells by lysis, releasing multiples of the input doses into surrounding cancer cells. Lytic cell death is immunogenic (Takasu et al., 2016), with the tumour specific infection promoting an anti tumour immune response. Furthermore oHSV can be armed with additional transgenes, either protein that enhance cell killing or enhance an anti tumour immune response.

Table 3 lists the principal oHSV in current clinical development. Oncolytic HSV have demonstrated excellent safety profiles and, in numerous studies, signals of efficacy. In 2015 Talimogene Laherparepvec (T-VEC), also known as IMMYLGIC became the first oncolytic virus to be licensed by the FDA as a cancer therapeutic (Andtbacka et al., 2015).

Table 1: Viruses that are being studied as potential anti cancer agents (oncolytic viruses).

Oncolytic Virus	Primary advantages	Primary disadvantages
Adenovirus	Possible to be produced at high titre Possibility of adding DNA transgenes	Dependent on receptor chimeric antigen receptor (CAR) expression dependent of loss of tumour protein 53 (*TP53)* Potential for significant local tissue inflammation /immune Rx
Coxsackie	Naturally preference for tumour cells	Infection depends on the presence of specific receptor molecules
HSV-1	M=Multimodal mechanism of action High yields and low viral antigen load Possibility of adding DNA transgenes Broad biodistribution of receptors Foes not integrate into the host genome Antiviral agents - acyclovir/gancyclovir	Potential for virus to return to a latent state in the peripheral nervous system (PNS) and therefore not enter lytic replication cycle
Maraba	High potency Strong anti tumour	Not well studied
Measles virus	Oncolytic	Pathogenic Narrow tropism
Myxoma	Non pathogenic to humans	Replicates only in cells with activated STAT1
Newcastle disease virus	Non-pathogenic in humans Moderate efficiency No permanent infection in host Oncolytic High potency	Unclear mechanism Not well studied Non-recombinant viruses used Transgene reduces viral replication
Parvovirus	Strongly oncolytic	Small – unable to insert transgenes
Polio virus	Oncolytic	Narrow tropism, Pathogenic, Difficult manipulation

Oncolytic Virus	Primary advantages	Primary disadvantages
Respiratory enteric orphan virus (Reovirus)	Mild pathogen Unable to infect normal cells Specific oncolytic activity	Previous antigens exist Infects only cells with activated Ras
Vesicular Stomatitis Virus	Relatively non-pathogenic Oncolytic	Difficult to manipulate Requires interferon-resistant cells
Vaccinia virus	High transduction efficiency Systemic dissemination - Resistant to clearance Possibility of adding DNA transgenes Long history of human use Antiviral agents - vaccinia Ig or cidofovir	Activated Ras dependent Different forms of the virus may affect production Immune response /adverse reactions to vaccination (1:50000)

Table 2: Features of Herpes Simplex Virus that lend it to being a potent oncolytic virus.

Feature	Advantage
Replicates only within tumour cells to generate multiples of the input dose	Infection results in cytolysis of tumour cells and propagation beyond the cancer cells infected initially. Self-limiting –the virus only replicates within cancer cells leaving normal cells unaffected.
Unique lytic mechanism of action	Decreases risk of resistance developing to oHSV therapy and of cross-resistance to other cancer therapies
Immunogenic cell death and tumour-specific infection promotes anti-tumour immune response	Lysis is an immunogenic form of cell death (ICD). This ICD stimulates an immune response to both virus and tumour. OVs are therefore important cancer immunotherapeutics
Emerging evidence of safety and synergy with other anti-cancer treatment modalities	OV may work synergistically with other forms of anti cancer treatments
Can be armed to enhance tumour-specific immunological reactions	OVs can be engineered to carry therapeutic or immuno-stimulatory genes. For example,, by arming viruses with immunomodulatory genes such as IL12(Toda et al., 1998, Varghese et al., 2006, Parker et al., 2005), IL2(Carew et al., 2001) , soluble B7.1- Ig (Todo et al., 2001) or Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) (Hu et al., 2006, Malhotra et al., 2007, Kaufman and Bines, 2010, Kaufman et al., 2010) to help promote the antitumor immune response the modified viruses are more efficacious.
Replication/lysis of cancer stem cells	Oncolytic viruses have been shown to replicate within and destroy cancer stem cells (Li et al., 2012)
Can be engineered to express additional transgenes that enhance tumour cell killing	Virus directed enzyme prodrug therapy (VDEPT) systems have also been utilised with oncolytic HSV. For example, HSV1yCD – a modified HSV coding for the yeast cytosine deaminase (CD) enzyme, which converts the non toxic 5-flurocytosine (5-FC) into 5-FU, a highly toxic chemotherapeutic agent, (Nakamura et al., 2001). rRp450 ,carrying rat cytochrome P450 (CYP2B1) which converts cyclophosphamide (CPA) into the alkylating toxin phosphoramide mustard(PM)(Chase et al., 1998) Nitroreducatase (NTR) which converts the prodrug CB1954 to an active alkylating agent (Braidwood et al., 2009). oHSV have also been armed to increase a cells sensitivity to radiation therapy (Sorensen et al., 2012)

Table 3: Oncolytic HSV's in clinical trials.

HSV strain	Genetic Modification	Phase	Indication	Status	Result	References/ Clinicaltrial.gov Reference
OncoVex GM-CSF (T-Vec) **IMMLYGIC**	Deletion in both copies of ICP34.5 + ICP47 disruption US11 expressed as an immediate early gene Encodes GM-CSF	I I/II II III	Solid Tumours SCCHN Melanoma Melanoma	Now approved and licences for the treatment of melanoma. Current trials ongoing in melanoma in combination with Keytruda	Evidence of virus replication in injected and adjacent uninjected tumours (head and neck). Regression of injected and uninjected tumors in late stage melanoma	NCT02658812 NCT02819843 (Liu et al., 2003), (Hu et al., 2006) (Harrington et al., 2010) (Sheridan, 2013, Andtbacka et al., 2015)
R7020 (NV1020)	Deletion of 1 copy of ICP34.5 + tk under ICP4 promoter control + deletion in UL24, 55 and 56.	I II	Colorectal cancer liver metastases	Completed	In phase II disease, stabilisation in 40-45% cases	(Kemeny et al., 2006) (Kelly et al., 2008) (Geevarghese et al., 2010) (Sze et al., 2012)
G207	Deletion in both copies of ICP34.5 + disruption of UL39	I/II	Recurrent brain cancer glioma, astrocytoma glioblastomas Recurrent brain tumours	Completed Ongoing	Well tolerated. Evidence of viral replication, radiographic and neuropathological signs of anti tumor activity	(Yazaki et al., 1995) (Mineta et al., 1995) (Hunter et al., 1999) (Todo et al., 2000), (Markert et al., 2000) (Markert et al., 2009) (Aghi and Chiocca, 2009) NCT02457845
G47Δ	Third generation HSV, ICP47 null	I/II	Glioma	ongoing		(Todo, 2012)

M032	Deletion in both copies of ICP34.5, expresses IL-12	I	Glioma	Ongoing	Safe in preclinical models	NCT02062827 (Roth et al., 2014)
HSV1716	Deletion in both copies of ICP34.5	I I/II a	Glioma Melanoma HNSCC Non-CNS solid tumours Malignant pleural mesotheliom a	Ongoing Ongoing	No toxicity. In phase I/II (recurrent glioblastomas) 3 of 12 patients showed disease stabilization. No toxicity in melanoma or HNSCC	NCT01721018 NCT00931931 (Harrow et al., 2004, Papanastassio u et al., 2002, Rampling et al., 2000, McKie et al., 1996), (Mace et al., 2007)
HF10	Spontaneou s generation of HSV-1 variant	I	Pancreatic cancer Recurrent breast cancer Bladder cancer HNSCC	Ongoing in solid tumours. Active in melanoma . Complete HNSCC	No adverse events and possible therapeutic potential	NCT02428036 NCT02272855 NCT01017185 (Nakao et al., 2011)
rQNestin -34.5	Expresses ICP34.5 under a synthetic Nestin promoter	I	Malignant Glioma	Ongoing		(Ning and Wakimoto., 2014)

References are given but in many cases open trials will not have published data. In these cases the clinical trial identifier (from cliniicaltrials.gov) is given.

1.2 Oncolytic Herpes Simplex viruses

Oncolytic herpes viruses (oHSVs) are attenuated, replication competent herpes simplex type 1 viruses that selectively infect, replicate within and lyse cancer cells. Among the promising oncolytic HSV-1 mutants is HSV1716.

HSV1716, like most oncolytic viruses, directly kills host tumour cells. This oncolytic activity is influenced by a number of factors including efficiency of cell receptor targeting, viral replication and host cell antiviral response elements, as well as the susceptibility of the cancer cells to the different forms of cell death (apoptosis, necrosis, pyrotosis and autophagy).

HSV1716 (SEPREHVIR®) is a herpes simplex oncolytic virus and lead product from Virttu Biologics, a University of Glasgow spin out company. HSV1716 is a deletion mutant of herpes simplex virus type 1 (HSV-1), strain 17+. The deletion removes the RL1 gene encoding infected cell protein 34.5 (ICP34.5), a specific neurovirulence determinant (MacLean et al., 1991, Valyi-Nagy et al., 1994). The deletion is shown figuratively in Figure1B. HSV1716 has been studied extensively over the last 25 years and is the subject of numerous scientific publications. HSV1716 forms the body of the work described in this thesis and as such OV's from other virus families will not be generally discussed.

To date (August 2016), 100 cancer patients have been treated with HSV1716 in clinical studies. The first of these clinical studies of HSV1716 involved a single intratumoural (i.t) injection of virus at doses of 10^3 to 10^5 infectious units (i. u) 9 patients with primary or recurrent glioblastome multiforme (GBM) were treated: 3 at 10^3 i.u., 3 at 10^4 i.u, and 3 at 10^5 i.u. No adverse clinical symptoms attributable to HSV1716 were identified (Rampling et al., 2000). No induction of encephalitis or any re-activation of latent wild type HSV was observed. Although patients in this study were immuno-compromised as a result of previous anti-tumour therapy and corticosteroid treatment, there was no evidence of replication of HSV1716 within normal brain. Buccal swabs showed no evidence of HSV shedding. Biopsy material was obtained from 3 of the patients (at 3.5 weeks, 2 months and 3 months). No HSV antigen was detected in the samples and no HSV DNA was detected by PCR. Post mortem material obtained from two patients who died 2 and 6 months after

HSV1716 injection showed no evidence of encephalitis but in each case the site of the injected virus could be identified as a cyst. One patient was still alive at 182 months following HSV1716 injection (as of Aug 2013 unpublished, Virttu Biologics). This study was extended with the recruitment of an additional 12 patients; 3 received a single dose of 10^5 i.u and 9 received a single dose of 10^6 i.u. No toxicity was observed in any patient. One patient from this subgroup was still alive at 45 months after HSV1716 injection (as of Dec-2006, unpublished, Virttu Biologics).

A second clinical study of HSV1716 assessed the potential for efficacy in GBM patients. 12 patients with biopsy-verified primary or recurrent malignant glioma received a single i.t injection of 10^5 i.u. HSV1716. 4-9 days following virus injection, tumours were resected and analysed for evidence of viral replication. Of the 12 subjects, 2 were HSV seronegative before treatment and sero-converted. In both cases, HSV1716 in excess of the input dose was recovered from tumour at the injection site (Papanastassiou et al., 2002). Given the low chance that the bulk of the input virus could be retrieved during sampling, this offers strong evidence that HSV1716 replicates in malignant glioma.

In the third glioma study, a further 12 patients (4 recurrent GBM, 6 *de novo* GBM, 1 anaplastic astrocytoma and 1 anaplastic oligodendroglioma) were treated. HSV1716 was injected into the brain surrounding tumour immediately following tumour resection (Harrow et al., 2004). No toxicity due to HSV1716 was observed.1 patient showed remarkable clinical improvement and there was imaging evidence of reduction in residual tumor over a 22 month period despite no further medical intervention. His clinical response was of particular note given his very poor pre-operative condition, the size of his tumour and that he declined all other adjuvant treatments. Another patient (newly diagnosed GBM) from this group was still alive 141 months following HSV1716 injection (as of Aug 2013, Virttu Biologics, unpublished).

Two further clinical studies of HSV1716 have been completed: a study in melanoma patients involved 5 patients with metastatic melanoma and accessible soft tissue tumour nodules. Patients received i.t injections of HSV1716 at a dose of 10^3 i.u. per injection: 2 patients received 1 injection, 2

received 2 injections, and 1 received 4. No local or systemic toxicity associated with HSV1716 was observed (MacKie et al., 2001). In 1 patient, flattening of previously palpable tumour nodules was observed 14 days after 2 direct injections of HSV1716. In virus-injected nodules in the 3 patients who received two or more injections, there was evidence of tumour necrosis with no morphological evidence of damage to surrounding tissues. Immunohistochemical staining of injected nodules demonstrated evidence of virus replication confined to tumour cells.

The second of these additional studies involved 20 patients with resectable squamous cell carcinoma of the head and neck (HNSCC). Patients received a single preoperative i.t injection (either at 1, 3 or 14 days prior to surgery) with HSV1716 at a dose of $1x10^5$ i.u. (5 patients) or $5x10^5$ i.u. (15 patients). No toxicity was experienced by any of the patients and evidence of virus in tumour tissue was observed (Mace et al., 2008).

There are 2 ongoing clinical studies of HSV1716: a phase I/IIa study sponsored and funded by Virttu Biologics (in UK) to assesses the safety, tolerability and biological effect of single and repeat intrapleural administration of HSV1716 in patients with inoperable malignant pleural mesothelioma. To date 12 patients have been treated, 3 with a single dose of $1x10^7$ i.u. HSV1716 as a loco-regional injection into the pleural cavity via an indwelling pleural catheter, 3 have received 2 doses and 6 patients have received 4 doses. Results for this study are as yet unpublished.

The second ongoing study is phase I dose escalation study in paediatric/young adult patients with refractory and actively progressing non-CNS solid tumors (in USA). To date 3 patients have received a i.t dose of $1x10^5$ i.u. of HSV1716; 2 patients have received a single i.t dose of $2x10^6$ i.u; 1 patient has received 2 dose via i.t administration of $2x10^6$ i.u. and 2 patients have received a single i.t administration of $1x10^7$ i.u. There have been no dose limiting toxicities with HSV1716 being well tolerated with minimal side effects. The protocol has been expanded to include an intravenous (IV) administration with 4 IV patients have received a single systemic administration of $2x10^6$ i.u. HSV1716. Again, as this study is ongoing, results are as yet unpublished.

1.3 HSV infection and host cell defence mechanism against infection

HSV-1 virion and genome.

HSV1716 is a deletion mutant of HSV-1 (strain 17+), a human neurotropic virus. The morphological structure of the infectious virus particle, the virion, is characterised by a central icosahedral capsid, containing the core dsDNA genome. The capsid is surrounded by the tegument, which is in turn surrounded by a protein-containing lipid bilayer, the envelope. The tegument is an electron dense material composed of at least 20 distinct viral proteins (Kelly et al., 2009) while the envelope is composed primarily of lipids derived from the host cell membrane, into which are inserted HSV glycoproteins. Membrane glycoproteins mediate HSV-1 entry into the cell, cell to cell spread, cell fusion and immune evasion.

The HSV-1 genome is a linear double stranded DNA duplex, 152 kb in length as shown illustratively in Figure 1A. There are two unique regions, long and short (termed U_L & U_S) which are linked in either orientation by internal repeat sequences (IR_L & IR_S). At the non linker end of the unique regions are terminal repeats (TR_L & TR_S). Most of the known genes are located in the long or short regions, and they are named according to their location within L or S. Three main classes of HSV-1 genes have been identified, namely the immediate – early (IE), early (E) genes or late (L) genes as described in Figure 1.

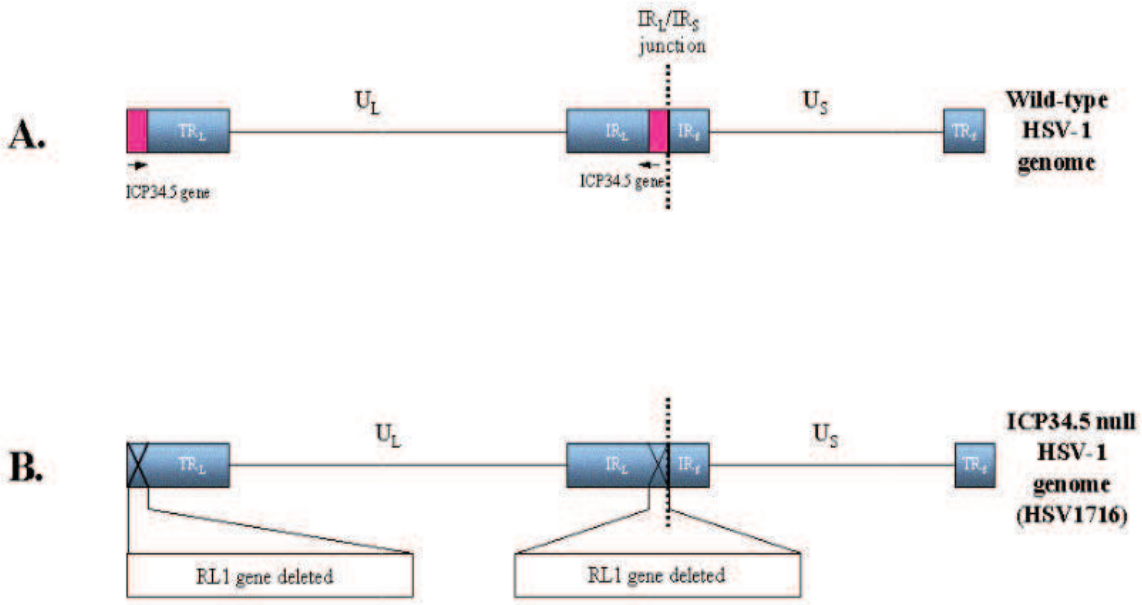


Figure1: (A) Wild type HSV 1genome and (B) illustrating the deletion in HSV1716

HSV viral entry into host cells

HSV1716 uses the same cellular receptors as wild type HSV-1 to initiate infection of cells. Entry of HSV-1 into the host cell involves interactions of several viral glycoproteins, namely gB, gD and the heterodimer comprising gH and gL (Campadelli-Fiume and Menotti, 2007). These glycoproteins, on the surface of the enveloped virus interact with receptors on the surface of the host cell.

Initial contact is between viral gB and cellular heparan sulphate. gD then interacts specifically with the cellular receptors for HSV-1 entry which include herpes virus entry mediator (HVEM), nectin-1, and 3-O-sulphated heparan sulphate. Membrane fusion requires the concerted activities of gB and gH/gL so that the nucleocapsid gains access to the cell and infection is initiated. Nectin-1 is the main entry receptor for infection of central and peripheral nerve cells, whereas HVEM expression is more restricted and limited to cells of lymphoid origin (Simpson et al., 2005). HSV-1 entry mediators have a wide bioavailability and many different human tumour cell types are permissive for HSV1716 infection.

The virus penetrates the cell by fusion of the virus envelope with the plasma membrane and the viral nucleocapsid is released into the cytoplasm of the cell. Following binding of the capsid to the nuclear pore the genome is then released into the nucleus where transcription, replication of viral DNA and assembly of progeny nuclear capsid takes place.

HSV replication

The expression of HSV-1 genes occurs through a highly regulated cascade beginning with the production of the or immediate-early (IE) proteins. The α regulatory proteins, ICP 0, 4, 22, and 27, cooperatively act to regulate the expression of all classes of viral genes. The ß or early (E) gene products, such as the viral thymidine kinase (TK), are synthesized next and are the proteins principally involved in viral DNA synthesis (reviewed in Roizman & Sears, 1996). The last set of viral proteins produced are the γ or late (L) proteins and are mainly associated with virion structure and assembly, such as the VP16, gD, and gC proteins (Batterson and Roizman, 1983,Fenwick and Walker, 1978) and Read et al., 1993).

The γ gene class is further subdivided into the γ_1 and γ_2 groups, where γ_2 expression is absolutely dependent on viral DNA synthesis. The completion of the HSV-1 replication cycle leads ultimately to the destruction of the cells.

Cell antiviral response elements

In normal cells, a variety of signalling pathways operate to detect and clear viral particles. The rapid detection of viral agents is essential for the effective initiation of host defence mechanisms against infection. The antiviral defence system starts to act through viral recognition by intracellular Toll like receptors (TLR) Single stranded (ss) RNA binds TLR-7 while double stranded (ds) RNA binds TLR-3 (Kawasaki and Kawai, 2014). TLRs induce intracellular signalling that leads to the activation of interferon (IFN) regulatory factors and activation of IFNα and IFNß. Released IFNs, through STAT3, lead to the transcription of the target genes, which include PKR; the double stranded RNA-activated protein kinase. Activated PKR can phosphorylate eukaryotic Initiation Factor-α (eIF2α). Phosphorylation of eIF2α inactivates it and results in inhibition of mRNA translation initiation, shut down of protein synthesis of the host cell and therefore blocking viral replication. Wild-type HSV-1 has evolved multiple mechanisms to prevent such shut down. The key determinant responsible for preventing this translational host cell shutdown, hence allowing virulence of HSV-1, was mapped to the viral protein ICP34.5 (Chou et al., 1990). ICP34.5 binds to Protein Phosphatase 1α (PP1α) resulting in dephosphorylation of eIF-2α allowing viral replication to proceed (Figure 2)As ICP34.5 deleted viruses are unable to recruit factors required for viral replication or circumvent this PKR-mediated host defence pathway, this results in no viral replication and no spread in normal tissues (Figure 2B). HSV viruses that lack ICP34.5 protein have a 10,000-fold reduction in replication and neurovirulence in normal mice (Leib et al., 1999). ICP34.5 null viruses such as HSV1716 fail to cause disease in animals sensitive to HSV-1 infection. (Valyi-Nagy et al., 1994).

Experiments in cancer cell lines and in human xenograft animal models demonstrated that ICP34.5 mutants are destructive to tumours(Varghese and Rabkin, 2002). The efficacy of tumour lysis in animal model systems, as measured by reduction in tumour volume or survival, directly correlates with the efficiency of viral replication (Smith et al., 2006).

STING (stimulator of IFN genes) has recently been identified as a key cytosolic DNA sensor for the detection of viruses. The presence of dsDNA in the cytosol is recognised by cyclic GMP-AMP synthase (cGAS). In the presence of ATP and GTP, cGAS catalyses the production of cyclic dinucleotide (CDN). A single CDN generated by cGAS binds to molecules of STING in the endoplasmic reticulum(ER). This binding changes STING conformation and it relocates to the perinuclear region of the cell where it phosphorylates transcription factors that in turn translocate to the nucleus to initiate innate immune gene transcription. The pathway is shown in Figure 3 (Barber, 2015). Colon cancers containing mutations in the STING-cGAS pathway are highly susceptible to DNA virus based oncolytic virus therapies (Xia et al., 2016) suggesting STING has an important role in innate responses to HSV. These factors activate the JAK-STAT (Janus kinase signal transducer and activator of transcription) pathway, resulting in IFN release (Randall and Goodbourn, 2008). The surrounding cells (which are uninfected by the virus) are induced into an antiviral state to limit the replication and spread of the invading virus.

IFN mediate a wide range of innate immune responses towards the invading virus. Interferons act as secreted ligands of specific cell surface receptors, eliciting the transcription of hundreds of interferon-stimulated genes (ISGs), whose protein products have antiviral activity, as well as anti microbial, antiproliferative/anti tumour and immunomodulatory effects (Schneider et al., 2014). IFNs, and their receptors have been extensively studied, and several detailed reviews have been dedicated to IFNs and their receptors (de Weerd et al., 2007, Pestka et al., 2004, Uze et al., 2007). As a brief overview, IFNs fall into the following categories:

Type I- IFN-α/ß. Nearly every cell is capable of producing IFN-α/ß; however, during the course of a viral infection, specialized immune cells known as plasmacytoid dendritic cells produce the vast majority of IFN-α (reviewed inLiu, 2005). There are several ways in which this induction occurs, most importantly by the recognition of double stranded RNA (Randall and Goodbourn, 2008).

Type II - IFNγ is secreted only by immune cells, specifically NK cells and T lymphocytes, but nearly all cell types care capable of responding to IFNγ, The

most well-characterized function of IFN-γ is the upregulation of the MHC class I molecules to aid in the priming and presentation of antigens to antigen-presenting cells such as dendritic cells (DCs) (Seliger et al., 2008).

Type III IFNs—IFNL1, IFNL2, and IFNL3 [also known as IFN-λ1, IFN-λ2, and IFN-λ3, or interleukin (IL)-29, IL-28A, and IL-28B, respectively] were described independently by two research groups in 2003, Kotenko et al., 2003 and Sheppard et al., 2003). This receptor complex signals through a similar JAK-STAT pathway as the type I IFN receptor complex and induces many of the same ISGs (Marcello et al., 2006, Bolen et al., 2014).

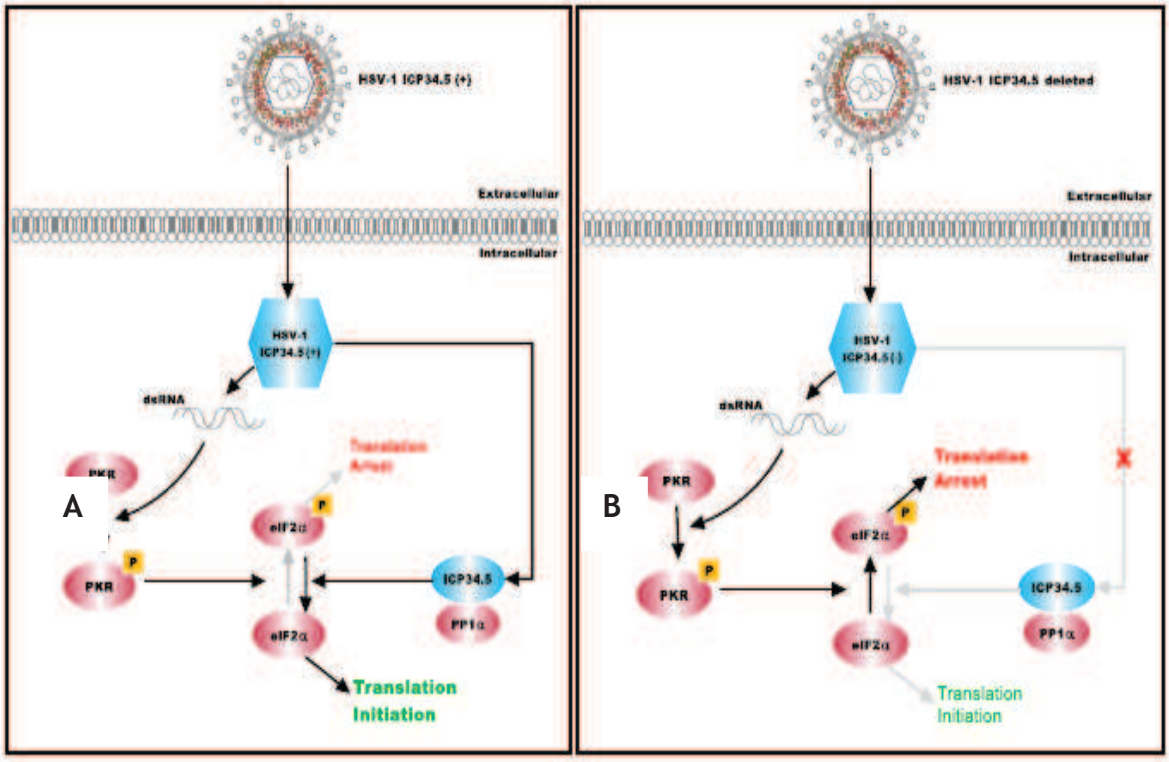


Figure 2: Wild type HSV-1 replication prevents host cell protein synthesis shutdown in normal cells. (B) HSV1716 replication does not prevent host cell proteins synthesis shutdown in normal cells

(A) In non-dividing cells wild-type HSV-1 (ICP34.5 +) virus enters the cell, begins replication and viral dsRNA is produced. The presence of the dsRNA in the cytosol induces and activates PKR. The viral ICP34.5 protein can bind to Protein Phosphatase 1 leading to dephosphorylation of eIF2α resulting in protein translation and viral replication, and hence the virus can escape host defence.

(B) (B): In non-dividing cells ICP34.5 deleted viruses, such as HSV1716 are not capable of dephosphorylating eIF2a since ICP34.5 is absent, and so PKR-mediated inhibition of translation initiation blocks virus replication in non-dividing cells.

Figure used with permission of Virttu Biologics.

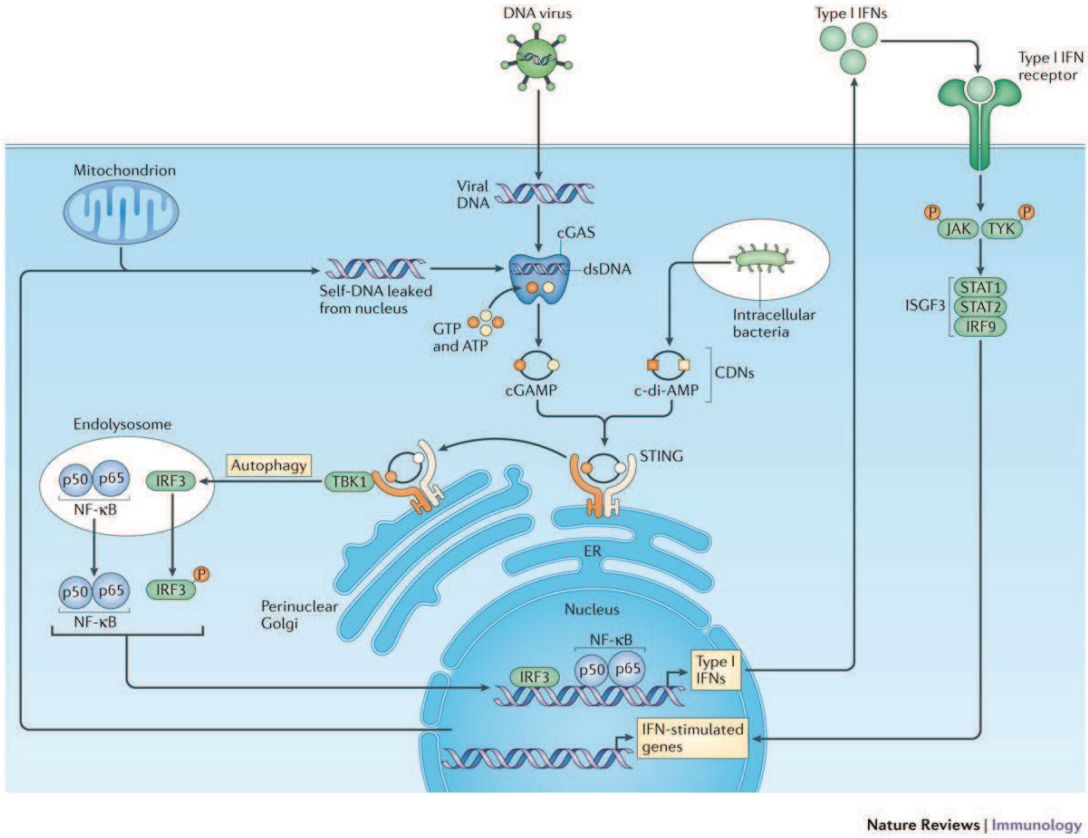


Figure 3: Initiation of innate immune system by virus infection

Stimulator of interferon genes (STING) is activated by cyclic dinucleotides (CDNs). Viral DNA in the cytosol of the host cells is recognised by cyclic GMP–AMP synthase (cGAS), which in the presence of ATP and GTP catalyses the production of the CDN cGAMP (cyclic GMP–AMP) .When cGAMP binds to STING, STING forms a complex with TANK-binding kinase 1 (TBK1). This complex traffics to the perinuclear Golgi to deliver TBK1 to endolysosomal compartments where it phosphorylates the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB). Stimulation of the IRF3 and NF-κB signalling pathways leads to the induction of cytokines and proteins, such as the type I interferons (IFNs) that exert anti-pathogen activity. c-di-AMP, cyclic di-AMP; dsDNA, double-stranded DNA; ISGF3, interferon-stimulated gene factor 3; JAK, Janus kinase; STAT, signal transducer and activator of transcription; TYK, tyrosine kinase.

Image downloaded with permission from Nature review: Immunology

MEK pathway

ICP34.5 mutants can infect cells from diverse tumour tissue types equally well, as demonstrated by the equivalent expression of early viral proteins expressed after viral entry (Smith et al., 2006). This suggests that the observed variability in viral yields across different tumour cell types might be a function of differences in overall viral protein synthesis, rather than differential infectivity. The presence of mechanisms in tumour cells that circumvent the PKR-mediated antiviral response may be essential for replication of ICP34.5 mutants. In permissive tumour cells, PKR is quiescent and viral protein synthesis proceeds uninhibited with efficient viral replication. In several studies, the differential susceptibility of various human tumour cell lines to ICP34.5 mutant infection was dependent on the activation status of the endogenous MAPK kinase (MEK) which can block the activation of PKR (Smith et al., 2006)(Figure 4).

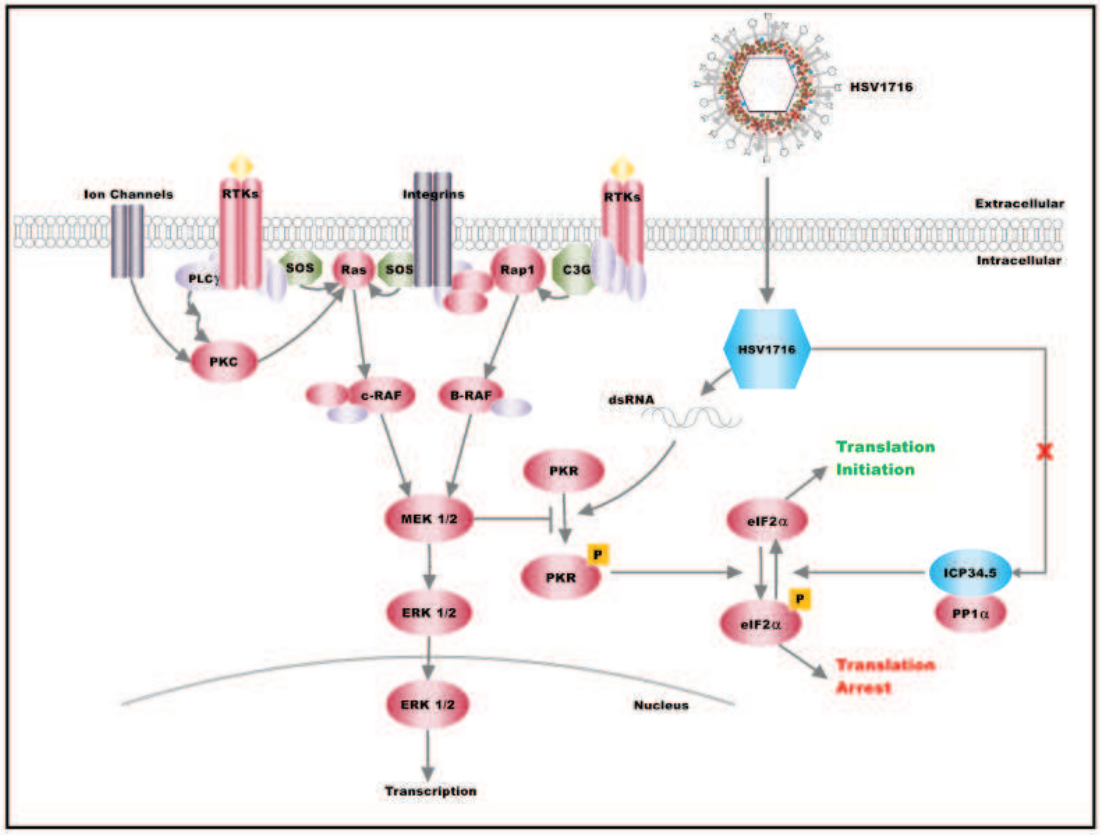


Figure 4: Constitutive MEK activation in cancer cells prevents the host protein synthesis shutdown in the presence of virus infection.

In ICP34.5 (-) mutants, the MEK activated pathway in tumour cells will block PKR activation, and in the absence of ICP34.5 protein, will result in translation initiation and viral replication.

Image is from Virttu Biologics and used with permission.

PCNA

Proliferating cell nuclear antigen (PCNA) has been identified as another molecular mechanism that contributes to the selectivity of HSV1716. PCNA plays a critical role in cellular proliferation and its tight association with cancer transformation has resulted in the frequent use of PCNA as a diagnostic and prognostic cell-cycle marker. PCNA levels are normally very low in non-cycling cells, but levels can be much higher in cycling cells, for example active tumour cells.

The HSV neurovirulence factor ICP34.5 was shown to form a complex with PCNA *in-vitro* and *in-vivo* through its 63-amino-acid carboxyl domain that is conserved in mouse MyD116, and hamster GADD34 (Brown et al., 1997, Harland et al., 2003).

Tumour expression of PCNA may be a component of oncolytic specificity and may determine its efficacy. The *in-situ* PCNA profiles, in histological sections of tumour biopsies obtained from patients undergoing craniotomy, were examined (Detta et al., 2003). Biopsies of 10 metastatic tumours were positive for PCNA expression by IHC and supported the replication of HSV1716. In tumour cells, where PCNA is already engaged in DNA replication, ICP34.5 is not required to activate the cellular replication machinery to allow viral DNA replication to commence, and so HSV1716, even though it lacks ICP34.5, can still replicate effectively. Although encouraging, correlation between the levels of PCNA and sensitivity to HSV1716 in a large cohort remains to be demonstrated.

Autophagy

Autophagy is a basic cellular maintenance mechanism involving the lysosomal degradation of dysfunctional or unnecessary proteins and organelles. The autophagy process can ensure cellular survival during starvation by providing essential cellular energy. Autophagy has an antiviral role with autophagy proteins targeting viral components or virions for lysosomal degradation as well as playing a role in initiating innate and adaptive immune responses to viral infection (Alexander et al., 2007). During wild-type HSV-1 infection, ICP34.5 interferes with autophagy by binding Beclin-1. Beclin-1 is a critical component of several highly regulated complexes that control the formation

and maturation of autophagosomes (Alexander and Leib, 2008). Tumour cells often display defects in autophagy and this is associated with increased tumourgenesis. As oncolytic HSV1716 is ICP34.5 deleted and thus cannot bind Beclin-1 to block autophagy, HSV1716 is more susceptible to autophagy-mediated inactivation in cells that have 'normal' functioning autophagy such as neurons and fibroblasts. US11, a late gene product of HSV-1 is another viral protein known to inhibit autophagy through its interaction with PKR, although it does not bind to Beclin-1 itself (Lussignol et al., 2013).

The fact that HSV-1 encodes two anti-autophagic proteins suggests that autophagy has a strong anti viral effect: however *in vitro* HSV-1 replicates as well in autophagy deficient cells as it does in wild type cells (Alexander et al., 2007). Orvedahl produced an HSV-1 mutant that had a mutation in ICP34.5 that abrogates binding to Beclin-1 and found that it was neuro-attenuated in mice, suggesting that the inhibition of autophagy by HSV-1 contributes to the neurovirulence of wild type HSV as a result of the ICP34.5 protein interacting with Beclin-1(Orvedahl et al., 2007).

Anna Claudia Lima, in a University of Strathclyde MSC project in collaboration with Virttu Biologics, investigated autophagy in a number of human cancer cell lines from the Virttu cell line panel (see M&M) during both wild-type HSV-1 and oncolytic HSV1716 infection. The autophagy response to HSV infection in the *in vitro* human cancer cell lines studied was variable and independent of ICP34.5 status (Results were presented as a poster at The 8th International Oncolytic Viruses meeting-see Appendix).

Apoptosis

Apoptosis is a form of programmed cell death that is triggered in normal development and as a response to stress stimuli. In normal cells, the initiation of apoptosis is tightly regulated by activation mechanisms, because once apoptosis has begun, it inevitably leads to the death of the cell. There are two well defined pathways for the induction of apoptosis – the intrinsic (also called the mitochondrial pathway) and the extrinsic pathway (Figure 5) shows both pathways, with the important key caspases highlighted in yellow.

In the extrinsic pathway, as the name suggests, the signal is initiated at the cell surface. Death ligands, such as TNFα or Fas ligand, bind to their death

receptors, type 1 TNF receptor (TNFR1) and a related protein called Fas (CD95), respectively. These death receptors have an intracellular death domain that recruits adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD). The binding of the death ligand to the death receptor results in the formation of death-inducing signalling complex (DISC) comprising the death receptor and its adaptor protein. The DISC formation activates a specific set of cysteinyl aspartate proteases, called caspases, but specifically caspase 8. Active caspase 8 cleaves and initiates downstream caspases 3 and 7. These caspases cleave cellular proteins which maintain the integrity of the cell, an irreversible process.

The intrinsic pathway is activated by intracellular signals. Internal stimuli such as irreparable DNA damage, hypoxia, extremely high concentrations of cytosolic Ca^{2+} and severe oxidative stress increase mitochondrial permeability and consequent release of pro-apoptotic molecules, such as cytochrome-c, into the cytoplasm. The intrinsic pathway is heavily regulated by proteins of the Bcl-2 family, named after the BCL2 gene originally identified at the chromosomal breakpoint of the translocation of chromosome 18 to 14 in follicular non-Hodgkin lymphoma. There are two main groups of Bcl-2 proteins, the pro-apoptotic proteins such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk and the anti-apoptotic proteins such Bcl-2, Bcl-X_L, Bcl-W, Bfl-1 and Mcl-1. The anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c whereas the pro-apoptotic proteins act by promoting its release. The balance between the pro- and anti-apoptotic proteins governs whether apoptosis will be initiated (Nguyen and Blaho, 2007). Other apoptotic factors can be released from the mitochondrial intermembrane space into the cytoplasm including apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP Binding protein with Low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2) (Wong, 2011). Cytoplasmic cytochrome c combines with Apaf-1 and caspase 9 to form the apoptosome whereas Smac/DIABLO or Omi/HtrA2 promotes caspase activation by releasing sequestered caspase 3 or 9 from inhibitor of apoptosis proteins (IAPs). Once caspase 3 or 9 is released

from sequestration, it becomes activated and apoptosis proceeds (Wong, 2011).

Both extrinsic and intrinsic pathways converge on the execution phase of apoptosis which involves a series of caspases. Activated caspase 9 initiates the executioner phase for the intrinsic pathway whereas caspase 8 is the central node for the extrinsic pathway. Both activate caspase 3 which then cleaves the inhibitor of the caspase-activated deoxyribonuclease, which is responsible for nuclear apoptosis. Other downstream cleavage targets are protein kinases, cytoskeletal proteins and DNA repair proteins.

The intrinsic endoplasmic reticulum (ER) pathway is less well understood and involves caspase 12 and is independent of the mitochondria. Briefly, ER injury via hypoxia, free radicals or glucose starvation, causes unfolding of proteins and reduced protein synthesis. Consequently, the adaptor protein TNF receptor associated factor 2 (TRAF2) dissociates from procaspase-12, resulting in its activation (Nakagawa et al., 2000). This apoptotic pathway may be important in OV therapy, with evidence of increased levels of ER stress, induced apoptosis when B-raf inhibitors were given in combination with a oncolytic reovirus type 3RT3D (Roulstone et al., 2015)

Apoptosis and HSV

HSV-1 infection triggers the host cell apoptotic pathway as a defence mechanism – the aim being to contain the spread and replication of the pathogen. Induction of host cell apoptosis by HSV-1 requires expression of the first class of viral genes (IE genes) (Sanfilippo and Blaho, 2006).

While productive HSV-1 replication induces major biochemical changes in infected cells, collectively referred to as cytopathic effect (CPE), the virus also triggers apoptosis in transformed or tumor cells, but not primary cells. Synthesis of virus ICPs during an apoptotic-prevention window (Aubert et al., 1999) delays the apoptotic process from killing the virally infected cells, presumably to allow productive viral replication to take place. A number of viral proteins which act to modulate apoptosis during infection have been identified. They include the immediate early proteins ICP27, ICP24 and ICP4. Deletion of any of these viral proteins results in virus that triggers apoptosis.

In addition, loss of either ICP4 or ICP27 also attenuates expression of early and late viral gene products, suggesting it also has regulatory functions (Su et al., 2016).

Other early HSV gene products including Glycoprotein D(Zhou and Roizman, 2001), US3 (Leopardi et al., 1997), R1 (Langelier et al., 2002) and latency associated transcripts (LAT) (Nguyen and Blaho, 2009) are involved in preventing apoptosis. Single deletions of either of these late viral genes do not cause apoptosis to the same extend as the ICP27 or ICP4 deleted viruses suggesting that the late viral genes may have redundant functions or act in concert to prevent apoptosis during a wild type HSV infection.

Cells infected with recombinant viruses with mutations in the anti apoptotic viral gene products die through a process called Herpes Simplex Virus-Dependent Apoptosis (HDAP). Early studies of HDAP were done *in vitro* using transformed cell lines but when studies were expanded to include non transformed lines fundamental differences were revealed (Aubert and Blaho, 2001). Primary, non transformed cells are resistant to HDAP whereas transformed, tumourigenic cells were sensitive to HDAP. Two cellular proteins, P53 and telomerase, two key oncogenes, have so far been identified as regulators of HDAP sensitivity (Nguyen et al., 2007a). As this HDAP only occurs in transformed, or cancerous cells, HSV viruses, could cause increased cell death in cancerous, but not normal cells through HDAP. HDAP is caspase dependent. Using specific caspases inhibitors, Aubert et al, 2007 showed that inhibitors of caspase 9 suppressed HDAP while caspase 8 inhibitors did not, indicating that HDAP occurs through the intrinsic pathway of apoptosis (Aubert et al., 2007).

Potentially, this difference in the ability of HSV virus to cause apoptosis in cancerous but not in normal cells could be exploited as a cancer therapy. Generally cancer cells are resistant to apoptosis, so a mechanism that specifically targets cells that are transformed is rare. HSV1716, like wild type HSV-1 retains a full complement of viral proteins that inhibit apoptosis of the host cell. In terms of production of progeny virions prevention of apoptosis by HSV1716 is positive, as it allows complete viral replication before the host cell is killed. However, HSV1716 infection could still result in cancer cell death, by

HDAP, if the production of the HSV1716 viral proteins that inhibit apoptosis was prevented. Therefore, anticancer drugs that inhibit viral replication, combined with HSV1716 could enhance cancer cell death by HDAP.

As HDAP is caspase dependent and occurs via the intrinsic pathway, one way of measuring this enhanced HDAP would be to look for increased levels of specific caspases. Caspase 3/7 levels could be measured to look for increased apoptosis, and caspase 8 or 9 could be used to differentiate between intrinsic and extrinsic pathways.

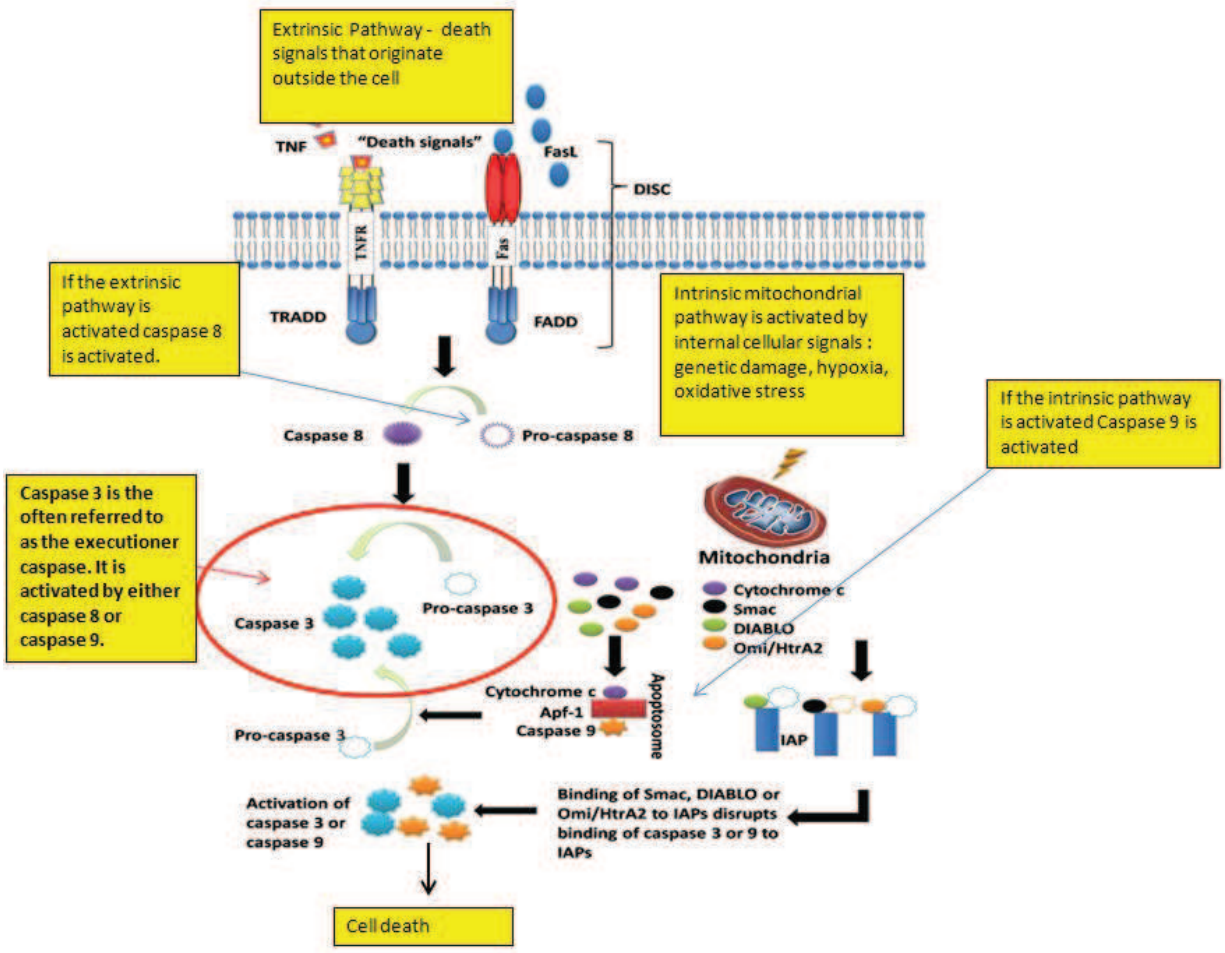


Figure 5: The extrinsic and intrinsic routes to apoptosis.

The extrinsic pathway is initiated by factors outside the cell, like death signals such as TNFα. These bind to the death receptors on the surface of the cell and form a death-induced signalling complex (DISC) which initiates the activation of pro-caspase 8 into caspase 8. This in turn cleaves the executioner caspase 3 downstream. The intrinsic mitochondrial pathway is initiated within the cell by internal stimuli.

Diagram adapted from Wong, 2011.

1.4 Induction of anti-tumour immune response

When oncolytic viruses were first described it was assumed that their primary mechanism of action was through direct oncolysis of tumour cells. Certainly within the *in vitro* setting, and within immune compromised xenograft models, the primary mode of action in terms of cell death is direct oncolysis of the cancer cell usually by a mixture of apoptosis, necrosis, pyrotosis and autophagic cell death (Bartlett et al., 2013). Evidence is accumulating, however, that although direct oncolytic effects are important, the induction of a systemic innate and tumour-specific adaptive immune response is critical for tumour eradication. The initial oncolysis causes an immunogenic cell death (ICD) that can activate innate and tumour-specific immune cells (Melcher et al., 2011),(Prestwich et al., 2008) generating an anti-tumour immunity vaccination effect to eliminate the uninfected cancer cells in primary and metastatic nodules (Bartlett et al., 2013).

ICD is defined as a type of cell death that engages the adaptive arm of the immune system. The ICD induced by OVs provide danger signals and a natural repertoire of tumour associated antigens (TAA) to DCs that triggers an adaptive immunity (Matzinger, 2002). These danger signals include Damage associated molecular pattern (DAMPs) and pathogen associated molecular pattern (PAMP) molecules derived from the OVs. PAMPs were first described in the late 1980s by Charles Janeway as a way that the immune system protects itself from infectious agents such as viruses(Janeway, 1989). They consist of essential components of the invading pathogen, for example nucleic acids (DNA, dsRNA, ssRNA), proteins and components of the cell surface and membrane that can be recognised by the host as 'non self'(Tang et al., 2012, Kono and Rock, 2008). DAMPs are molecules derived from normal host cells. They can be proteins, DNA, RNA or metabolic products. Among the protein DAMPS are high mobility group box 1 proteins (HMGB1), heat shock proteins (HSPs) and proteins in the inter-cellular matrix such as hyaluronan fragments that are generated following cellular injury (Krysko et al., 2012). Both PAMPs and DAMPs stimulate the innate immune system through pattern recognition receptors including the Toll like receptors (TLRs) and retinoic acid inducible gene 1 (RIG-1) like receptors. DCs express a wide repertoire of these PRRs –it is the binding of PAMPs and DAMPs to these PRRs on the antigen presenting

cells (APC) that promote the maturation of antigen presenting cells such as DCs. They, in turn, activate CD4+ and CD8 + T cell responses. Once activated CD8+ T cells expand into cytotoxic effector T cells. The T cells mediate anti-tumour immunity upon antigen recognition (Figure 6).

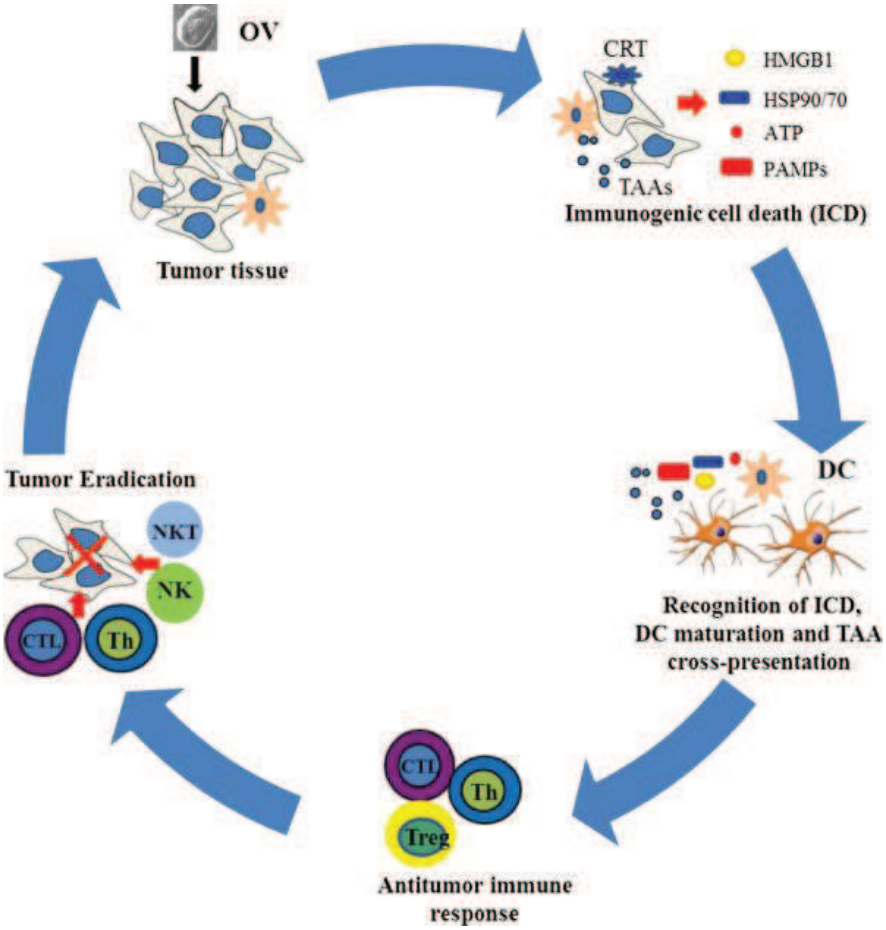


Figure 6: ICD of cancer cells induced by OVs leads to anti-tumour immunity.

An OV, delivered either intra-tumourally or systemically, reaches tumour tissue and selectively replicates in tumour and/or stromal cells. This leads to induction of death of these cells, presenting “eat me” DAMP and PAMP signals on the cell surface and later release of danger signals from necrotic cells. Apoptotic bodies are engulfed by APC, and TAAs are processed and presented along with MHC complex and co-stimulatory molecules. The released DAMPs (and PAMPs) activate and mature DCs and TAAs are cross-presented to naive T-cells. The resulting cytotoxic immune response against tumour, involving CD4+ and CD8+ T cells, may help in complete eradication of tumour mass. Additional immunotherapies targeting DCs, T cells, and the immunosuppressive TME can further enhance this antitumor immune response. Figure fromBartlett et al., 2013.

Evidence that HSV1716 induces an anti-tumour immune response

The role of the immune response during HSV1716-mediated tumour destruction has been studied in a syngeneic murine intracranial melanoma model (Miller and Fraser, 2000). The authors reported a significant prolongation in survival in the HSV1716 group compared with mock-treated mice. Additionally, 60% of the animals treated with HSV1716 had complete regression of their tumours. When SCID mice were tested rather than immunocompetent animals, no difference was observed in the mean survival rates between HSV1716 and mock treated groups. Similarly, when cyclophosphamide was used to deplete leukocytes in the syngeneic model before and during HSV1716 administration there was no significant difference observed in the survival times of the mock vs. HSV1716 treated mice (Miller and Fraser, 2000). The immune cell infiltration into the tumour after viral administration (when little or no immune cells were present) was also examined –CD4+ T cells and macrophages were the main early infiltrating cells, but polymorphonuclear leukocytes (PMN), CD8+ T cells, B cells, NK cells, and microglia cells were also present (Miller and Fraser, 2000). Significant Natural Killer cell (NK) infiltration was seen on day 7, with significant CD4+ T cells again present on day 12. HSV-1 antigen staining was found throughout the tumour mass. MHC class I expression was down-regulated 3 days after viral therapy in treated mice when compared with mock-treated mice, in accordance with previous reports on the ability of HSV-1 to down-regulate MHC class I expression through ICP47 (Jugovic et al., 1998).

The down-regulation of MHC class I expression also corresponds with the concurrent shift from CD4+ and CD8+ T cells to NK cell and PMN infiltration. This correlates with the proposed escape from CTL recognition of tumours and the importance of NK cells in tumour clearance (Ockert et al., 1999).

As the natural hosts for HSV-1 are humans many rodent tumour cell lines are resistant to HSV-1 infection. Miller and Fraser, 2003 stably transfected murine cell lines and used these HSV1716 replication-competent cell lines to form tumours in syngeneic C57/BL6 mice, and 4 strains of knockout mice (RAG2 -/-, CD4 -/-, CD8 -/-, and NK-/-). Only immunocompetent C57/BL6 mice showed an increase in survival when treated with HSV1716, suggesting that all

components of the immune system are necessary to mediate the prolongation in survival seen following HSV1716 therapy (Miller and Fraser, 2003).

HSV1716 therapy has also been shown to reduce the growth of primary tumours and increase survival time in the highly malignant 4T1 mouse mammary carcinoma model. Coincident with this increase in survival was a reduction in metastases in the lungs. HSV1716 therapy of the primary tumour was also able to reduce the establishment of a second challenge of 4T1 tumours. Immunohistochemical analyses showed that as early as day 12 post-injection of tumour cells, inflammatory cells, such as neutrophils, could be detected throughout the mass of HSV1716-treated tumours. CD4+ and CD8+ T cells could also be detected throughout the HSV1716-treated tumours at higher levels than in mock-treated tumours. HSV1716 therapy did not reduce the growth of 4T1 tumours in SCID mice, suggesting a role for the T cell infiltrates (Thomas and Fraser, 2003).

A vaccination effect has also been demonstrated by intra-tumoural administration of HSV1716 in a murine model of ovarian cancer, where previously HSV1716 showed a significant reduction of tumour growth and a survival advantage. Upon HSV1716 infection, mouse ovarian tumour cells showed high levels of expression of gB and gD and were readily phagocytosed by dendritic cells (DCs). The increased phagocytosis of tumour-infected cells by DCs was impaired by heparin, and anti-HSV g B and gD, suggesting that viral infection enhances adhesive interactions between DCs and tumour apoptotic bodies (Benencia et al., 2008).

1.5 Hallmarks of Cancer

In parallel with an increased understanding that oncolytic virotherapy is much more complex than the simple idea of a lytic virus infecting and killing a tumour cell, understanding of the complexity of tumour biology has taken great strides. Tumours, once thought of as a mass of homogenous replicating cells, are becoming recognised as complex ecosystems with a range of cancerous and non cancerous cells, all with roles in allowing the tumour to grow and evade the immune system. In addition, no two tumours are likely to be the same, even tumours within the same patient. Even different areas within the same tumour are likely to have different microenvironments.

There are, however a number of commonalities that all tumours share. These are often referred to as the 'hallmarks of cancer' that distinguish a tumour cell from its non malignant counterpart (Hanahan and Weinberg, 2011) and are detailed below. All cancers must:

- Resist cell death
- Increase genome instability and mutation
- Evade growth suppressor signals / sustain growth signals
- Evade immune detection
- Enable replicative immortality
- Reprogramme energy metabolism
- Induce angiogenesis

It is increasingly important to understand that cancer progression is not so much a signalling pathway as it is a signalling web. The normal cellular processes involve signalling pathways that cross-talk with each other: the components of one pathway can regulate another. The 'hallmarks of cancer' described here are not isolated processes; each mutation or driver feeds the others, amplifying their effects and driving cells towards the uncontrolled growth that results in cancer.

Resisting cell death- One of the hallmarks of cancer is the ability of malignant cells to evade apoptosis. Consequently, cancer cells tend to survive.

The process of apoptosis is complex and described in relation to HSV-1 infection above. There are a number of ways in which a cell can become resistant to apoptosis but they can be broadly divided into the following categories which are shown figuratively in Figure 7.

Defects/mutations in p53: P53 induces apoptosis by up regulating pro apoptotic proteins in response to substantial levels of DNA breaks and other chromosomal abnormalities reviewed in (Speidel, 2015). Loss of the p53 protein is the most common way of limiting or circumventing apoptosis, and the p53 pathway is defective in >50% of human cancers (Kunisaki et al., 2006)

Disrupting the balance of pro and anti – apoptotic proteins*:* Many proteins exert anti and pro apoptotic activities within the cell. It is not the absolute quantity that is important but rather the balance of the pro and anti apoptotic members of the BCL-2 family. BCl-2 along with Bcl-x_l, Bcl-w, MCL-1 and A1 are inhibitors of apoptosis, while BAX and BAK and pro apoptotic proteins (Letai, 2008).

Reduced caspase function: Caspases are central to all routes of apoptotic death; they can function as both initiators and executioners. Low levels of caspase function lead to a decrease in apoptosis and carcinogenesis. Shen et al., 2010 found that dowregulation of caspase 9 was a frequent event in patients with late stage colorectal cancer and correlated with poor clinical outcome. Caspase 3 has also been shown to be down regulated or lost in a significant proportion of breast cancers (Devarajan et al., 2002).

Impairs death signalling: Down-regulation of death receptors or impairment of their function will contribute to reduced level of signalling, hence reduced apoptosis (Fulda, 2010).

Increased expression of inhibitors of apoptosis proteins (IAPs): IAPs, as their name suggests, inhibit apoptosis. There are, to date, 8 IAPs identified all of which inhibit caspase activity by binding their conserved domains to the active sites of caspases, either promoting the degradation of caspases or keeping them from their substrates (Wei et al., 2008), and their dysregulation has been reported in many cancers (Krepela et al., 2009)

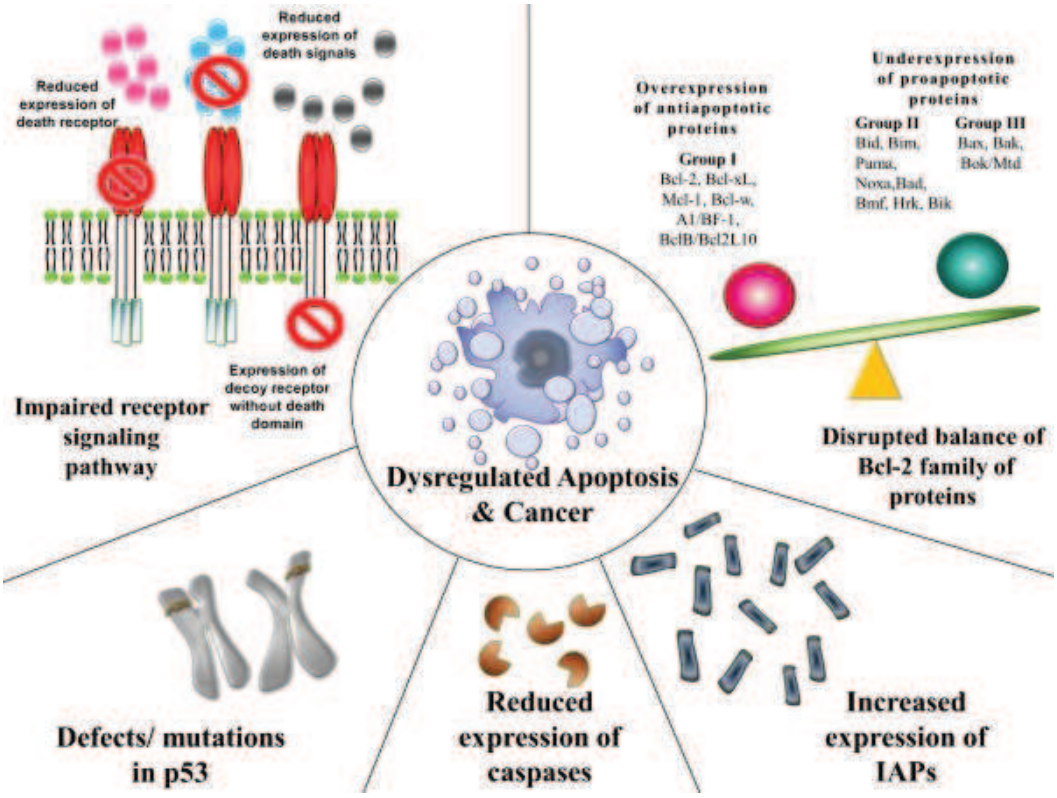


Figure 7: Deregulation of apoptosis in cancer cells

Cancer cells acquired resistance to apoptosis in a number of ways. (used with permission (Wong, 2011).

Evading growth suppressor signals / sustaining growth signals: Cell signalling is a complex system that governs basic activities such as cellular growth and division. When the ability of cells to perceive and correctly respond to their microenvironment is altered, then cells can continue to grow and divide in an uncontrolled manner, leading to malignancy. Cell proliferation in normal cells is a tightly controlled process wherein the pro- and antiproliferation signals coordinate their activities at the cell-cycle level. Growth in normal cells is blocked generally by inducing the cell to enter G_0 phase, blockage at the G_1/S phase boundary, or terminal differentiation of a cell. Cancer cells circumvent these normal growth suppressors in order to keep proliferating (Lehrmann et al., 2002).

Just as it is important for cancer cells to avoid growth suppressors, it is equally important for them to sustain proliferative signalling. These enabling signals are conveyed by growth factors that bind cell surface receptors, typically intracellular tyrosine kinase domains. These tyrosine kinases emit signals via a branched network of pathways affecting growth, proliferation, migration and angiogenesis. These include PI3K-Akt-mTOR and MAPK pathways which are often mutated in cancer cells to support unchecked cellular replication. 40% of human melanomas contain activating mutations affecting the structure of the B-raf protein, resulting in constitutive signalling of the mitogen activated protein (MAP) kinase pathway (Davies and Samuels, 2010), similarly 80% of glioblastomas have alterations in the PI3K (phosphoinositide 3 – kinase) signal pathways, including its key Akt-mTOR signal transducers (Engelman, 2009).

Immune detection: Immune surveillance is a proactive process that prevents tumour formation by recognising and eliminating most potentially cancerous cells before they can establish tumours (Prendergast, 2008). However, some tumour cells are not detected and escape immune surveillance and continue to divide and grow. For example, cancer cells may secrete IL-10 (Marques et al., 2004), which down-regulates T cell immune recognition and reduces cytokine production and impairs infiltrating effector T cells. Tumours may also express molecules that directly inhibit cytotoxic T cells, such as CTLA-4 (Contardi et al., 2005) or PD-L1 (Weber, 2010).

Induce angiogenesis: The formation of new blood vessels is critical for sustained tumour growth and metastasis. Tumor angiogenesis is a multistep process and involves signalling input from several pro-angiogenic growth factors (Bergers and Benjamin, 2003). The moment at which a tumor begins to over express pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), is generally referred to as the 'angiogenic switch'. By delivering oxygen and nutrients and producing growth factors angiogenesis enables tumor expansion and local invasion. Furthermore, the exit of tumour cells through the new tumour vasculature into the systemic circulation results in distant metastases being formed (Hicklin and Ellis, 2005).

Energy metabolism: In order to sustain their uncontrolled proliferation cancer cells require more energy than 'normal' cells. Normal respiration, under aerobic conditions, in normal cells processes glucose as the primary energy source. Cells break down glucose to pyruvate, to eventually form ATP (adenosine triphosphate) while releasing carbon dioxide as a waste product. If there is not enough oxygen cells can switch to anaerobic respiration, where glucose is incompletely broken down and lactic acid is produced rather than carbon dioxide. Aerobic respiration is far more efficient at producing ATP: 32 ATP molecules are produced per glucose molecule compared to anaerobic respiration which only yields 2. Otto Warburg (1956) first observed over 50 years ago a characteristic of cell energy metabolism of cancer cells (termed the Warburg effect(Warburg, 1956)). Even when oxygen is not limited, cancer cells preferentially get their energy requirements by aerobic glycolysis (see Figure 8). Despite the fact this process is 16 times less efficient than normal respiration, energy can be produced much faster – cancer cells can produce ATP almost a hundred times faster than normal cells. Furthermore glycolysis produces many biosynthetic intermediate precursors that can be used as building blocks for the production of the necessary proteins, lipids and DNA required by the rapidly dividing cancerous cells (Vander Heiden et al., 2009).

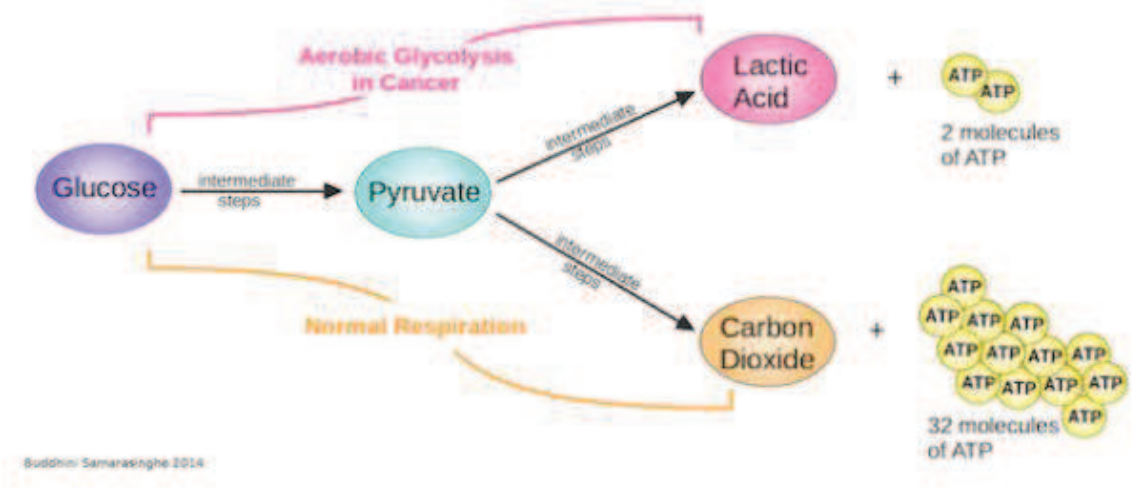


Figure 8: Cancer cells preferentially undergoing aerobic glycolysis

Cancer cells preferentially used aerobic glycolysis, despite in being far less efficient than normal aerobic respiration. Glucose gets broken down to pyruvate and the lactic acid, producing only 2 ATP molecules. In cells undergoing normal respiration (orange) glucose gets completely broken down into pyruvate, which is further processed into carbon dioxide producing 32 ATP molecules (image credit : Buddhini Samarasinghe).

Genome instability and mutation rate: Situations of genome instability are common in cancer cells, and they are considered a 'hallmark' for these cells. The unpredictable nature of these events also contributes to the heterogeneity observed among tumour cells.

Sporadic tumours (non-familial cancers) are thought to originate due to the accumulation of genetic errors (Storchova and Pellman, 2004). Within breast and colon cancers, Vogelstein et al., 2013 showed cancerous cells have 60- 70 protein altering mutations. Of these 3 or 4 are considered "driver" mutations, with the remaining mutations being acquired thereafter. These 'driver' mutations increase the mutation rate and, as a consequence, increase the acquisition of new mutations, further increasing the probability of tumourgenesis. Common mutations include those in genes responsible for maintaining genome integrity (caretaker genes), as well as in genes that are directly controlling cellular proliferation (gatekeeper genes)(Kinzler and Vogelstein, 1997).

Replicative immortality: Cancer cells require unlimited replicative potential in order to become large tumour masses. Most normal cells can only undergo a limited number of successive cell growth-and-division cycles. Cells can become terminally differentiated, an irreversible process that results in viable but non-proliferative cells. Otherwise, cells die. When primary cells are propagated *in vitro*, most cells in the population die. Rarely, a few cells will not die and continue growing. The surviving cells are termed immortalised cells, a trait that most established cell lines possess by virtue of their ability to proliferate in culture without evidence of either senescence or crisis. Telomeres, protecting the ends of chromosomes, are centrally involved in the capability for unlimited proliferation (Kipling et al., 1999, Martinez-Delgado et al., 2012). In normal, non-immortalised cells, telomeres, composed of multiple tandem hexanucleotide repeats, shorten progressively with every round of cell division and eventually the ability of these telomeres to protect the ends of chromosomal DNA from end-to-end fusions is lost resulting in loss of cell viability. Cancer cells over express telomerase, an enzyme that maintains telomere length, which protects the ends of the chromosomes, allowing cancer cells to escape replicative mortality (Artandi and DePinho, 2010).

1.6 Oncolytic viruses in combination with other anticancer drugs

Oncolytic viruses are emerging as a potential new way of treating cancers. They are selectively replication-competent viruses that propagate only in actively dividing tumour cells but not in normal cells and, as a result, destroy the tumour cells by consequence of lytic infection. Oncolytic cell killing is independent of many genomic alterations that lead to drug-resistant tumours so may be effective in drug-resistant cancers.

Intuitively, therapeutically beneficial interactions between oHSV and chemotherapeutic and targeted therapeutic drugs would be limited as the virus requires actively dividing cells for maximum replication efficiency and most anticancer agents are cytotoxic or cytostatic. However, since the initial studies by Toyoizumi et al,1999 with HSV1716 and four standard chemotherapeutic drugs, methotrexate, cisplatin, mitomycinC and doxorubicin(Toyoizumi et al., 1999), there have been many reports of the combinations of such agents displaying a range of responses, with antagonistic, additive, or synergistic enhancement of anti-tumour activity.

When synergistic interactions in cancer cell killing are observed, the clinical implications of this combination therapy are not limited to enhanced efficacy. The dose reduction index, the most relevant clinical parameter derived by Chou and Talalay analysis (Chou and Talalay, 1984), reveals the potential for significant dose reduction without compromising cell kill. Reducing the dose of drugs such as chemotherapeutics by giving them in combination with an oHSV would minimize the toxicity and may allow patients to remain on an otherwise intolerable regime, or increase their quality of life whilst still receiving treatment for their disease. Therefore, the combination of an oHSV with “standard-of-care” anti-cancer agents makes a logical and reasonable approach to improved current therapy, and merits further investigation, both preclinically and in the clinic.

Numerous publications have reported positive interactions between o HSV and anti-cancer agents, with many of these combinations shown in Table 4.

Table 4: HSV1716 in combination with wide range of chemotherapeutic agents that are currently approved and used in many cancer patients

oHSV	Drug	Cell line	Cancer type	In vitro	In vivo	Reference
HSV1716	Cisplatin	UM_SCC 14CUM-SCC 22A UM-SCC 22B	HNSCC HNSCC HNSCC	Additive Additive additive	ND ND ND	(Mace et al., 2007)
HSV1716	Cisplatin, Doxorubicin, MitomycinC, Methotrexate	NCI-H460	NSCLC	Additive	ND	(Toyoizumi et al., 1999)
NV1066	Cisplatin	H-2452, H-Meso, H-2373, H-28 JMN, Meso-9 MSTO-211H VAMT, H-2052 Meso-10	Malignant pleural mesothelioma (MPM)	Synergistic Synergistic Synergistic Synergistic Additive Additive Additive	ND ND ND ND ND ND ND	(Adusumilli et al., 2006)
G207	Cisplatin	SCC-25/CP Sq20B UMscc-38	HNSCC	No effect ND ND	ND No effect Additive to synergistic	(Chahlavi et al., 1999)
G47Δ	Cisplatin	LNCaP	Prostate cancer	Antagonistic	ND	(Passer et al., 2009)
OncoVex-GALV/CD	Cisplatin	EJ T24 TCCSUP-G KU19-9	Bladder transitional carcinoma	Antagonistic Antagonistic Antagonistic	ND ND ND	(Simpson et al., 2012)
rRp450 (CYP2B1)	Cyclophosphamide	Rh30	Alveolar rhabdomyosarcoma	ND	Enhanced	(Currier et al., 2008)
G47Δ	Doxorubicin	LNCaP	Prostate cancer	Antagonistic	ND	(Passer et al., 2009)
G207	Doxorubicin	KAT4 DRO90-1	Anaplastic thyroid cancer	Additive Additive	Enhanced ND	(Lin et al., 2008)
G47Δ G47Δ	Docetaxol Etoposide	LNCaP DU145 LNCaP	Prostate cancer Prostate cancer	Synergistic Synergistic Antagonistic	Enhanced ND ND	(Passer et al., 2009) (Passer et al., 2009)
G207	Fluorodeoxyuridin	HCT8	Colon cancer	Synergistic	ND	(Petrowsky et al., 2001)
G207	5-fluorouracil	KIGB-5 (murine) MKN45 (human)	Gallbladder Gastric cancer	Enhanced Enhanced (viral replication)	Enhanced (Syrian hamster)Enhanced (SCID mouse)	(Nakano et al., 2005)
NV1020	5-fluorouracil	HT29 WiDr HCT116 CT-26	Colon cancer Colon Colon Colon	Enhanced Enhanced Enhanced ND	ND ND ND enhanced	(Guterman et al., 2006)
OncoVex-GALV/CD	5-fluorouracil	A549, H460 CAPAN-1 MIA PACA-2, BXPC-3 HCT-116, HT-29, SW620 9L LacZ (rat)	Lung cancer Pancreatic cancer Colon cancer Gliosarcoma	Enhanced Enhanced Enhanced ND	ND ND ND Enhanced	(Simpson et al., 2006)
NV1066	Gemcitabine	Hs 700T PANC-1 aPaCa-2	Pancreatic cancer Pancreatic cancer	Synergistic Synergistic	ND ND	(Eisenberg et al., 2005)

R3616 hrR3	Gemcitabine	CAPAN1 PaCa-2 SW1990	Pancreatic cancer Pancreatic cancer	ND ND	Enhanced both cell lines) Not enhanced	(Watanabe et al., 2008)
OncoVex -GALV/CD	Gemcitabine	EJ T24 TCCSUP-G KU19-9	Bladder transitional carcinoma	Antagonistic Antagonistic Antagonistic Antagonistic	ND	(Simpson et al., 2012)
HF10	Gemcitabine	CT26	Murine colorectal model	Antagonistic if given together. Synergistic if GEM is pre-treatment	Enhanced effect in both injected tumour and distal tumour	(Esaki et al., 2013)
NV1020	Irinotecan (SN38)	HT29 and WiDr HCT-116	Colon cancer	Enhanced Enhanced	ND ND	(Guterman et al., 2006)
MGH2	Irinotecan (SN38)	Gli36□EGFR, U87□EGFR U251 T98G	Glioma	Enhanced Enhanced Enhanced Enhanced	Enhanced ND ND ND	(Tyminski et al., 2005)
G207	MitomycinC	OCUM-2MD3 MKN-45-P	Gastric cancer	Synergistic Synergistic	Enhanced ND	(Bennett et al., 2004)
NV1066	MitomycinC	KU19-19 SKUB	Bladder transitional carcinoma	Synergistic Synergistic	ND ND	(Mullerad et al., 2005)
OncoVex -GALV/CD	MitomycinC	EJ T24 TCCSUP-G KU19-9	Bladder transitional carcinoma	Synergistic Synergistic Synergistic	ND ND ND ND	(Simpson et al., 2012)
NV1020	Oxaliplatin	HT29 and WiDr HCT-116	Colon cancer Colon cancer	Enhanced Enhanced	ND ND	(Guterman et al., 2006)
G207	Paclitaxel	KAT4 DRO90-1	Anaplastic thyroid cancer	Synergistic Synergistic	Enhanced ND	(Lin et al., 2008)
NV1023	Paclitaxel	KAT4 DRO90-1	Anaplastic thyroid cancer	Synergistic Additive	ND ND	(Lin et al., 2008)
G47Δ	Paclitaxel	LNCaP DU145	Prostate cancer	Synergistic Synergistic	ND ND	(Passer et al., 2009)
MGH2	Paclitaxel	MDA-MB-435S	Mammary carcinoma	ND	Enhanced	(Nagano et al., 2008)
G207	Temozolomide	U87 U87-dnp53 U373 T98 U87MG	Malignant glioma	Synergistic (with O6-benzylguanine) Synergistic (with O6-benzylguanine)	Enhanced ND ND ND ND	(Aghi et al., 2006)
G47Δ	Temozolomide	GBM13, BT74, U87MG, T98, GBM4, GBM6, GBM8	Glioma Stem cells (TMZ resistant/MGMT+ve Glioma Glioma Glioma Stem cells (TMZ sensitive/ MGMT-ve)	No synergy No synergy No synergy Synergistic Synergistic Synergistic	Not enhanced in the presence of + O6-benzylguanine) ND ND Enhanced	(Kanai et al., 2012)
G207	Vincristine	KFR, KF-RMS-1	Rhabdomyosarcoma	ND	Enhanced Enhanced	(Cinatl et al., 2003)
NV1042	Vinblastine	CWR22 PC3	Prostate	Synergistic Synergistic	Enhanced ND	(Passer et al., 2013)

1.7 Mechanisms underlying synergistic interactions between oncolytic viruses and other agents.

There are a number of different ways in which an oHSV in combination with an anti-cancer drug can be synergistic. The simplest way of increasing the efficacy of viral therapy would be to combine it with a drug that increases viral replication.

O HSVs have selective replication competence in cancer cells and by increasing the replicative capacity of the virus within those cells, the number of progeny viruses produced during a cycle of infection is increased. Theoretically, replication of an oHSV within a tumour should result in much higher levels of virus than the input dose but studies have shown the gradual loss of virus over time in animal tumours (Lou et al., 2002). One reason for the loss of oHSV from the tumours is that not all cells within the tumour are cancer cells, many are stromal or cancer associated cells that do not support oncolytic virus replication. Furthermore, not all cancer cells (see table in materials and methods) support viral replication to the same extent. This could be partially, but not exclusively due to differential MEK expression (Smith et al., 2006). Another reason for the loss of oHSV from tumours is the anti-viral host response to an invading pathogen. One of the results is the induction of apoptosis in both infected cells and in surrounding non infected cells. By eliminating the non infected cells surrounding the infected cell, the host limits the spread of virus. Stanziale et al., 2004 reported that the increase in apoptosis in cells that neighboured oHSV NV1066 [derived from wild type HSV-1 F strain backbone, with single copy deletions of ICP4, ICP0 and ICP34.5 (Wong et al., 2002) infected cells could be prevented by treatment with an inhibitor of apoptosis, N-acetylcysteine (NAC), and this block improved the propagation of viral progeny, maximising the lateral spread of virus and potentially improving tumour destruction.

Wood and Shillitoe, 2011 reported on viral replication in the presence of zVADfmk, a pan-caspase inhibitor that has previously been shown to prevent apoptosis (Aubert et al., 2007). The authors showed that blocking apoptosis had no effect on wild type HSV replication in a number of cell lines but restored an ICP34.5 null mutant replication back to the levels of wild type

HSV-1. In terms of clinical relevance, it is unlikely that a drug that prevents apoptosis (hence makes cells, including tumour cells, less likely to die) could be used in cancer patients. Drugs that prevent apoptosis increase viral replication in ICP34.5 null mutants suggest an anti-apoptotic role for viral protein ICP34.5 in wild type HSV.

The differentiating reagent hexamethylene bisacetamide (HMBA) has also been shown to improve viral yield. Naito et al, (2006) reported up to 10000-fold increase *in vitro* for an ICP34.5 null virus, R849 (Andreansky et al., 1997), at low MOI. Mice treated with both HMBA and R849 virus had significantly smaller tumour burden and survived longer than either virus or HMBA treatment alone (Naito et al., 2006). HSV-1 immediate early, early and late gene expression was all increased in the presence of HMBA, suggesting that an increase in viral replication resulted in increased numbers of infected cells. HMBA was initially described as a drug that has some potential as a stand-alone anti-cancer agent, however the level of drug require for such activity could not be achieved in cancer patients (Egorin et al., 1987). There has recently been success in synthesising less toxic analogues of HMBA. In the study with oHSV, a much lower dose of drug was used; one which could easily be achieved in patients and potentially would act as a promoting agent for oncolytic therapy.

Eisenberg reported that hyperthermia potentiates oncolytic viral killing. After hyperthermic insult the heat shock protein Hsp72 (which inhibits cellular apoptosis) is upregulated, thereby allowing increased viral replication and, in turn, enhanced tumor kill. This finding has great potential, as in a clinical setting the application of heat is likely to be non- invasive and relatively toxicity free (Eisenberg et al., 2010).

Histone Deacetylase inhibitors (HDACis) are a class of compounds that may act to increase oHSV efficacy. HDACs are a class of proteins that have pleiotropic effects on cells through deacetylation of proteins, including histones, that then alter epigenome and transcription profiles (Lehrmann et al., 2002, Mai et al., 2005). There are numerous HDACs which have been targeted for drug discovery for cancer therapies, either for use as a single agent or in combination with chemotherapeutic agents (Xu et al., 2007). Pre-treatment

with the HDACi Valproic acid (VPA) was shown to enhance the o HSVs MGH2 (oHSV expressing 2 prodrug-activating transgenes, Kasai et al., 2013) and rQNestin34.5 (HSV engineered by expressing ICP34.5 under control of a synthetic Nestin promoter,Kambara et al., 2005a) replication and spread in tumours through inhibition of the IFN anti-viral response. VPA used in combination with rQNestin34.5 (with the VPA as a pre-treatment to oHSV) extended the survival of mice bearing intracerebral tumours (Terada et al., 2006). Alvarez-Breckenridge et al., 2012 showed that VPA also inhibits NK cells. NK cells are involved in viral clearance hence by inhibiting NK cells virus avoids clearance thus enhancing its therapeutic effect.

The effects of HDACis on other OVs are also of interest in this respect. Nguyen et al., 2008 reported on a VSV that replicates efficiently only in cells lacking an intact IFN response. The IFN responsive prostate cell line PC3 is refractory to VSV. However when the cells were pre-treated with HDACis the cells become permissive to VSV infection and the combination showed strong synergy as measured by the Chou & Talalay method both *in vitro* and *in vivo*. HDACi-induced sensitisation of tumour cells to non-HSV OVs has been shown in adenoviruses (Kitazono et al., 2002), Semliki Forest virus (Nguyen et al., 2008) and vaccinia virus (MacTavish et al., 2010). Similarly compounds that abrogate innate and adaptive responses to a virus such as cobra venom factor (Ikeda et al., 2000) and cyclophosphamide (Currier et al., 2008, Fulci et al., 2006, Kambara et al., 2005b) show synergy with OVs both *in vitro* and *in vivo*. Other compounds, such as Rapamycin, an immunosuppressant drug used to prevent rejection in organ transplantation, have been shown to significantly prolong survival of malignant glioma-bearing rats when given in combination with VSV (Tyminski et al., 2005, Alain et al., 2010).

It has been reported that rapamycin, an mTOR inhibitor can increase both the yield and spread of the oHSV in tumour cells in which oHSV only replicates poorly (Fu et al., 2011).

In addition, work carried out at Virttu Biologics as a undergraduate project by Leigh McGibbon, suggested that levels of Lactate dehydrogenase (LDH) in the medium of human cancer cells treated with HSV1716 + AZD8055 (a dual mTOR inhibitor) were higher compared to the levels of LDH when either HSV1716 or

AZD8055 treatment was given alone. LDH is only released from cells into the surrounding medium upon cell death, indicating levels of cell death are higher than expected when HSV1716 is given in combination with AZD8055, suggesting the two modalities may be working synergistically to increase cancer cell death. MTOR inhibitors are not generally cytotoxic to cancer cells, but are cytostatic and prevent further growth and division of the cancer cell. Oncolytic viruses on the other hand replicate in actively dividing cells. There are multiple redundancies within these signalling pathways, for example rapamycin only reduces mTOR activity for 12 hours before another kinase substitutes and re-engages the mTOR network (Kudchodkar et al., 2004). Potentially, there is enough redundancy in the system to subvert the action of one particular drug but, if combined with oncolytic virus assault, the cell may not have the option to recruit the alternative pathway thus potentiating the action of the drug.

Upregulation of DNA damage pathways benefits oncolytic viral therapy

Many chemotherapeutic drugs are DNA damaging agents and following exposure to such agents' cells up-regulate their DNA damage repair pathways. Up-regulation of DNA repair genes appears to be beneficial for OV replication; mitomycin C (Bennett et al., 2004), temozolomide (Kanai et al., 2012, Hadjipanayis et al., 2008) and 5FU (Eisenberg et al., 2005) have all been shown to increase o HSV replication.

Growth Arrest and DNA Damage inducible protein (GADD34) is induced by stressful growth arrest conditions and treatment with DNA damaging agents. The carboxyl terminal of GADD34 bears significant homology with the virulence factor ICP34.5, which is deleted in some oHSV, e.g. HSV1716, NV1066 (Stiles et al., 2003), and T-Vec (Kohlhapp and Kaufman, 2016). Previous studies (Roizman, 1996) have shown that the carboxyl terminus of GADD34 can substitute for ICP34.5 in preventing premature shutoff of protein synthesis and ICP34.5 null mutants can use the host cell GADD34 protein for viral replication. Thus the presence of GADD34 in tumour cells following treatment with a DNA damaging agent would increase the number of cells that oHSV can replicate in, and increase the viral spread through the tumour. Indeed when GADD34 siRNAs were added to block GADD34 expression after treatment with a DNA damaging agent (cisplatin), the previously observed

synergy with the oHSV NV1066 and cisplatin was abolished (Adusumilli et al., 2006).

HSV DNA replication occurs in discrete compartments in the nucleus that assemble as pre-replicative sites with viral DNA and the HSV DNA binding protein ICP8. HSV DNA polymerase and cellular factors are then recruited to these compartments for use in viral replication. The DNA Damage and Repair (DDR) pathways repair the damage to the cancer cell DNA caused by treatment with DNA damaging drugs such as temozolomide (TMZ). However in the presence of oHSV infection, key components of these pathways have been sequestered into discrete compartments for use in viral replication and the cell is unable to repair the damage. Thus the damage, in terms of number of cancer cells killed by a specific amount of drug, is greater in the presence of oHSV (Kanai et al., 2012).

Another potential mechanism for synergy with some oHSVs is up-regulation of cellular ribonucleotide reductase (RR) by DNA damaging chemotherapeutic agents (Petrowsky et al., 2001). High throughput screening has been reported to identify small-molecule compounds that augment the replication of HSV G47Δ (Passer et al., 2010) and of the 2460 compounds screened, 6 compounds were identified and subsequently validated for enhanced G47Δ replication. Two of these compounds, dipyridamole and dilazep, interfered with nucleotide metabolism by potently and directly inhibiting the equilibrative nucleoside transporter-1 (ENT-1) and were dependent on HSV mutations in ICP6, the large subunit of RR. ENT-1 antagonists are thought to augment oHSV replication in tumour cells by increasing cellular RR activity (Passer et al., 2010). Oncolytic HSV such as G207, hrR3 and rRp450 has mutations in the UL39 gene which encodes ICP6, the large subunit of viral RR. As oHSV's with UL39 deletions can only replicate in cells with active cellular RR, increasing cellular RR will improve viral replication. Nakano et al, 2005 reported an up-regulation in RR in tumours mediated by 5FU which augmented the therapeutic effect of G207 (Nakano et al., 2005). Gutermann et al, (2006) also found synergy both *in vitro* and *in vivo* with oHSV NV1020 and 5FU, despite the fact that 5FU actually reduced viral yields (Gutermann et al., 2006). The authors speculated that the synergy was in part due to the cells being sensitised to 5FU as the virus caused the cells to arrest in S phase. They

further speculated that the reduction in viral progeny could be due to the immune IFNγ response as well as the 5FU-induced up-regulation of cell death-molecules such as TRAIL and Fas ligand.

Compounds that modulate the immune system

The immune response to oncolytic viral therapy is an essential factor determining the success of oHSV as an anti tumour agent; it could be a hindrance if it causes premature viral clearance, but it is becoming increasingly recognised that the TAAs liberated by oncolysis, and the recognition of DAMPs and PAMPs by the innate immune system drive an anti-tumour immune response. In order to magnify such a response immunomodulatory genes have been inserted into a number of OV's. IMLYGIC, for example has the immunomodulatory gene granulocyte-macrophage colony stimulating factor (GM-CSF) inserted into its genome (Andtbacka et al., 2015).

In parallel with clinical development of OVs, the field of cancer immunotherapy has, and likely will continue, to revolutionise treatment options for cancer patients. Recently, monoclonal antibodies (mAb) targeting immune checkpoint molecules CTLA-4 (e.g. Yervoy, Bristol Myers Squibb (BMS)) and PD1 (Keytruda (Merck) and Opdivo (BMS)) have been approved in a number of cancer indications. These immune checkpoint inhibitors (ICIs) effectively 'take the brakes off' pre-existing anti tumour immunity by interrupting the negative feedback loops within a tumour (Pardoll, 2012). Results to date with these ICIs are extremely impressive, with as many as 20-30% of patients (depending on the indication) receiving this treatment as a monotherapy showing a durable long term response (Topalian et al., 2012). For the remaining patients these ICIs have limited efficacy, due to either a lack of anti-tumour immune response or other immune suppressive aspects of the tumour microenvironment that still needs to be corrected before ICIs can provide benefit. Tumour cell infection by OV's leads to an inflammatory response with localised production of cytokines, all of which that favours an immune response (Breitbach et al., 2007). OVs would appear to be a perfect complement to ICIs. Indeed, an early trial with IMLYGIC and Keytruda suggest this is the case, with 44% of patients who received the combination reported to have a durable response lasting longer that 6 months, compared to either IMLYGIC or Keytruda alone(Puzanov et al., 2016). The oncolytic virus

CAVATAK, a Coxsackie Type A21 is also in clinical trials with Keytruda and Yervoy. The trial is ongoing but initial data showed an impressive 67% response rate in the first six patients treated with CAVATAK and Yervoy (http://www.viralytics.com).

Compounds that alter the tumour microenvironment

Tumours need blood vessels to grow and spread thus inhibitors of angiogenesis which prevent the formation of new blood vessels, are being investigated as agents that prevent or slow the growth and spread of tumours. Unlike chemotherapeutic agent, angiogenesis inhibitors do not kill cancer cells directly but instead prevent tumours from growing, therefore in order to completely eradicate a tumour an anti-angiogenic drug would have to be given in combination with a modality that kills cancer cells, such as an OV.

VEGF is a key component in tumour angiogenesis and is over expressed in many human tumours. Inhibitors of VEGF, such as Avastin, Sorafenib and Sunitinib appear to 'normalise' tumour vasculature, potentially enhancing localisation of systemically delivered OV.

Tumours receiving the dual therapy of both oHSV and Avastin were significantly smaller than either treatment alone in several studies using different xenograft models (Eshun et al., 2010 suggesting Avastin does indeed improve replication and spread of the oHSV within a tumour.

Vinblastine, a microtubule disrupting agent that has been shown to inhibit angiogenesis in humans (Albertsson et al., 2008) in combination with oHSV NV1042 (as 2nd generation mutant of NV1020 expressing the cytokine IL-12Varghese et al., 2006) showed increased anti-tumour and anti-angiogenic effects *in vivo* in prostate cancer models (Passer et al., 2013), providing further evidence that the combination of an antiangiogenic agent and an oncolytic virus may have clinical benefit. Although Sunitinib has been investigated in combination with other oncolytic viruses (in VSV (Jha et al., 2013,Breitbach et al., 2012)and reovirus (Kottke et al., 2010), there are no published studies of preclinical oHSV in combination with small molecule VEGF receptor inhibitors such as Sorafenib or Sunitinib. Heo et al., (2011) reported on a clinical trial with JX-594 (an oncolytic vaccinia virus) in which a number of patients treated with JX-594, and then Sorafenib up to 8 weeks

later had objective tumour responses (i.e. tumour shrinkage) compared to 0 of/15 untreated patients matched for age, stage and gender. Furthermore, they also reported a complete cure in one patient treated with Sunitinib, 8 weeks after JX594 treatment. As the virus is likely to be cleared from the patient by 8 weeks the mechanism by which the oncolytic virus can sensitise tumours to these inhibitors is unclear. Interestingly the patients who have the best responses to Sorafenib are those patients who have Hepatitis C related HCC (Cabrera et al., 2013) suggesting that there may be a therapeutic class effect, where viruses sensitise tumours to VEGFR inhibitors.

Conclusions – oHSV in combination

Numerous preclinical studies have shown that oHSV can synergise with a variety of chemotherapeutic, monoclonal antibodies and small molecule targeted agents. The outcome of a specific drug oHSV effect on cells varies depending on the virus, the drug, the dosing schedule and the cell itself. Even within cell lines of the same cancer type there is variation, with synergy in some lines but not in others (Kulu et al., 2013). It is conceivable that the drugs can inhibit virus replication but the combined effects of virus and drug act in concert to enhance cell death and seemingly conflicting results serve to illustrate our poor understanding of such interactions.

Each oHSV, even those with similar deletions, are subtly different and may impact differently on different pathways. Many oHSVs have a deletion in ICP34.5 (including HSV1716). Likewise different cell lines have different mutations and have different expression of multiple gene sets. The majority of virus: drug combinations listed on Table 11 show synergistic, enhanced or additive effects, but this may in part reflect the fact that antagonistic combinations might be unlikely to be submitted for publication. Potentially gene expression profiling of the synergistic and non synergistic combinations could reveal patterns that correlate with and predict treatment efficacy.

Furthermore, the sequence in which the drug and oHSV are given may impact on cell killing. For example gemcitabine and HDACis such as VPA are synergistic when given as a pre-treatment to the virus, thus sensitising the tumour to virus, whereas Sorafenib appeared to work better given after oncolytic virus, thus the virus is acting as the sensitizer. Similarly when oHSV

rRp450 (Currier et al., 2008) was given before Avastin (bevacizumab) there was a significantly prolonged survival compared to the same combination in reverse order (Eshun et al., 2010).

Many of the combination studies examined the effects of combinations *in vitro*. These identify combinations that enhance cancer cell cytotoxicity. However, many of the interactions between oHSV and drugs either affect the tumour or host biology, and these interactions will only be seen *in vivo*. The immune system is a key player in the efficacy of any combination treatment – it appears that the initial suppression of the innate immune response in order to allow the virus to undergo initial replication, followed by up-regulation of the immune system to clear the virus and tumour would be a rational strategy in terms of reducing tumour burden.

Anti-angiogenic drugs are gaining support for cancer treatment, and combining these with oHSV appears to be beneficial. Tyrosine kinase inhibitors as anti-cancer therapies are currently of great interest, but, to the best of our knowledge, there are limited published studies of their interactions with oHSV. However, the picture here will probably be complicated as not all viruses appear to have the same effect in one particular cell line and different cells with different mutation profiles will probably behave differently.

It's worth noting that synergy may not be necessary for clinical translation, even an additive effect would be of benefit, particularly if the effective chemotherapy dose could be reduced to decrease the toxic side effects. However careful consideration must be paid to the scheduling regime as the mechanism by which the synergy occurs will determine whether pre or post-treatment will be more efficacious.

As preclinical studies progress into the clinical setting major progress in the understanding of oHSV in combination with other treatments is likely to occur. Early clinical trials usually involve patients who have already exhausted all the available standard treatment options, and even later phase III trials will often compare standard of care versus standard of care + oHSV. Such studies should help confirm pre-clinical findings on useful virus/drug combinations and hopefully bring benefit to cancer sufferers.

As the mechanisms for synergy with oHSV are different for different drugs it is likely that combining more than one drug with oHSV may result in even more synergistic effects. For example, pre-treating tumours with gemcitabine, which down-regulates innate immune responses, then treating with oHSV, and then treating with an anti-angiogenic agent may result in an even greater anti-tumour effect.

Project limitations

All *in vitro* systems, not just the assay developed in this thesis, have a number of limitations. The first and most obvious limitation is that *in vitro* assays are carried out using tumour cell lines grown on a monolayer in dishes, in laboratories – a system far removed from an actual, naturally occurring tumour. The benefits however of using such an *in vitro* system are the cost, both in terms of finances and time. The initial assay set up in this thesis allowed 2 different drugs to be tested in ~10 cell lines every week, meaning that screening a large number of drugs could be carried out in a few months. *In vitro* assay systems and tumour cell lines have been established and used in scientific research for over 40 years and despite their inherent limitations, have been used in most of the seminal work in the cancer field since that time. Rarely are they used in isolation – more often they are used in early experiments that lead to larger, most sophisticated studies. By carrying out initial *in vitro* screens in a rapid *in vitro* screening programme, combinations of interest that show the most promise can be selected for further *in vivo* screening.

There are concerns about the authenticity of the tissue origin and tumour type of many cell lines. Also, cell lines at high passage numbers experience alterations in morphology, response to stimuli, growth rates, protein expression and transfection efficiency compared to low passage cells (Wenger et al., 2004).

To this end, the Virttu cell bank panel was created in-house to attempt to harmonise and validate all work carried out within Virttu laboratories. The first step was obtaining cells from well known biological resource centres (mainly ECACC and ATCC), which were grown up in bulk to create a bank of low passage cells. Cells used throughout this thesis were never used after

passage 10 (based on passage 1 being designated when cells were received from an appropriate repository). Consistency in the environment is a key factor in maintaining the integrity of a cell line – cell passage time, media and sera, control buffers, gases & temperature were all maintained to minimise any selection pressure to cells in culture.

The second limitation of *in vitro* assays is they do not fully reflect what is happening within a tumour. Cells within a cell culture environment are evenly spread out over a dish and all have an equal chance of being infected by the substance being tested – whereas a tumour is a 3D structure. To this end, a project to make better *in vitro* models, such as using raft type 3D modelling systems is underway.

Thirdly, tumours are made up of not only cancer cells but also non malignant cells, each with roles in enabling tumour growth and persistence. The tumour microenvironment, which varies by tumour type and location, even within an individual patient, is often composed of stromal cells such as vascular endothelial cells, pericytes, tumour associated fibroblasts, hematopoietic cells & innate immune cells such as macrophages, neutrophils and myelocytes. Even within a single tumour the environment is heterogeneous; there are often necrotic regions within a tumour & regions of hypoxia. These regions are hard to reach - they have a poor blood supply and often high interstitial pressure meaning delivery of any agent to these areas is challenging. None of these factors can be modelled *in vitro*, and even a 3D modelling system will not account for these factors.

Lastly, it is becoming increasing apparent that the immune system has a vital role to play in cancer therapy. Again, none of these factors can be assayed using an *in vitro* assay but have huge impact on the efficacy of any therapy, not just oncolytic virotherapy. In order to look at the effects of HSV1716 as an immunotherapy, whole animal systems are still necessary. It is interesting also to consider the rapidly evolving therapeutic landscape in cancer treatments, when this project was initiated; kinase inhibitors were at the forefront of treatment paradigms whereas now they have largely been supplanted by immunotherapies

Despite such limitations, *in vitro* assays have a key role to play - relatively quickly, cheaply and reproducibly, they can reveal key aspects of the mechanisms of action that OVs and drug combinations use to kill cells, and allow selection of the most likely synergistic candidates to take forward for translational *in vivo* work.

Background to this thesis

I have been employed by Virttu Biologics since 2004, in this time I have been involved in many projects involving HSV1716 and new variant viruses. I am the head of *in vivo* research and previous to this thesis have published a number of papers of HSV1716, including two first author papers. The first paper concerns the HSV1716 variant HSV1790, a variant that expressed the E.Coli enzyme nitroreducatase. The presence of this enzyme converts the relatively inert pro drug CB1954 into an active chemotherapeutic (Appendix 1).

My second first author paper, in the Journal of Hepatocellular Carcinoma (Appendix 1), assesses HSV1716 in preclinical studies with two human hepatocellular carcinoma cell lines. This work was carried out as Virttu has approval to move ahead with a clinical trial in Hepatocellular Carcinoma. The plan for this trial was to administer HSV1716 via a transcatherter infusion in combination with the chemotherapeutic agent doxorubicin and a chemo embolisation agent which essentially blocks the veins in the liver allowing the doxorubicin (and HSV1716) to remain in the liver for longer periods to enhance the efficacy of the therapy. As HSV1716 had not previously been used in combination with doxorubicin either in the clinic, or in the lab, a project to look at the combination was undertaken. This in turn, lead to the wider question of what effects would other agents has on HSV1716, or what effect would other agents have on HSV1716, and after setting up a collaboration with Professor S Graham at the University of Glasgow, the basic aims of this thesis were set up.

Aims of this thesis:

To investigate HSV1716 in combination with anti-cancer agents

1. Develop a high throughput screen to look at HSV1716 in combination with a number of anti cancer drugs across a number of different classes. (i.e. chemotherapeutics, targeted agents, and receptor tyrosine kinases).
2. Identify a number of drugs or classes of drugs that are synergistic with HSV1716 and determine the mechanism of action behind this synergy

Chapter II -Materials & Methods

2.1 Virttu cell line panel

Cell lines shown in Table 5 were used for all experiments. Cells had been bought from the source shown in Table 5.

2.2 Cell line media composition:

The medium used in all experiments, both routine cell passaging, plate set up and titrations are summarised in Table 6. All plastic ware was sourced from Greiner.

2.3 Viruses

HSV1716 GFP for combination studies - An HSV1716 variant expressing green fluorescent protein (GFP) is used for combination analysis and subsequent caspase and apoptosis assays. HSV1716GFP was produced from the parental HSV1716 by insertion of a CMV-GFP expression cassette in the UL-43 gene. The virus stock used was originally created on 23.07.99 (Conner, Virttu Biologics) and was titrated by plaque forming assay to reconfirm titre in December 2012 (1x10e9 pfu/ml).

Virus was diluted to generate 200 aliquots of 1x10e6 pfu/ml working stock aliquots to ensure consistency across combination experiments and stored at -70oC. Virus was stored in the same conditions as Virttu Biologics clinical grade virus which is subject to stability testing yearly to determine if the virus loses titre over time. To date, the clinical grade stocks, stored under the exact same conditions as the virus described here, and used throughout this thesis, is stable for at least 120 months (10 years) (unpublished data, Conner & Braidwood) . For each combination experiment a fresh aliquot was used and prepared using the dilution serial dilutions of 1e. MOI are based on cell counts between 8000-10000 cells per well.

Table 5: Virttu Cell line panel

Name	Source	Details
Hep3B	ECCAC 86062703	Hepatocellular carcinoma derived from an 8 year old male and cells contain integrated Hepatitis B virus genome. However there is currently no evidence that this cell line produces infectious Hepatitis B virus. Doubling time 29 hrs ((Sagawa et al., 2008)
HuH7	ECACC JCRB0403	HuH-7 is a well-differentiated, hepatocyte-derived cellular carcinoma cell line that was originally taken from a liver tumour in a 57-year-old Japanese male. HuH-7 is epithelial-like tumourigenic cells which are able to form subcutaneous xenografts in nude mice. COSMIC: HuH7 cells have mutated FAM123B andTP53genes. Doubling time 51 hrs
HepG2	ECACC 85011430	Hep G2 cell line itself was isolated from a liver biopsy of a male Caucasian aged 15 years, with a well differentiated hepatocellular carcinoma. The cells secrete a variety of major plasma proteins e.g. albumin, alpha2-macroglobulin, alpha 1-antitrypsin, transferrin and plasminogen but Hepatitis B virus surface antigens have not been detected. Doubling time 29hrs
HepG2-luc2 Bio ware	Calliper HT1080-*luc2*	HepG2-luc2 is a luciferase expressing cell line which was stably transfected with firefly luciferase gene (luc1). The cell line was established by transducing lentivirus containing luciferase 2 genes under the control of human ubiqution C promoter.
A2780	ECACC - 93112519	Human, ovarian cancer derived cell line established from tumor tissue of an untreated ovarian cancer patient. According to the COSMIC entry there is a mutation in the Kinase insert domain receptor (KDR) which is also known as vascular endothelial growth factor receptor 2 and has been linked to various cancers.
CP70	ECACC 93112517	Human, ovarian cancer derived cell line. The CP70 cell line is a cisplatin-resistant derivative of A2780 cells and the cells have approximately 13-fold more resistance to cisplatin than the parental A2780 line. The A2780 human ovarian cancer cell line was established from tumour tissue from an untreated patient. According to their entry in COSMIC (Catalogue of Somatic Mutations in Cancer) they have a mutated PTEN gene
Ovcar3	ATCC – HTB-161	Adherent, epithelial cells derived from the ascitic fluid from a 60 year old Caucasian female with an ovarian tumour. Reported by ATCC to be tumourigenic but cells established in Virttu cell bank did not form xenografts in 10/10 nude mice injected subcutaneously with approximately 5e6 cells (unpublished data, Braidwood). The cell line is aneuploid human female, with chromosome counts in the sub to near-triploid range. COSMIC entry indicates somatic mutation in TP53.
Skov3	ECCAC - 91091004	Adherent, epithelial cells derived from the ascitic fluid from a 64 year old Caucasian female with an ovarian tumour that form moderately well-differentiated adenocarcinoma consistent with ovarian primary cells. Cells have a hypodiploid to hypotetraploid karyotype. COSMIC entry indicates somatic mutations in CDKN2A, CDKN2a (p14), MLH1, PIK3CA and TP53. Doubling time 35hrs (http://physics.cancer.gov/docs/bioresource/ovary/NCI-PBCF-HTB77_SK-OV-3_SOP-508.pdf)

U87MG	ECCAC - 89081402	Epithelial like cells derived from a malignant glioma from a female patient by explant technique and reported to produce a malignant tumour consistent with glioblastoma in nude mice. Karyotype is 2n (=46). COSMIC entry indicates somatic mutations in CDKN2A, CDKN2C, CDKN2a (p14) and PTEN
UVW	ECCAC - 86022703	Cell line established from an anaplastic astrocytoma of normal adult brain and forms xenografts in nude mice. No entry in COSMIC
One58	ECCAC 10092313	This cell line was derived from the pleural fluid of a patient with malignant mesothelioma. The patient had known exposure to crocidolite asbestos. Cells express cytokeratin and epithelial membrane antigen (EMA) but not mucin. Cells are epithelial-like and spindle-shaped with few vacuoles. No entry in COSMIC. Doubling time 24 hrs (Manning et al., 1991)
SPC-111	ECCAC 11120716	SPC111 was derived from the pleural effusion of a 55-year old male patient, prior to treatment, with a known history of exposure to asbestos. The cells are Epitheloid/mesenchymal. No entry in COSMIC.
Vero	ECACC 84113001	Established from the kidney of a normal adult African Green monkey. Susceptible to a wide range of viruses hence used for titration were obtained from the VIRTTU Biologics Ltd cell bank. No entry in COSMIC
BHK	ECACC 85011433	Sub clone of parent line derived from 5 1-day-old unsexed hamster kidneys. Used extensively for virus replication studies i.e. poliovirus, rabies, foot and mouth disease, VSV (Indiana strain), herpes simplex, Ad25 and arboviruses.
3T6	ECACC 86120801	Established from disaggregated Swiss mouse embryos in 1963. 3T6 cells are not permissive to HSV1176 replication – failure to express ICP34.5 results in a defect in virus maturation and egress from the nuclei to the extracellular space (Brown et al., 1994). No entry in COSMIC Doubling time 16hrs (Rath et al., 1984)
A431	ECACC - 85090402	Human squamous carcinoma derived from the epidermal carcinoma of the vulva taken from an 85 year old woman. The cells carry large numbers of EGF binding sites. COSMIC entry shows a mutation in PTCH1.Doubling time 24 hrs (Bonner et al., 2009)

Table 6: Cell medium, supplements, titration and overlay medium.

Cell lines	Medium	Supplemented
All except Hep3B	For normal passaging and plate set up Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12 no glutamine, Invitrogen),	50 ml Newborn Calf Serum NBCS, 16010159, Invitrogen)
		5.5 ml Penicillin-Streptomycin-Glutamine 10378-016, Invitrogen)
		2.5 ml Fungizone Antimycotic (15290-026, Invitrogen
Hep3B	For normal passaging and plate set up Advanced RPMI 1640 (, 12633-012, Invitrogen)	100 ml Newborn Calf Serum NBCS, 16010159, Invitrogen)
		5.5 ml Penicillin-Streptomycin-Glutamine 10378-016, Invitrogen)
		2.5 ml Fungizone Antimycotic (15290-026, Invitrogen
All titrations	Overlay 100 ml 10 X GMEM medium	Glasgow's MEM powder (11710-035, Invitrogen) is mixed with Baxter's water (UKF7114), + 7.5% Sodium Bicarbonate solution (25080102, Invitrogen). The solution is mixed using a magnetic stirrer until dissolved. Ph the solution to pH 7.2-7.3. The medium is then filter sterilised using 0.45uM bottle top filter (Nalgene 296-4545) using a vacuum into sterile 50 ml tubes. These can be stored for up to 6 months at -20°C.
	300 ml Methyl cellulose	4.2 g Carboxymethyl cellulose sodium salt (9004-32-4, VWR) is added to 300 ml Baxter's water (UKF7114). The powder should be fully dissolved in the solution. The lid of the bottle is left loose and then autoclaved at 121°C for 15mins. The lid is tightened then bottle allowed to cool before being stored at 4°C for up to three months
	50 ml Newborn Calf Serum	Newborn Calf Serum(NBCS, 16010159, Invitrogen)
	5.5 ml Penicillin-Streptomycin-Glutamine	(10378-016, Invitrogen)
	2.5 ml Fungizone Antimycotic	(15290-026, Invitrogen
	33 ml Tryptose Phosphate Broth	11.8 g Tryptose Phosphate Broth powder (Sigma T9157) is dissolved in 500ml Baxter's water (UKF7114). The lid of the bottle is left loose and then autoclaved at 121°C for 15mins. The lid is tightened then bottle allowed to cool before being stored at 4°C for up to three months

2.4 Drugs for combination studies

Drugs used in combination studies are shown in Table 7.

2.5 Cell line panel for HSV permissivity

Virttu has been working with the cell panel shown above for a number of years, so prior to the work described in this thesis the permissivity of the cell lines to HSV1716 and wild type virus have been tested. The expected yields are shown in Table 8.

2.6 Culturing, passaging and setting up plates

All tissue culture was performed in a microbial safety cabinet, adhering to aseptic techniques at all times. Cells were maintained, passaged and 96 & 6 well plates set up as described in Freshney ISBN: 978-0-470-52812-9

2.7 Plaque assay

Determination of infectious particle titre by plaque forming assay is described in Harland & Brown (1999) with the basic premise shown in Figure 9.

Table 7: Drugs used in combination studies.

Drug	Selleck Chem cat no.	Target	Preclinical/Clinical Indications
Temsirolimus	S1044	mTOR	Approved for the treatment of renal cell carcinoma and mantle cell lymphoma
AZD8055	S1555	mTORC1/mTORC2	Completed phase I trials in recurrent glioma, liver cancer and advanced tumours.
Ku0063794	S1226	mTORC1/mTORC2	Inhibits tumour growth in xenograft model of renal cell carcinoma
GSK690693	S1113	pan Akt1/2/3 inhibitor	Evidence of inducing apoptosis and inhibiting cell growth in leukemic cell lines
LY294002	S1105	PI3K	Inhibits cell proliferation and induces apoptosis
Sunitinib	S7781	RTK	Approved for the treatment of renal cell carcinoma and imatinib resistant gastrointestinal stromal tumour.
Sorafenib	S7397	Raf-1, B-Raf and VEGFR-2	Approved for the treatment of renal cell carcinoma, hepatocellular carcinoma and iodine resistant advanced thyroid carcinoma
Pazopanib	S3012	TKI	Approved for the treatment of renal cell carcinoma and soft tissue sarcoma
Cabozantinib	S S1119	VEGRR-2	Approved for the treatment of medullar thyroid cancer and advanced renal cell carcinoma
Nintedanib	S1010	VEGFR1/2/3, FGFR1/2/3 PDGFRα/ß	Approved for the treatment of idiopathic pulmonary fibrosis and some forms of non-small-cell lung cancer
Crizotinib	S1068	c-Met ALK	Approved for the treatment of some non-small-cell lung carcinoma, undergoing phase I and II trials in advanced cancer, metastatic breast cancer, solid tumours and anaplastic large cell lymphoma
Dovitinib	S1018	RTK	Undergoing phase II/III and phase II trials for solid tumours and prostate cancer
Gefitinib	S1025	EGFR	Approved for the treatment of breast and non-small cell lung cancer
Erlotinib	S1023	EGFR	Approved for the treatment of non-small cell lung cancer, pancreatic cancer and other cancers
GSK1120212 (Trametinib)	S2673	MEK1/2	Approved for the treatment of metastatic melanoma, recruiting for a phase I trial in neuroblastoma and a phase II trial in recurrent non-small cell lung cancer
LY2228820	S1494	P38 MAPK	Recruiting for a phase II trial in metastatic breast cancer and a phase I trial in advanced or metastatic cancer
Doxorubicin	S1208	DNA topoisomerase II	Approved for the treatment of breast and ovarian cancer, multiple myeloma and Kaposi's sarcoma

All powders were prepared as per manufacturer's instructions and stored in aliquots at -70^{o}C until required.

Table 8: Permissivity of cell line panel to HSV1716 and wild type HSV17+ infection.

Cell Line	HSV17+ yield @ 72hrs	HSV1716 yield @ 72 hrs	Replication Competence ratio (HSV17+ compared to HSV1716)
Hep3B	14430	4820	0.33
Huh7	3250	28500	9
HepG2	40670	60030	1.5
Cp70	223	57	0.26
Ovcar3	95543	179009	1.9
Skov3	27849	913	0.03
U87MG	43100	8806	0.2
UVW	72234	78369	1
One58	13650	12650	1
3T6	400	0.5	0.00125
Spc111	11920	2710	0.23

In Hep3B, Cp70, U87MG, Skov3 & SPC111 HSV1716 doesn't replicate as well as HSV17+. In UVW, one58 & HepG2 HSV1716 replicates approximately as well as 17+. Huh7 is unusual in that HSV1716 replicates to a higher titre than HSV17+.

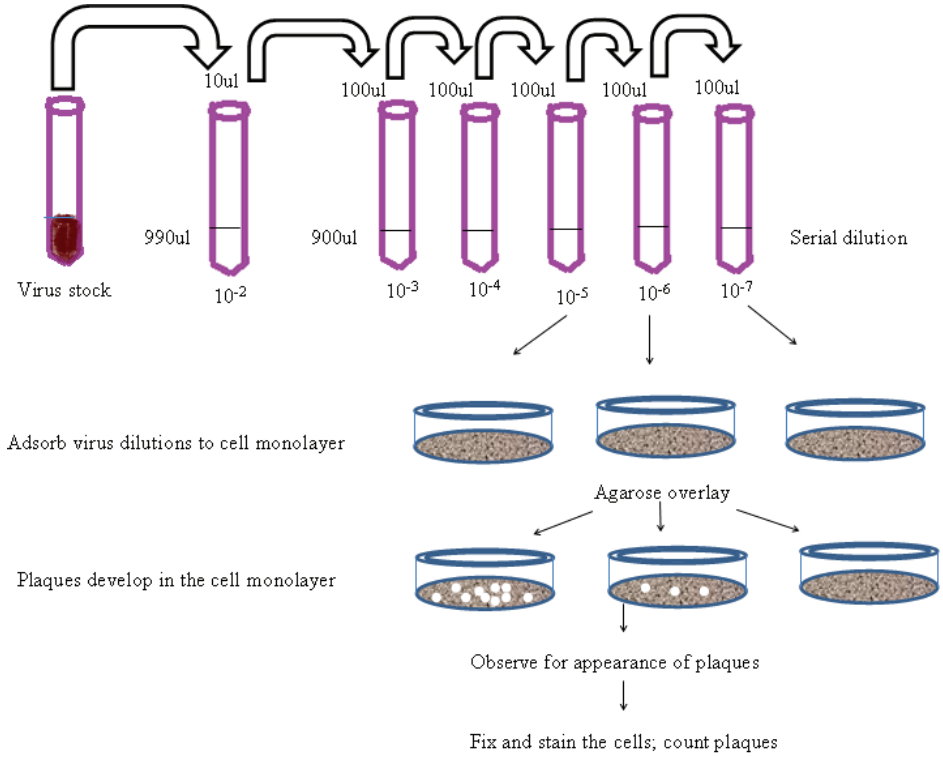


Figure 9: Determination of viral titre by plaque assay

Virus is serially diluted and the dilutions added to the confluent monolayers of Vero cells. Overlay medium is added and the cells are incubated at 37°C for 72 hrs. At 72hours plaques are visible on the monolayer. Plates are stained using Giesma stain and counted using a stereo tactic microscope. The plate with between 100-300 plaques is counted and the PFU/ml is worked out using the dilution factors

2.8 Dead Cell Protease (DCP) Assay

DCP was assayed using the CytoTox-Glo Cytotoxicity kit from Promega. The kit provides a luminogenic peptide substrate, AAF-Glo, to measure dead cell protease activity in the media. DCP is released from cells which have lost membrane integrity (Figure 10A). The peptide substrate cannot cross the intact cell membrane of a live cell and will only be cleaved (Figure 10B) when dead cell protease has been released into the media as cells die. The assay then uses the Ultra-Glo recombinant luciferase, which can use the released aminoluciferin as substrate, to generate a readily detectable luminescence signal. Light emission from the DCP assay was detected using a Perkin Elmer 1420 multilabel counter Victor 3 in luminometer mode for 0.1s/well.

2.8.1 DCP plate assay set up

Plates for Chou Talalay combination analysis were set up as described in Table 9.

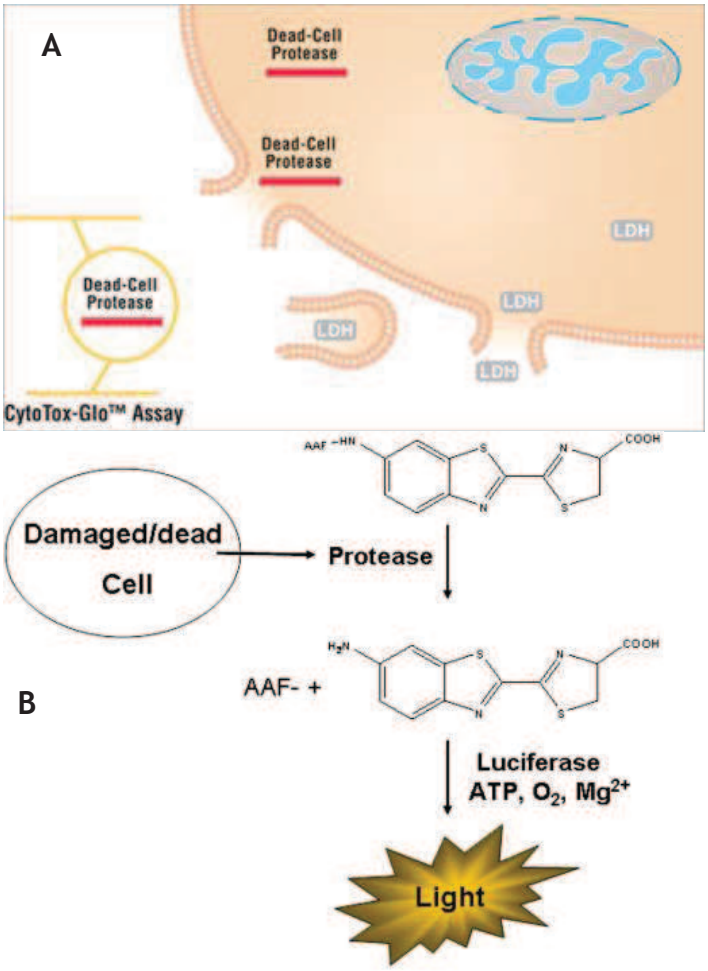


Figure 10: DCP assay

(A): DCP is leaked when membrane integrity has been compromised.

(B)**:** DCP coupled reaction for measuring cell death. The AFF-Glo peptide is a substrate for dead cell protease and cleavage releases aminoluciferin. Aminoluciferin is a substrate for a modified recombinant luciferase but not for wild-type luciferase. Figure taken with permission from www.promega.com

A

A1	2	3	4	5	6	7	8	9	10	11	12
B	Drug Dose 1	Drug Dose 2	Drug Dose 3	Drug Dose 4	Drug Dose 5	Drug Dose 6	Drug Dose 7	Drug Dose 8	No Drug		
C											
D											
E											
F											
G											
H											

B

A1	2	3	4	5	6	7	8	9	10	11	12
B	1	MOI 0.5	2	MOI 0.5	3	MOI 0.5	4	MOI 0.5		MOI 0.5	
C	Drug Conc.		Drug conc.		Drug conc.		Drug conc.		No drug		
D		MOI 0.05		MOI 0.05		MOI 0.05		MOI 0.05		MOI 0.05	
E		Drug conc. 1		Drug conc. 2		Drug conc.3		Drug conc. 4		No drug	
F		No virus		No virus		No virus		No virus		No virus	
G		Drug conc. 1		Drug conc. 2		Drug conc.3		Drug conc. 4		No drug	
H											

Table 9: Plate set up for IC50 determination of drug toxicity and combination analysis

(A) Plate set up for determination of drug toxicity. (B) Combination analysis plate set up – grey wells round the outside the plates are filled with HBSS only. Each virus +/- drug combo is set up in quadruplicate and each plate has its own no virus/no drug controls.

2.9 Caspase Assay

The Caspase-Glo 3/7 (G8090), Caspase 8 (G8200) and Caspase 9 (G8210, all Promega, UK) assays are methods of quantifying the amount of a specific caspase, either 3/7, 8 or 9 as a measure of apoptosis. Figure 5 describes the different actions of each of the caspases within the apoptosis pathways. Caspase 3/7 is referred to as an executioner caspase and is the key caspase where the intrinsic and extrinsic apoptosis pathways converge. Caspase 8 is only activated if the extrinsic pathway is activated and caspase 9 only when the intrinsic pathway is activated (Figure 5).

The kits contain a substrate that lyses cells, releasing any caspase 3/7, 8 or 9 present within the cell into the surrounding medium. This released caspase cleaves the luminogenic substrate producing a light signal proportional to the amount of caspase present. The luminosity is measured a using Perkin Elmer Victor^3 machine.

2.9.1 Caspase Assay- Optimisation and Validation

Assay validation and optimisation was carried out to identify the optimal conditions for the analysis of apoptosis in cells treated with HSV1716 + drug.

To identify a positive control for apoptosis, the caspase 3/7 assay was carried out on cells from the Virttu panel (Table 5) incubated with drugs described in the literature as inducers of apoptosis, namely vincristine, carboplatin, Etoposide & docetaxel. Caspase 3/7 activity determined after 72 hours drug exposure. Docetaxel was the only chemotherapeutic tested which increased caspase 3/7 levels in all cell types tested relative to non-drug treated cells and was used as a positive control for apoptosis in all subsequent experiments (results not shown).

2.9.2 Assay set up

96 well plates were seeded with cells using the format illustrated in Table 10 with three cell lines being used per plate. Each cell line was assayed in quadruplicate. Control wells had equal volumes of medium added and docetaxel was used as a positive control to validate each plate. After caspase activity, the CytoTox-Glo total lysis method was used (to estimate the total number of cells present) to correct the caspase 3/7, 8 &9 values for the number of cells in each well.

For analysis, the caspase 3/7 or caspase 8 light output readings were divided by the total DCP value for the respective well to correct for the number of cells in each well. GraphPad Prism was used to graph the relative caspase 3/7 or caspase 8 activities. Student T tests were used to compare the groups of interest. If the caspase ratio in the HSV1716 + drug combination was significantly ($P<0.05$) greater than the caspase level in both the HSV1716 alone AND the drug alone, then the combination was said to have significantly increased caspase levels relative to controls

2.10 Virus free Conditioned Medium (VF_CM) +/- drugs

Production of virus free conditioned medium (VF_CM)

Figure 11 shows a schematic representation of the production of VF-CM. T175 flasks were set up using the cell line of interest. Once confluent monolayers had formed they were infected with HSV1716 MOI 1 and the medium harvested after 24 hours. To remove any live virus the medium was passed through a 0.1uM filter (Millipore syringe filters, McQuilkin SLVV033RS) to produce the VF-CM. In order to confirm that all viruses were removed from the VF-CM by the 0.1μM filter plaque forming assays (section 2.7) were carried out on a number of test samples before and after filtration. The results of these are shown in Figure 11B

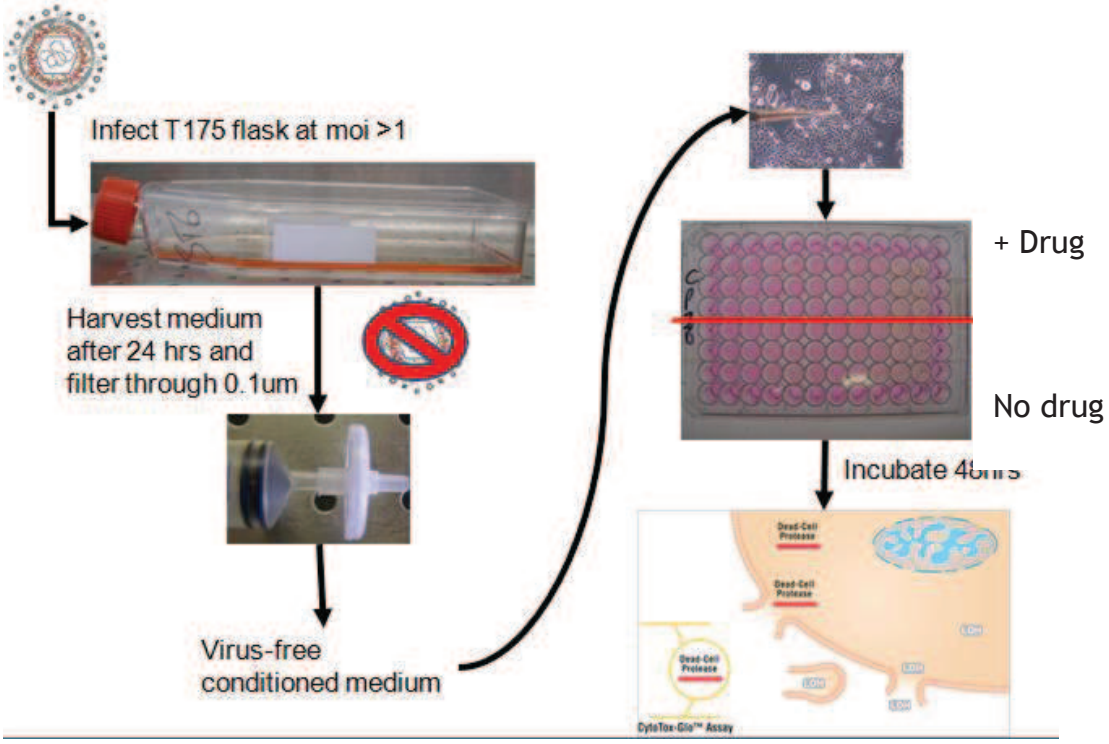
2.10.1 Production of UV -VF-CM The production of UV VF-CM used exactly the same protocol as VF-CM except prior to infection of the T175 flasks, HSV1716 was placed under UV light (wavelength 260 - 270nm) at room temperature for 15 mins. A sample of the UV treated virus was titrated as described in Materials and Methods section 2.7 and no plaque forming units were detected.

Table 10: Plate set up for caspase assays

	Control	HSV1716	Drug alone	HSV1716 + drug	Docetaxol	
	Cell line 1					
	Cell line 2					
	Cell line 3					

Each cell line was assayed in quadruplicate.

A



(B) Titre of CM before filtration	Titre of VF-CM after filtration
$6x10^5$ pfu/ml	0
$3.2x10^6$ pfu/ml	0
$5.5x10^6$ pfu/ml	0
$9.1x10^6$ pfu/ml	0
$3.5x10^7$ pfu/ml	0

Figure 11: Production of virus free conditioned medium (VF-CM).

(A) Monolayers were infected with HSV1716 and the medium harvested at 24hours. The medium was then passed through a 0.1μM filter to remove virus. The resultant VF-CM was used in 96 well plate DCP assays to look for increased cell death in recipient cells that had been exposed to VF-CM +/- drug.

(B) (B) Filtration using a 0.1um filter removed all HSV1716. *The limit of detection using this assay is approximately 100 pfu/ml.

2.10.2 Plate set up- VF-CM +/- Drugs

96 well plates of various cell lines were seeded in the wells at volumes of 100µl. After 24hours, when the VF-CM + drug were being added, medium was first removed. 100ul VF-CM (or uv VF-CM) was added and either 100µl of fresh medium (no drug) or 100µl of medium with the drug before being incubated for 48 hours at which point the amount of dead cells was quantified using the DCP assay (described in M&M section 2.8).

2.11 PCR

PCR reagents used per reaction were: 12.5ul Quick Load taq 2X master mix (New England Bio labs M0271S), 10µM forward primer (in 0.5µl) 10µM reverse primer (in 0.5µL), 1µl template + nuclease free water (to final reaction volume of 25µl). PCR was performed in a Techne Genius Thermal cycler under the following conditions: 95^{o}C for 2 minutes (hot start) then 30 cycles x (94^{o}C for 15 s 62^{o}C for 60s, 68^{o}C for 60s); 2 mins at 68^{o}C followed by a 4^{o}C incubation. After PCR, 20µl of the reaction was electrophoresed on a 1% agarose gel containing Ethidium Bromide alongside DNA markers of known concentrations to confirm band size. The gel was visualised using a UV lamp and photographed.

Primer sequences:

UL42: forward (5'-ACGACGACGTCCGACGGCGA3') reverse: (5'-GTGCTG GTGCTGGACGACAC3')

gH forward(5'-CGACCACCAGAAAACCCTCTTT3') reverse: (5'-ACGCTCTCGTCTAGATCAAAGC3')

Chapter III - Results

3.1 HSV1716 in combination with targeted anti cancer agents

OVs kill cancer cells while sparing normal cells. They utilize viral gene products to facilitate immune evasion, and commandeer cellular biosynthetic machinery to replicate, while manipulating cell death programs. Many of the pathways that viruses manipulate are the same pathways that tumour cells must deregulate in order to become tumourigenic, and as a consequence, these same pathways are the targets for anticancer drug development. Thus is seems reasonable to expect that certain types of chemical, radiological or biological therapy could enhance or synergize with OVs in terms of improving tumour cell killing. HSV1716 is currently a clinical stage OV. In clinical trials new treatments are compared to 'standard of care', therefore it is important to determine if the standard of care, usually chemotherapy or radiotherapy, has a negative effect on HSV1716 efficacy.

This premise behind this thesis was to study the effect of currently approved and potential new therapies on HSV1716. This was both to look for synergistic combinations but also to look for any antagonistic combinations that could then be avoided in the clinical setting.

Due to the vast numbers of current and potential anti-cancer drugs that are currently either approved or in late stage clinical trials it was recognised that in order to test them, a high throughput screen required to be designed. As this screen is an *in vitro* screen carried out on cell lines over 72hours, no immunotherapeutic agents were tested.

It is not the intent of this thesis to detail the interaction between HSV1716 and every drug tested; instead this chapter will summarise the results of the screening. Chapters IV and V then investigate a smaller number of drugs identified within this screen as synergistic with HSV1716 in order to try and elucidate the mechanism of the observed HSV1716/drug interaction.

96 well plates were seeded with cells and treated with HSV1716, HSV1716 plus drug or drug alone at various concentrations. After 72hours DCP (Materials and Methods section 2.8), a measure of cell death, was

determined. Validation of the assay was carried out looking at a number of variables. The results are detailed below:

3.1.1 Validation of DCP assay in measuring virally mediated cell death

HSV1716 infection over 72 hours caused an increase in DCP leakage from the various cell lines used which was dependent on the HSV1716 MOI (Figure12A).The DCP basal level varied with the different cell lines and probably reflected the intrinsic DCP amounts present in each cell line. Even the lowest MOI of 0.001 HSV1716 increased DCP levels above base line (base line ~ 0.0001 Figure 12A) and DCP levels increased in a dose-dependent manner to reach a maximum at MOI 10. In all cell lines the increase at MOI 1 was >50% of base line. In order to study the effect of HSV1716 in combination with another drug, it was necessary to pick a MOI of virus that had some effect, but did not kill all cells. If the virus alone killed all cells then it would not be possible to measure any further increase in cell death. Therefore MOI of 0.5 and 0.05 were chosen for future studies.

3.1.2 Validation of DCP assay in measuring drug mediated cell death

Drug toxicity could also be detected using the DCP assay as shown in Figure 12B. Increasing concentrations of the c-met inhibitor XL-184 caused increased leakage of DCP from CP70, one58, HepG2 (Figure 12B). As with virus, UVW cells displayed the highest basal levels of DCP (0.01 on log scale) which increased by ~300% at 50μM XL-184. One58 had the lowest basal levels and smallest increase at 50μM (~50%). Basal levels of DCP were intermediate in CP70 and HepG2 cells (0.01 on log scale) and exposure to 50μM XL-184 increased DCP leakage by ~100% in these cells lines. This indicates the DCP assay is sensitive enough to be able to measure dose-dependent changes in cell death.

3.1.3 Validation of DCP assay- cell density dependent.

Another assay variable that was investigated was the effect of different cell densities on the DCP assay. If cells are overgrown there will be higher basal DCP levels. The results for the two cell lines UVW and Ovcar3 cells incubated for 72 hrs with HSV1716 (C and D), or XL-184 (Figure 12E and 12F) are shown. Ovcar3 and UVW were plated out at different cell densities ranging from 8000 cells/well to250 cells/well and, 24 hours after plating out at these densities,

they were treated either with XL-184 at 50, 10 or 1μM or HSV1716 at MOI 10, 1 or 0.1 for 72 hours. Although the basal DCP levels at higher cell densities were higher per se, the difference between HSV1716 or drug-treated cells and control cells was greatest at 5000 cells/well and this density was used in all subsequent studies.

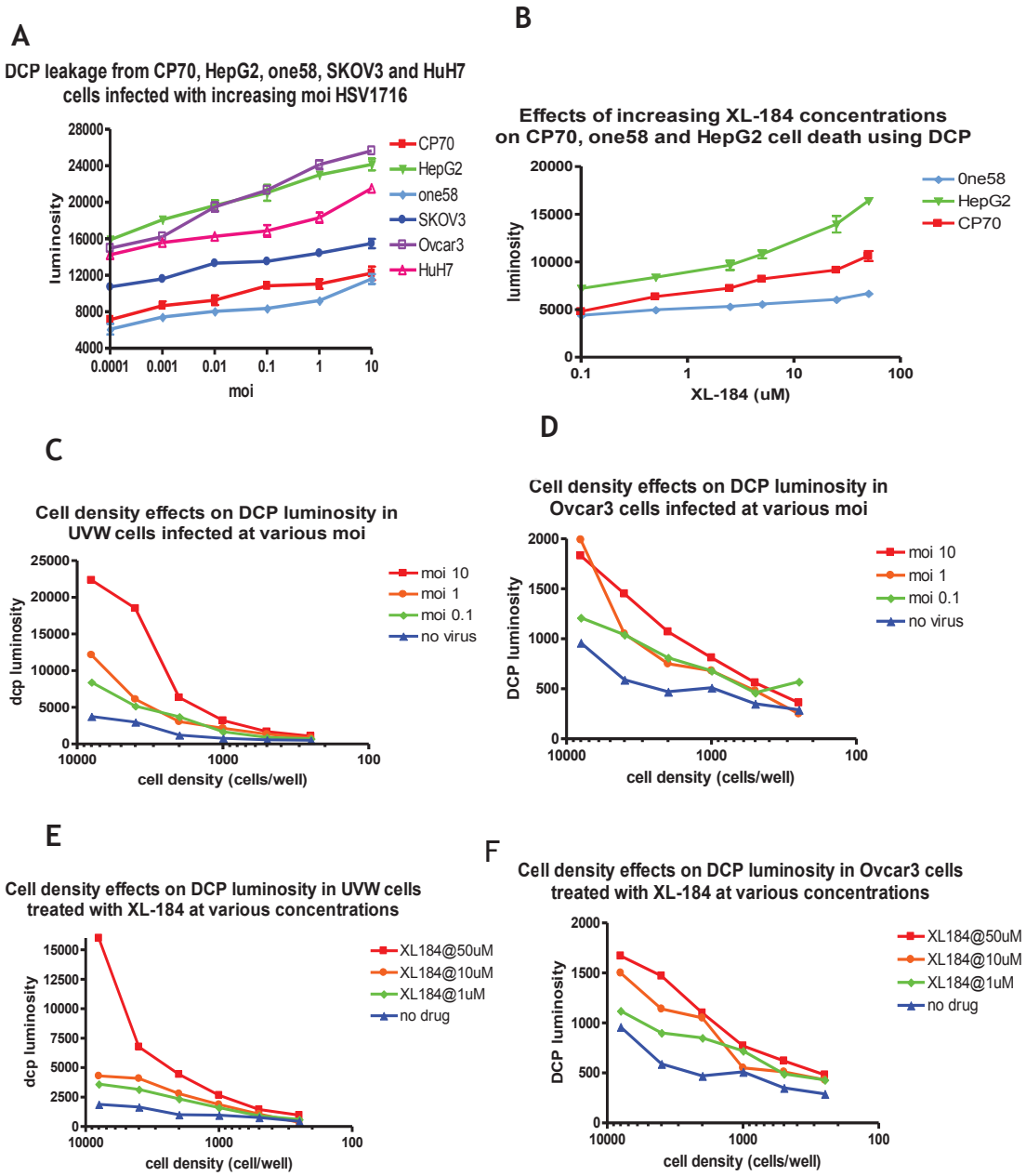


Figure 12: Validation of DCP assay

(A): MOI-dependent DCP leakage from various cell lines infected with HSV1716. (B): Dose-dependent toxicity of XL-184 in CP70, one58 and HepG2 cells using DCP assay. (C): Effects of UVW cell density on DCP readings after treatment with HSV1716 at MOI 10, 1 or 0.1 for 72 hours. (D): Effects of Ovcar cell density on DCP readings after treatment with HSV1716 at MOI 10, 1 or 0.1 for 72 hours. (E): Effects of UVW cell density on DCP readings after treatment with XL-184 at 50μM, 10μM or 1μM for 72 hours. (F): Effects of Ovcar3 cell density on DCP readings after treatment with XL-184 at 50μM, 10μM or 1μM for 72 hours.

3.1.4 DCP assay to determine drug toxicity values and plot median effect.

As a prerequisite to Chou Talalay analysis of treatment combinations, the effect of each modality (i.e. drug or virus) must first be assessed on its own. Drug toxicity for each individual drug was assessed. 96 well plates of all cell lines were set up as described in Materials and Methods 2.6 and treated with serial dilutions of drug. Serial dilutions were used to determine a range where the high doses would induce death in almost 100 % of cells, and low doses would have an effect close to the baseline of untreated cells. Drug doses were chosen based on the data available from the manufacturer (www.Selleckchem.com) with a starting doses at least 100 fold higher than any suggested dose. The dose effect curve was plotted. In order to use the sigmoidal dose effect curve for analysis it must first be transformed into the corresponding linear form, termed the median effect plot. Examples of the median effect plot (Figure 13B) for the dose effect curve (Figure 13A) are show in Figure 13.

The dose effect curve graphs the dose on the X axis and the Fa (fraction affected) on the Y axis. Fraction affected means the amount of cells (as a ratio of the total proportion) that is killed by the drug dose. For example, if 75% of the cells are killed by a particular dose of drug, the Fa would be 0.75. The Fu is the fraction unaffected. In this example the Fu would be 0.25 (25% or cells are not killed by this dose of drug). LD_{50}, ID_{50}, ED_{50} or CI_{50} are often used interchangeably as a measure of how toxic a drug is. Although often used interchangeably IC_{50} is the maximal concentration of drug to cause 50% inhibition of biological activity of cancer cells, ED_{50} refers to the dose of the drug which causes 50% response in a biological system or which treats effectively 50% of the population and LD_{50} is the concentration causing 50% cell death (LD = lethal dose). Within this thesis the term IC_{50} will be used, in terms of the dose of drug required to cause a 50% increase in cell death.

There are three features of the linear median effect plot that are important for subsequent combination analysis: M, which indicates the slope of the line (three examples are shown where m =2, 3 and 5 in Figure 13B). The second parameter is the point at which the line intercepts the x axis (log (Fa/fu=0); and R^2 . R^2 indicates how well the real data from the actual experiment fits the trend line an R^2 value of 1 means the experimental data fits the line

perfectly. An R^2 value of >0.9 is considered good. If the R^2 value was less than 0.85 then the data was considered not being accurate enough for Chou Talalay analysis and repeated. If an accurate median effect plot could not be obtained, the drug + HSV1716 combination was analysed by the enhancement of data analysis.

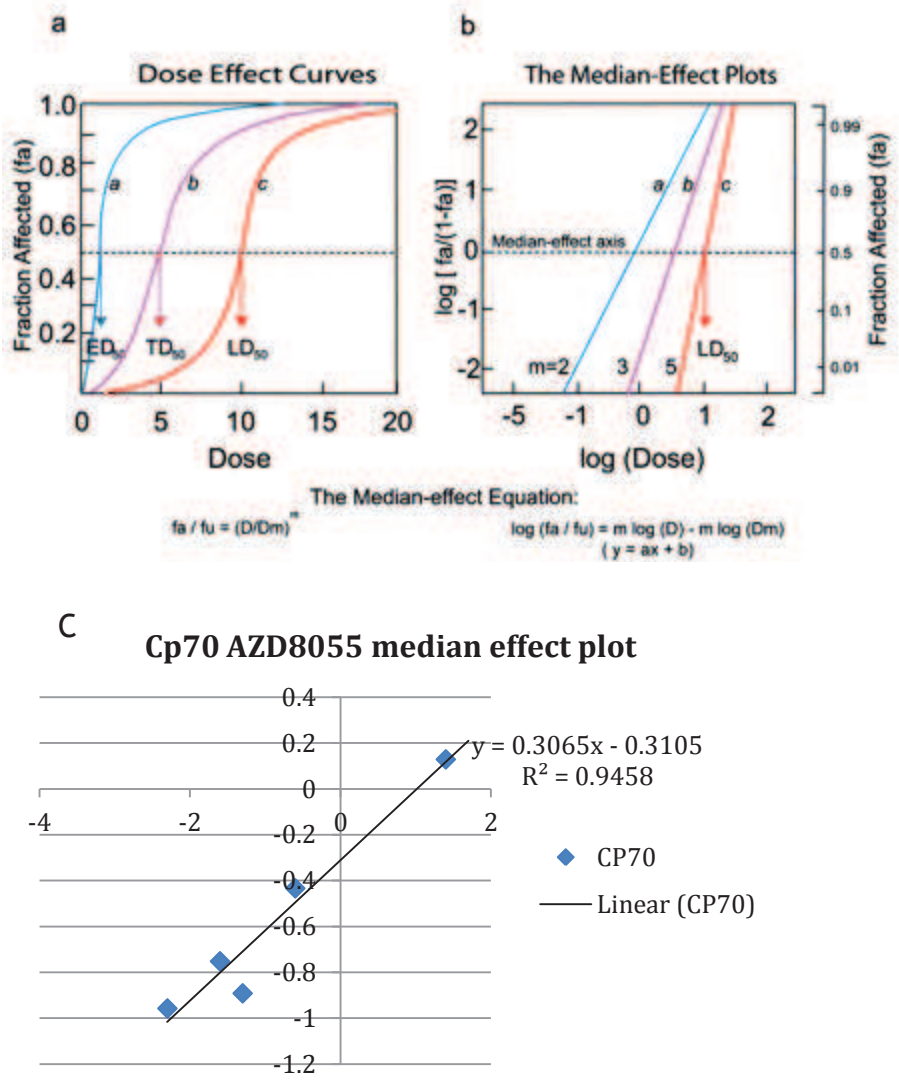


Figure 13: Transformation of various sigmoidal dose effect curves.

(A) Into the corresponding linear forms.

(B) By the median-effect plot, where y = log (f_a/f_u) versus x = log (D). The slopes (in this case, equal to 2, 3, and 5 for curves *a*, *b*, and *c*) signify the degree of sigmoidicity, and the antilogs of the x-intercepts on the axis, where $f_a/f_u = 1$ [or $\log(f_a/f_u) = 0$], give the D_m values, which signify the potency of each drugs.

(C) An example of the median plot produced, for Cp70 cells treated with AZD8055. The R^2 value in (c) is 0.9458. The M value is 0.3065and the Dm is-0.3105. Figures A and B are from (Chou, 2006).

3.2 Combination analysis

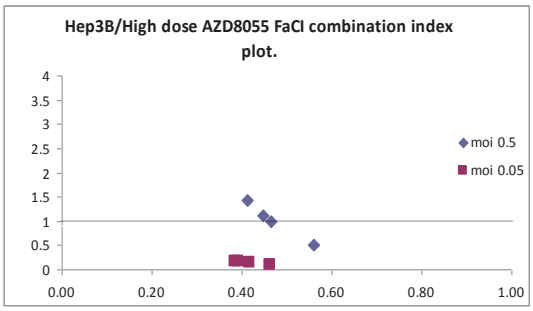
Chou Talalay is the most widely used method of studying drug/drug (or virus/drug) interactions between two modalities *in vitro* (Chou and Talalay, 1981, Chou and Talalay, 1984).This type of analysis is one of the few available that identifies beneficial interactions based on an extrapolated equation. The possibility of predicting a false positive is minimized as the analysis takes account of both the potency (the IC_{50}, LD_{50} or D_M value) and the shape of the dose effect curves (M values) in the precise analysis of two therapeutic combinations. The method defines the expected additive effect of two (or more) agents and quantifies synergy or antagonism by way of how different the measured effect is from the expected additive effect. The equations are detailed elsewhere (Chou and Talalay, 1981, Chou and Talalay, 1984, Chou, 2006). Interpretation of the CI (combination Index) values are defined as: CI=1 indicates an additive effect; a CI of <1 indicates synergy; and a CI > 1 indicates antagonism. Synergy is the working together of two agents to produce a result greater than the sum of their individual effects, while antagonism is less than that of an additive effect. A negative Fa value occurs when the test DCP value is less than the control without any drug, which indicates a decreased cell death, and is therefore scored as antagonistic. Initially the commercially available Compysyn software was used to analyse the data, but in order to streamline the analysis an Excel spreadsheet was designed where the raw DCP values could be pasted in and the spreadsheet would then automatically calculate Fa and CI values and graph the corresponding results from the raw data..

Figure 14 shows an example of Chou Talalay analysis, where synergy betweenHSV1716 + the mTOR inhibitor AZD8055 were assessed in Hep3b and Huh7 cells. Combinations were set up at two HSV1716 MOIs (0.5 and 0.05) and 8 AZD8055 doses. Results were divided into high dose (25, 12.5, 2.5 and 1.25 μM) and low dose (0.25, 0.125, 0.025 and 0.0125 μM) AZD8055 and separate plots along with their respective Fa and CI table of values are presented. HSV1716 in combination with AZD8055 in Hep3B cells (Figure 14A & B) was highly synergistic with 14/16 drug/virus combinations generating CI values <1. The only two combinations not synergistic were 25μM and 12.5μM AZD8055 with HSV1716 at MOI 0.5. HSV1716 in combination with AZD8055 in HuH7

(Figure 14 C& D) cells was highly synergistic with 14/16 drug/virus combinations generating CI values <1. The only two combinations not synergistic were 25μM and 12.5μM AZD8055 with HSV1716 at MOI 0.05.

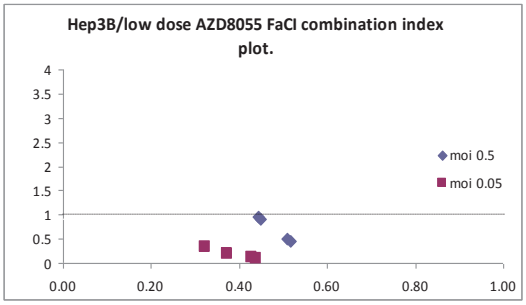
For the initial round of screening, looking at the mTOR inhibitors AZD8055, Ku003 and Temsirolimus, 8 drug doses with 2 MOI of HSV1716 resulting in 16 different combinations were tested. For the rest of the drugs tested, (listed in Table 7) 4 drug doses and 2 virus doses were used, resulting in 8 different combinations points. Synergistic points are scored as a percentage of the total measured, so for example if 6/16 combinations were synergistic this would be given a score of 37.5%.

A



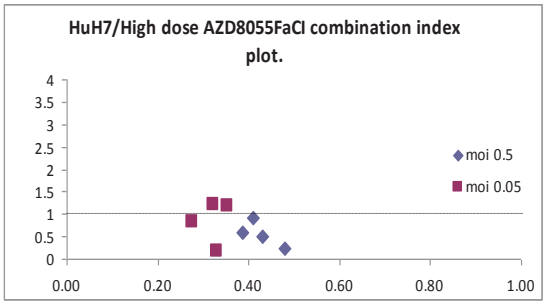
virus moi	drug (uM)	Fa	CI
0.50	25	0.41	1.44
0.50	12.5	0.45	1.12
0.50	2.5	0.46	0.99
0.50	1.25	0.56	0.50
0.05	25	0.39	0.17
0.05	12.5	0.42	0.14
0.05	2.5	0.38	0.18
0.05	1.25	0.46	0.10

B



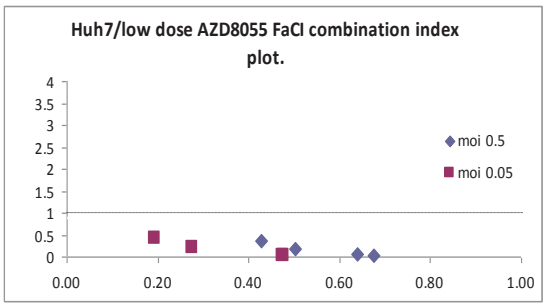
virus moi	drug (uM)	Fa	CI
0.50	0.25	0.44	0.95
0.50	0.125	0.52	0.46
0.50	0.025	0.45	0.90
0.50	0.0125	0.51	0.50
0.05	0.25	0.32	0.34
0.05	0.125	0.43	0.11
0.05	0.025	0.37	0.19
0.05	0.0125	0.44	0.10

C



virus moi	drug (uM)	Fa	CI
0.50	25	0.41	0.76
0.50	12.5	0.43	0.46
0.50	2.5	0.39	0.56
0.50	1.25	0.48	0.23
0.05	25	0.35	1.12
0.05	12.5	0.32	1.12
0.05	2.5	0.28	0.74
0.05	1.25	0.33	0.18

D



virus moi	drug (uM)	Fa	CI
0.50	0.25	0.50	0.19
0.50	0.125	0.68	0.04
0.50	0.025	0.64	0.06
0.50	0.0125	0.43	0.36
0.05	0.25	0.28	0.21
0.05	0.125	0.48	0.02
0.05	0.025	0.48	0.02
0.05	0.0125	0.19	0.41

Figure 14: Chou Talalay plots for HSV1716 in combination with AZD8055

(A& B) Hep3B, (C&D) HuH7. The relevant tables of Fa (x axis) and CI values (y axis) for the individual AZD8055 concentrations and HSV1716 MOI accompany each Chou Talalay plot. If the Fa value was negative then the corresponding CI value could not be determined and CI values above 4 are not presented in the graphs.

3.3 Enhancement of cell death

Chou Talalay analysis depends on both modalities (both drug and virus) being cytotoxic to cells. Many of the drugs that were examined in combination with HSV1716 were cytostatic, preventing cell growth rather than cytotoxic. Despite looking for toxicity across a wide range of concentrations, median effect plots could not be derived within the confines of this screen. As an alternative, another method of analysing the data in which the drug itself was not toxic to the cells, termed 'enhancement of cell death' was designed. Measuring enhancement is based on the drug alone not having any effect in terms of increasing DCP (or cell death) levels on its own. Enhancement as described in this thesis is defined as any increase in cell death seen in the presence of a non toxic drug when given in combination with HSV1716, above the cell death levels seen with HSV1716 alone.

Comparison was made between the percentage of cell death relative to untreated control cells (no virus, no drug) resulting from increasing concentrations of drug alone or in combination with HSV1716. The results are presented graphically and points (referring to each combination point) were scored for enhancement (greater than control) or antagonism (combination less than control). Figure 15 shows a schematic illustration. The drug X alone (blue line) doesn't increase DCP levels above the basal level of untreated cells. HSV1716 is shown at two different MOI, 0.5 and 0.05. Both MOI increase the DCP levels. At MOI 0.05 the level is increased to 2 (double the background) when virus is given alone (no drug). With MOI 0.5 the DCP level increases to 3 (x the background level in the absence of drug). When drug X is given in combination with HSV1716 the levels of cell death increase, in this example to 2.3 and 3.4 respectively, despite the drug having to effect on its own. The drug is therefore assumed to be enhancing the virally mediated cell death.

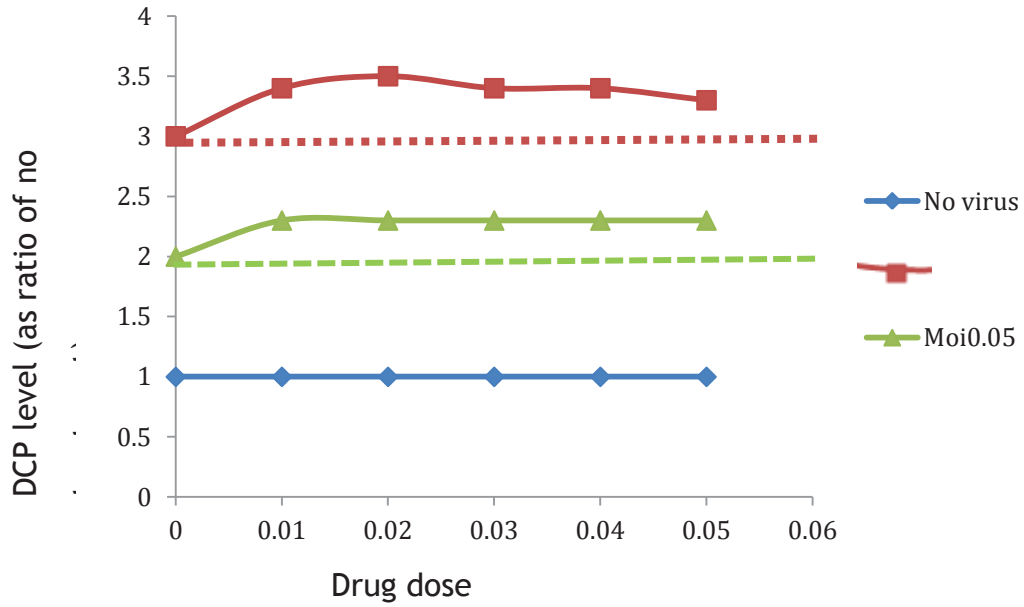


Figure 15: Schematic illustration of enhancement of cell death analysis.

Drug X has no effect alone (blue line), however when given in combination with HSV1716 increased cell killing above the levels expected by HSV1716 alone (dotted lines).

3.4 Heat map of combination analysis between HSV1716 + targeted agents

It was identified early in the process that the results varied from cell line to cell line; hence in order to try and build up as complete a picture as possible, each combination was tested in a number of difference cell lines from the Virttu Cell line panel. A total of 17 agents were assessed for synergy/enhancement with HSV1716 in between 8-10 cell lines, resulting in approximately 170 Chou Talalay /enhancement plots. Rather than detail every drug and cell line separately a 'heat map' (Table 11) was produced. The heat map gives the percentage of synergistic/enhancement of cell death' hits' – for example if a drug had been studied at 4 concentrations using 2 different HSV1716 MOI i.e. = 8 combination points then if 6/8 points were synergistic/enhanced cell death then they would be given a score of 75%. The heat map does not take into account if the synergies were seen at low or high drug concentration, or at low or high MOI.

When taken as a group, mTOR inhibitors + HSV1716 had the most 'hits'. AZD8055 + HSV1716 combination generated some 'hits' in every cell line. In terms of least 'hits' Dovitinib, a FLT3/cKIT inhibitor, and Erlotinib and gefitinib (both EGFR inhibitors) had almost no 'hits', with only 1/8 combination points in Hep3B being synergistic with dovitinib.

VEGFR inhibitors, Sunitinib and Sorafenib, all had 'hits' in 8/10 cell lines, while pazopanib, another similar VEGFR inhibitor had 'hits' in 9/9 lines, although Huh7 only had 1/8 points synergistic.

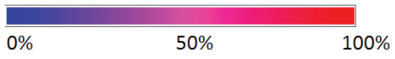
The heat map gives a percentage of the number of combination points measured that were 'hits'. Generally synergies were seen at some drug dose with some virus doses.

Drug sensitivity was charted (Table 12). There does not appear to be any correlation between sensitivity of the cell line to the drug and whether or not it synergises/enhances cell death when given in combination with HSV1716.

drug	target	HuH7	Hep 3B	Hep G2	U87	UVW	one58	SPC111	Ovcar3	SKOV3	CP70
Temsirolimus	mTOR	50	60	30	0	0	10	0	30	90	60
AZD8055	mTOR	88	88	30	75	50	69	63	35	100	30
Ku0063794	mTOR	50	25	13	38	0	50	13	25	45	30
GSK690693	AKT	75	88	nd	0	100	0	0	13	0	13
LY294002	PI3K	13	50	nd	50	100	38	38	50	0	25
Sunitinib	VEGFR	13	38	75	100	0	75	63	63	13	75
Sorafenib	VEGFR	75	63	88	100	0	0	50	63	50	50
Pazopanib	VEGFR	13	nd	50	100	88	63	38	50	75	100
Cabozantinib	cMET/VEGFR	63	50	13	63	63	0	25	38	63	13
Nintedanib	FGF/VEGFR	25	50	68	25	nd	13	25	50	25	nd
Crizotinib	ALK/ROS	38	nd	50	100	75	0	38	13	38	75
Dovitinib	FLT3/cKIT	0	13	0	0	0	0	0	0	0	0
Gefitinib	EGFR	0	63	nd	0	0	nd	0	0	0	0
Erlotinib	EGFR	0	0	nd	50	0	0	13	0	13	0
GSK1120212	MEK	13	0	50	68	100	0	0	0	0	38
LY2228820	p38MAPK	50	0	nd	75	63	0	13	0	63	75
Doxorubicin	chemo	100	13	63	63	63	25	13	nd	38	63

Table 11: Heat map of synergistic points observed by ChouTalalay or enhancement of cell death.

Analysis of 3 HCC, 2 glioma, 2 Mesothelioma and 3 ovarian cell lines when HSV1716 was given in combination with a targeted therapy. The therapy, alongside the target it inhibits is shown. Colours represent the percentages of synergistic/enhanced (shown underlined cell death points measures, from blue where there was no synergy to red where all combinations looked synergistic/enhanced cell death.



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IC50 values		HuH7	Hep 3B	Hep G2	U87	UVW	one58	SPC111	Ovcar3	SKOV3	CP70
Temsirolimus	mTOR	16	nd	0.25	25	25	15	19	25	8	11
AZD8055	mTOR	3	12	25	25	25	25	0.3	4	3	6
Ku0063794	mTOR	1.5	25	25	25	25	25	4	6	7	5
GSK690693	AKT	25	3		7	25	10	10	25	25	12
LY294002	PI3K	3	8		25	25	25	10	8	10	10
Sunitinib	VEGFR	7	5	7	12	15	8	20	13	13	10
Sorafenib	VEGFR	3	3	4	1.5	0.1	8	3	1	2	9
Pazopanib	VEGFR	11	6	3	6	0.3	18	40	40	40	10
Cabozantinib	cMET/VEGR	2.8	4	2.6	15	11	5	8	1.4	15	5
Nintedanib	FGF/VEGFR	40	10	40	20	15	10	15	15	15	40
Crizotinib	ALK/ROS	12	20	5	18	2	13	25	30	20	7
Dovitinib	FLT3/cKIT	0.5	5		2	40	2	5	40	2	2
Gefitinib		4	15		30	15	10	30	20	10	30
Erlotinib	EGFR	1	25		20	1	15	40	17.5	25	10
GSK1120212	MEK	0.6	0.1		25	0.2	25	7	8	25	25
LY2228820	p38MAPK	25	30		17.5	30	15	20	10	6	30
Doxorubicin	MEK	0.2	0.2	0.02	0.7	0.2	0.08	0.15	25	0.6	0.08

Table 12: IC50 values for each drug in µM.

The shading refers to the sensitivity of the cell line with blue representing the lines most sensitive to the drug while red indicates the cell lines which are least sensitive. The shading for each drug is relative to the other cell lines with the same drug.

3.5 HSV1716 + anti cancer agents reduce HSV1716 replication

Within the combination analysis described above, a HSV1716 variant that expresses GFP was used. GFP expression was used as a marker of viral replication. It was noted that despite seeing synergy when HSV1716 and another drug was used in combination, GFP levels (hence viral replication) were decreased in most combinations, suggesting that viral replication was inhibited (data not shown).

In order to confirm the effects of such drugs on the replication efficiency of HSV1716, single step growth kinetic curves were set up at selected drug/HSV1716 combinations as described in the Materials and Methods section 2.7. For virus/drug combinations, the virus yield (input virus dose/output progeny) was determined and compared to virus alone.

3.5.1 MTOR inhibitors effectively inhibit HSV1716 replication

The effect of AZD8055, Ku0063794 and temsirolimus on HSV1716 viral replication was tested in U87MG, Cp70 and SPC111 cells. For SPC111 cells the results are shown graphically in Figure 16. The viral yields are shown in Table 13. In all cases the presence of the drug substantially and significantly decreases viral replication (P values comparing HSV1716 alone vs. HSV1716 + drug are shown in Table 13.In some instances, such as in SPC111 and CP70 cells at 10uM and 1uM AZD8055 and Ku0063794, there was no virus replication (the yield was less than 1, indicating that each input virion did not produce any progeny virions).

3.5.2 Doxorubicin effectively inhibits HSV1716replication

Doxorubicin also inhibited HSV1716 replication in both UVW, which are sensitive to doxorubicin (IC_{50} of 0.2μM Table 12) and the resistant Ovcar 3 cell lines. At 1μM, doxorubicin reduced HSV1716 (input MOI 0.5) replication by 99% in UVW cells and 96% in Ovcar3 cells (Table 13B).

The IC_{50} of doxorubicin in UVW cells was estimated to be approximately 0.2uM, hence all UVW cells at 1μM Doxorubicin would be likely to be killed, hence viral replication is unlikely to occur (as all the cells are dead). However, 1μM doxorubicin in Ovcar3 cells had no effect (in terms of causing cell death) thus the reason for the lack of viral replication is not due to the lack of live cells the virus needs for replication.

3.5.3 Sunitinib effectively inhibits HSV1716replication

HSV1716 replication (at MOI 0.5 only) was tested in the presence of Sunitinib at concentrations 1 & 10μM as described in M&M section 2.7. At these concentrations synergy was observed with HSV1716 (Table 11). 6 cell lines were chosen for analysis, UVW and Skov3 had shown very few synergistic points when Sunitinib was given in combination with HSV1716, while SPC111, one58, Cp70 and Ovcar3 cells had shown a high number of synergistic points. The results are shown in Table 13B. All cell lines HSV1716 replication in the presence of 10μM Sunitinib completely abrogated viral replication. With the lower dose of 1μM Sunitinib was substantially and significantly reduced HSV1716 replication in all lines. In SPC111, Cp70, Ovcar3 and Skov3 the HSV1716 yields in the presence of 1μM Sunitinib decrease by between 50-80 fold, for example in SPC111 the yield decreased from 9300 virions per input virion, to 113 per input virion. The fold decrease in yield in one58 and UVW was lower, with only a tenfold decreased in viral replication. As HSV1716 + Sunitinib combination had a high number of synergistic points, while UVW did not , this fold difference in yields did not correlate with whether Sunitinib is synergistic or not in the cell lines tested.

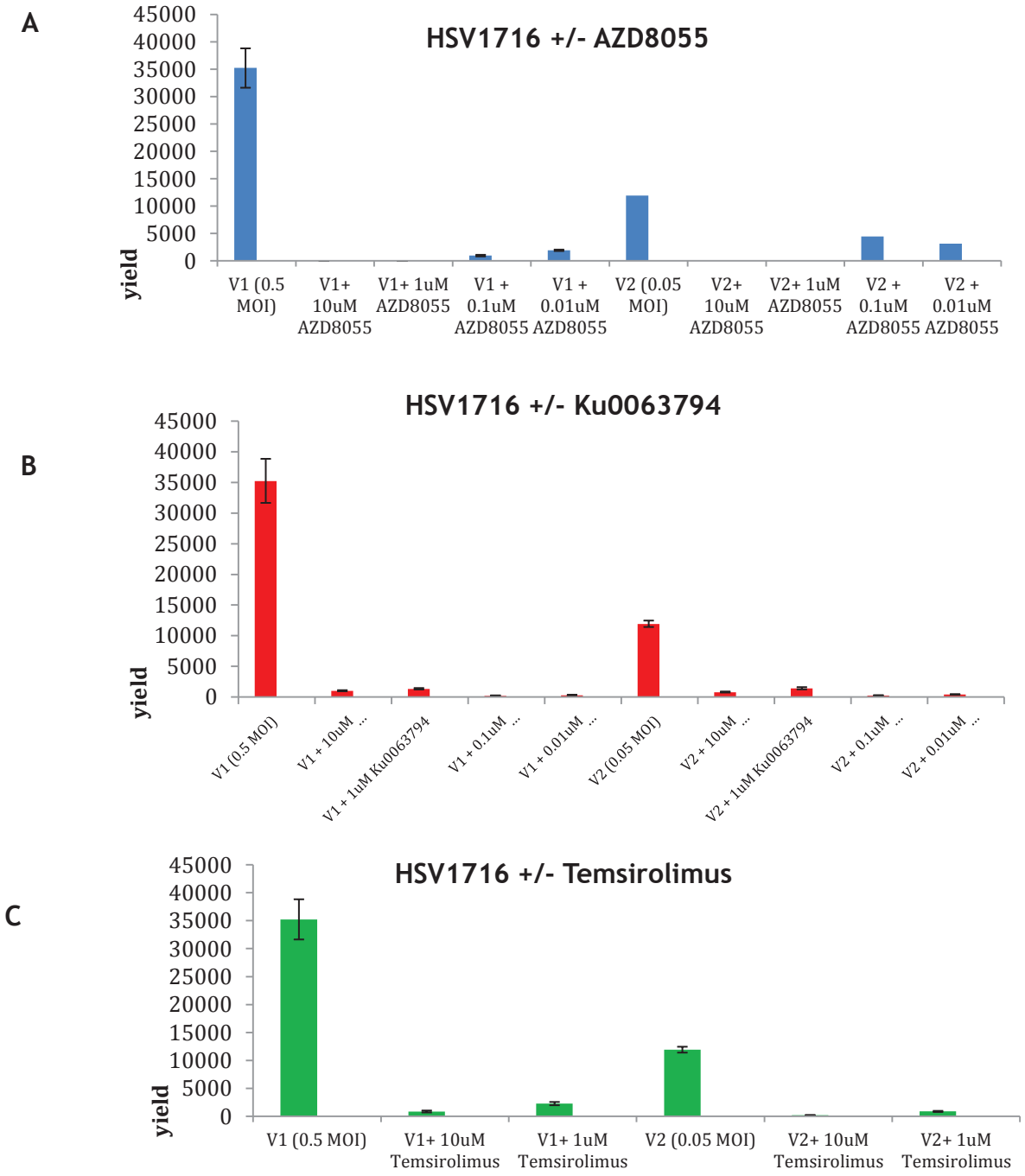

Figure 16: Yields of HSV1716 +/- drugs.

SPC111 cells infected with HSV1716 at MOI 0.5 (V1) or 0.05 (V2) alone or in combination with (A) AZD8055 at 10μM, 1μM, 0.1μM or 0.01μM. (B) Ku0063794 at 10μM, 1μM, 0.1μM or 0.01μM. (C) Temsirolimus at 10μM or 1μM.

Table 13: Yields from SPC111, Huh7 and Cp70 cells infected with HSV1716 +/- Drugs.

Treatment	Average Yield SPC111	P value	Average Yield Huh7	P value	Average Yield Cp70	P value
V1 (0.5 MOI)	35238		88095		2888	
V1+ 10 μM AZD8055	1	<0.0001	292	<0.0001	0.39	0.0004
V1+ 1 μM AZD8055	1	<0.0001	461	<0.0001	0.69	0.0004
V1 + 0.1μM AZD8055	984	<0.0001	9698	<0.0001		
V1 + 0.01μM AZD8055	1952	<0.0001	3444	<0.0001		
V2 (0.05 MOI)	11920		75066		1493	
V2+ 10μM AZD8055	1	<0.0001	840	<0.0001	0.36	<0.0001
V2+ 1μM AZD8055	1	<0.0001	1120	<0.0001	0.78	<0.0001
V2 + 0.1μM AZD8055	4466	<0.0001	9600	<0.0001		
V2 + 0.01μM AZD8055	3133	<0.0001	9333	<0.0001		
V1 (0.5 MOI)	35238		88095		2888	
V1 + 10μM Ku0063794	998	<0.0001	998	<0.0001	0.87	0.0004
V1 + 1μM Ku0063794	1317	<0.0001	1317	<0.0001	0.92	0.0004
V1 + 0.1μM Ku0063794	193	<0.0001	9984	<0.0001		
V1 + 0.01μM Ku0063794	284	<0.0001	13174	<0.0001		
V2 (0.05 MOI)	11920		75066		1493	
V2 + 10μM Ku0063794	786	<0.0001	786	<0.0001	0.73	<0.0001
V2 + 1μM Ku0063794	1400	<0.0001	1400	<0.0001	1.69	<0.0001
V2 + 0.1μM Ku0063794	213	<0.0001	7866	<0.0001		
V2 + 0.01uM Ku0063794	398	<0.0001	14000	<0.0001		
V1 (0.5 MOI)	35238		88095		2888	
V1+ 10μM Temsirolimus	857	<0.0001	1285	<0.0001	71	<0.0001
V1+ 1μM Temsirolimus	2269	<0.0001	2714	<0.0001	113	<0.0001
V2 (0.05 MOI)	11920		75066			
V2+ 10μM Temsirolimus	186	<0.0001	1746	<0.0001	72	<0.001
V2+ 1μMTemsirolimus	866	<0.0001	2973	<0.0001	170	<0.001

Cells were infected with HSV1716 at MOI 0.5 (V1) or (V2)0.05 alone or in combination with 10μM, 1μM 0.1μM or 0.01μM AZD8055, between10- 0.01μM Ku0063794 and 10μM or 1μM Temsirolimus. The average yield of three replicates is shown. The difference between the yield of virus alone vs. virus + drug was analysed by Students T test, with P values shown.

Table 13B: Titres from HSV1716 alone were compared to HSV1716 + drug

Cell line	Treatment	Yield	P value		Yield	P value
Cp70	no drug	553				
	Sunitinib(1μM)	11	<0.002			
	Sunitinib(10μM)	0	<0.002			
one58	no drug	3196				
	Sunitinib(1μM)	282	<0.004			
	Sunitinib(10μM)	0	<0.002			
Ovcar3	no drug	86830		No drug	83333	
	Sunitinib(1μM)	1070	<0.005	Doxorubicin(0.1uM)	60	<0.002
	Sunitinib(10μM)	13	<0.002	Doxorubicin(1uM)	27	<0.002
Skov3	no drug	3370				
	Sunitinib(1μM)	42	<0.003			
	Sunitinib(10μM)	0	<0.002			
Spc111	no drug	9300				
	Sunitinib(1μM)	115	<0.002			
	sunitinib(10μM)	14	<0.002			
UVW	no drug	11016		No Drug	7476	
	sunitinib(1μM)	660	<0.002	Doxorubicin(0.1uM)	6	<0.002
	sunitinib(10μM)	0.	<0.002	Doxorubicin(1uM)	3010	<0.002

These were analysed using Students T test. P values are shown. Values of <0.05 are considered statistically significantly different.

Conclusions chapter III – combination analysis

The first aim of this thesis was to set up a screen that could be used to look at the effect of HSV1716 in combination with a wide variety of drugs in a number of cell lines in cancer types that were of clinical interest to Virttu Biologics. Table 11 summarises the results of HSV1716 in combination with 17 drugs in 9-10 cell lines.

The agents tested were mostly targeted therapeutics that act upon kinase signalling networks frequently upregulated as part of tumourgenesis and therefore block drivers of growth within the cancer cell. Doxorubicin, a chemotherapeutic, is included in the analysis. At the time of this project Virttu was planning a phase I clinical trial in hepatocellular carcinoma. HSV1716 was going to be injected by intra-arterial injection in combination with TACE. TACE or trans-catheter arterial chemo-embolisation combines chemotherapy and small embolic particles that blocks tumour blood supply, acting by both keeping the chemotherapeutic agent at the tumour site, but also stalling tumour growth due to blood supply restriction. As HSV1716 was going to be given in combination with TACE doxorubicin, it was important to study the effects of this agent on viral efficacy.

As most of the agents investigated block drivers of growth within the cancer cell it is perhaps not surprisingly that, in the presence of all drugs examined and detailed in this chapter, these agents almost always completely abrogated production of progeny virions. Despite this, synergy or enhanced cell death was observed with many of the drugs and the reason for such increased cell death is not increased virus mediated lysis of the cells. Other mechanisms of cell death were therefore investigated and are described in subsequent chapters.

At the inception of this project, it was envisioned, perhaps somewhat naively, that the screen may reveal particular cell types where HSV1716 synergised with a specific class of drug. However the results reveal a much more complex picture. Even between similar drugs (for example between mTOR inhibitors temsirolimus, AZD8055 and Ku0063794) the results varied. There are two classes of mTOR inhibitor (Figure 17); rapamycin and its closely related rapalogues including Temsirolimus inhibit the mTOR complex (mTORC) 1 only

via binding to FKBP12 whereas dual mTOR inhibitors (AZD8055 and Ku0063794) bind directly to mTOR in both mTORC 1 and 2. In this thesis there was no synergy between HSV1716 and Temsirolimus in U87MG, UVW, one58 or SPC111 cells, but with HSV1716 + AZD8055, synergy was observed at more than 50% of the points measured in these 4 cell lines. However another dual mTOR inhibitor, Ku0063794 was analysed and the results were different from both AZD8055 and Temsirolimus.

Rapamycin and mTORC1 inhibitors have been reported to induce autophagy, (Sudarsanam and Johnson, 2010), and there are several reports of non HSV oncolytic viruses in combination with mTORC1 inhibitors enhancing autophagy,(Yokoyama et al., 2008, Zhuang et al., 2011). In parallel with this thesis, Anna Claudia Lima, and Leigh McGibbon (both University of Strathclyde MSc/BSC students respectively) carried out projects in collaboration with Virttu Biologics to investigate autophagy both in terms of HSV1716 as a single agent and in combination with other agents such as mTOR inhibitors, in a number of cell lines from the Virttu cell line panel. No potent induction of autophagy by the mTOR/HSV1716 combination was observed and results were presented as posters and shown in Appendix 1. We therefore discounted increased autophagic cell death induced by HSV1716 in combination with mTOR inhibitors as a source of synergy. It also seems more likely that the differences between the three mTOR inhibitors are due to variable off-target effects associated with the kinase inhibitors themselves. The various cell lines will have different dependencies on different signalling networks and therefore be more or less susceptible to inhibition by off target kinases.

Similarly, Sunitinib, Sorafenib and Pazopanib are three kinase inhibitors that principally target VEGFR2, (Table 7). In combination with HSV1716 all three drugs were synergistic at all combinations in glioma U87MG cells. However in the other glioma line in the panel, UVW, only Pazopanib was synergistic while both Sunitinib and Sorafenib displayed no synergy in any HSV1716 + drug combination. Dovitinib is another multi targeted kinase inhibitor that was tested in combination with HSV1716. Dovitinib targets FGFR/cKIT but also has activity against VEGFR/PDGFR and therefore surprisingly, was antagonistic with HSV1716 in all cell lines. The variations between cell lines and the variations between similar drugs suggest that the synergistic action between

oncolytic virus and drugs is not universal, and depends on the cell type and targeted agent The finding that multi targeted tyrosine kinase inhibitors are not all equivalent is not novel. Canter et al., 2011 examined the *in vitro* cellular effects of Sunitinib and Pazopanib (which are used interchangeably in the clinical setting) in a panel of renal cell carcinomas (RCCs) and found that Sunitinib, but not Pazopanib, induced apoptosis and was cytotoxic across the panel tested, while Pazopanib was cytostatic. This indicates that the two agents may have a profoundly different activity, dependent upon the context in which they are used.

As many of these small molecule kinase inhibitors target evolutionary conserved ATP binding sites within the target kinase, many inhibitors are promiscuous and also inhibit off target kinases. Reaction Biology Corp (http://reactionbiology.com/webapps/largedata/) (Anastassiadis et al., 2011) assayed 178 commercially available kinase inhibitors against a panel of 300 protein kinases and found many off target interactions occurred with seemingly unrelated kinases. The resulting Kinase Inhibitor Resource (KIR) data set is available in the public domain and allows users to retrieve the activity of a single inhibitor against the entire kinase panel to look for off target inhibition. Unfortunately, AZD8055 and Ku0063794 are not profiled by Anastassiadis et al., 2011; however Sunitinib, Sorafenib, Pazopanib and Dovitinib all feature. Table 14 shows the kinases that are inhibited by more than 80% by each of the four inhibitors. It is therefore conceivable that the combinations of different kinase inhibitors with HSV1716 generate synergies across the various cell lines via action on an off-target kinase rather than inhibiting the primary target. This would explain the variation amongst closely related targeted agents in cell lines from the same cancer indication. For example, the only kinase that is inhibited by the synergistic inhibitors Sunitinib, Sorafenib and Pazopanib, but not by Dovitinib (which is antagonist with HSV1716) is FMS. FMS, first discovered as the proto-oncogene responsible for Feline McDonough Sarcoma, encodes the tyrosine kinase transmembrane receptor for colony stimulating factor 1 receptor (CSF1R). FMS is homodimeric, contains a kinase insert domain and is a member of the CSF1R/PDGF receptor family of tyrosine-protein kinases. FMS mediates most if not all of the biological effects of CSF1, which control the production,

differentiation and cell function of the monocyte/macrophage lineage (Sherr, 1990). Mutations in FMS have been associated with sustained signals for cell growth and a predisposition to myeloid malignancy (Follows et al., 2005). There are a number of commercially available FMS inhibitors; these have not been tested in combination with HSV1716 yet, but such combinations warrant further investigation.

Thus, further data mining of the KIR resource would hopefully identify off-target kinases consistently inhibited by synergistic targeted agents and these could be screened in the cell line panel. Such analysis would hopefully identify key nodes to target in order to generate synergy with HSV1716. Since these kinase inhibitors also block virus replication, identification of such an important node(s), capable of regulating successful oncolysis will be advantageous in the design of novel next generation variants. For example proteins or microRNAs that augment the key activity could be expressed by an HSV1716 variant, leading to better oncolysis.

The cell lines used in this study could also be useful in identifying the key synergy axes. The mutations within a number of cell lines used in this study are listed in COSMIC (catalogue of somatic mutations in cancer; http://cancer.sanger.ac.uk/cosmic) and this may give information on pathways/signalling networks upregulated in specific cell lines.

Therefore, although a number of additional studies will identify key interactions that generate synergies, further insights will be gained from identifying the underlying mechanisms whereby inhibition of replication in a cancer cell leads to enhanced cell death.

Table 14: Off target kinases that are inhibited by 80% or more by Sunitinib, Sorafenib, Pazopanib or Dovitinib

Sunitinib	Sorafenib	Pazopanib	Dovitinib
ALK			
	ARAF		
ARK5/NUAK1			ARK5/NUAK1
	BRAF		
			BLK
CAMK2a			
CAMK2d			
CHK2			
CK1g2			
c-Kit		c-Kit	c-Kit
			c-SRC
CLK2			
	DDR2		
DAPK2			
FGR			FGR
			FGFR1
			FGFR3
FLT3	FLT3		FLT3
FLT4/VEGFR3		FLT4/VEGFR3	FLT4/VEGFR3
FMS	FMS	FMS	
	HIPK4		
HGK MAP4K4			HGK MAP4K4
KHS MAP4K5			KHS MAP4K5
LCK			LCK
LRRK2			
		KDR/VEGFR2	
			LYN
			LYN B
MELK			MELK
MINK/MINK1			MINK/MINK1
MLCK2/MYLK2			MLCK2/MYLK2
		MLK1/MAP3K9	
		MLK3/MAP3K11	
MST1/STK4			MST1/STK4
PDGFRa	PDGFRa	PDGFRa	PDGFRa
PDGFRb	PDGFRb		PDGFRb
PHKg1			
			PKN1/PRK1
PKCnu/PRKD3			
	RAF1	RAF1	
RET	RET		RET
		ROS/ROS1	
RSK3			RSK3
RSK4			RSK4
TAK1			TAK1
TBK1			
TRKA			TRKA
TRKB			TRKB
TRKC			TRKC
ULK1			
YES/YES1			YES/YES1
	ZAK/MLTK		

Results from queries on (http://reactionbiology.com/webapps/largedata/)

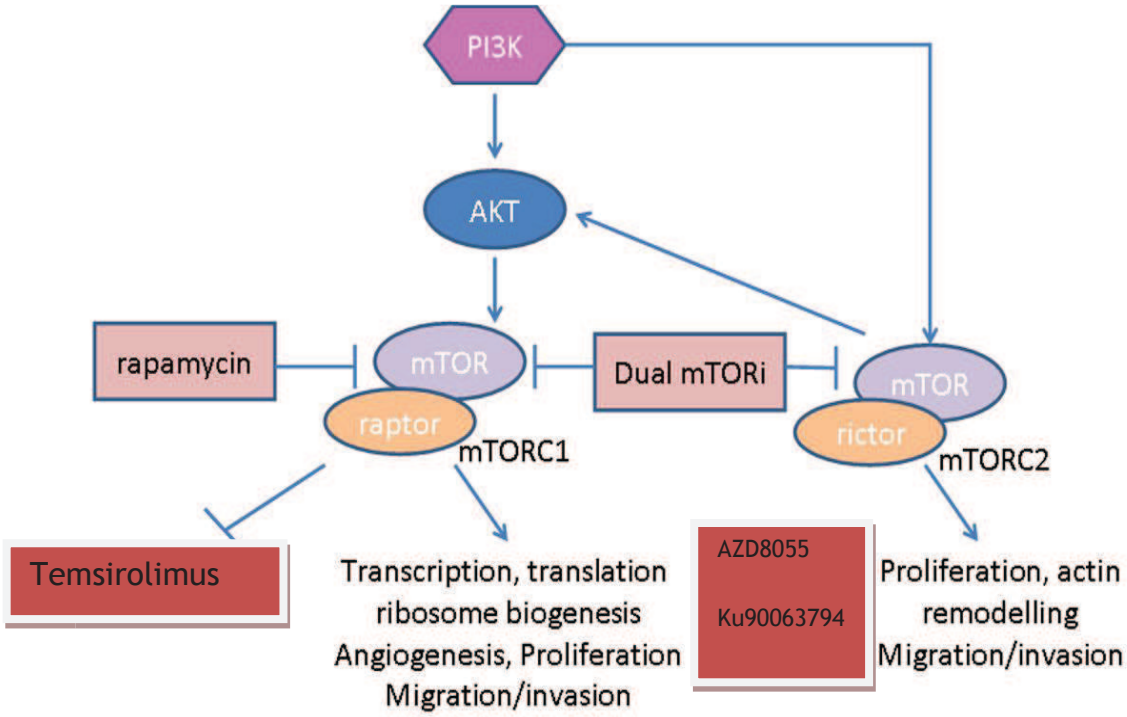

Figure 17: mTOR inhibitors and their targets.

There are two classes of mTOR inhibitors, those which act only on mTOR1 and those that act on both mTORC1 and mTORC2. The mammalian target of rapamycin is a protein kinase of the PI3K/AKT signalling pathway with a central role in controlling cancer cellular growth.

Chapter IV - Modulation of apoptosis

Introduction

Results from the previous chapter show that there is greater than expected amount of cell death when HSV1716 is given in combination with a number of drugs. AZD8055, Doxorubicin and Sunitinib were initially chosen for further study to elucidate the mechanism behind such synergy, based on the fact that all three had shown synergy in a significant proportion of the cell lines. Furthermore, these synergies were not due to increased oncolysis, indeed the opposite, viral replication was substantially reduced in the presence of these drugs. MTOR inhibitors, like AZD8055 are largely cytostatic and exert their anti tumour effect by preventing cells from proliferating. On their own they do not increase cell death.

If late stage viral replication is blocked, the viral proteins that usually would be produced to prevent cell apoptosis are not produced. Therefore the presence of the virus entering the cell may stimulate the cell to die by HSV dependent apoptosis (HDAP) (Nguyen and Blaho, 2009). This HDAP had previously been shown to occur only in transformed cells, and occurs by upregulation of the intrinsic apoptotic pathway (Nguyen et al., 2007a). Using specific caspases inhibitors, Aubert et al, 2007 showed that inhibitors of caspase 9 suppressed HDAP while caspase 8 inhibitors did not, indicating that HDAP occurs through the intrinsic pathway of apoptosis (Aubert et al., 2007).

HSV1716, like wild type HSV-1, retains the ability to express a full complement of viral proteins that are able to inhibit apoptosis of the host cell. In terms of production of progeny virions this prevention of apoptosis by HSV1716 is positive, as it allows complete viral replication before the host cell is killed. However, HSV1716 infection could still result in cancer cell death, by HDAP, if the production of the HSV1716 viral proteins that inhibit apoptosis was prevented. Therefore, anticancer drugs that inhibit viral replication, combined with HSV1716, could enhance cancer cell death by HDAP.

As HDAP is caspase-dependent and occurs via the intrinsic pathway, one way of measuring this enhanced HDAP would be to look for increased levels of specific caspases. Caspase 3/7 levels could be measured to look at increases

in apoptosis, and caspase 8 or 9 could be used to differentiate between intrinsic and extrinsic pathways.

4.1: HSV1716 + AZD8055 – synergistic combinations of HSV1716 and AZD8055 correlates with enhanced caspase 3/7 activity

In order to test if the combination of mTOR inhibitors and HSV1716 are increasing apoptosis, caspase assays (as described in the Materials and Methods section 2.9) were set up. Briefly 96 well plates were set up with 2 rows of each cell type at ~5000 cells/well. After 24 hours in culture, cells were treated with HSV1716 (MOI 1), AZD8055 (5μM) or both and left for 48 hours (in quadruplicate). Replicate plates were set up to measure caspase 3/7 and caspase 8 activities.

Both caspase 3/7 and 8 assays were performed in order to confirm apoptosis and to separate out the intrinsic and extrinsic pathways of apoptosis in 8 cell lines of the Virttu cell line panel. Caspase 3/7 activity was analysed in Huh7 (Figure 18A), Hep3B (Figure 18B) One58 (Figure 18C), UVW (Figure 18D), Skov3 (Figure 19A), U87MG (Figure 19B), Ovcar3 (Figure 19C) and Cp70 (figure 19D).

The difference in the combination treatment was considered significant if it was different to both treatments alone. In all 8 cell lines the caspase 3/7 levels were significantly higher than either treatment alone indicating that the HSV1716 + AZD8055 combination enhances cell death by increasing apoptosis.

The combination of HSV1716 + AZD8055 failed to significantly augment caspase 8 activities relative to virus alone in any of the cell lines. Graphs of the data obtained for each of the cell lines are shown in Figure 20 and Figure 21. In all 8 cell lines HSV1716 + AZD8055 did not increase caspase 8 activity relative to AZD8055 alone. The results are summarized in Table 15. Neither AZD8055 nor HSV1716 alone were strong apoptotic stimulants in tumour cell lines that were tested, based on caspase 3/7 and caspase 8 activity assays. When used in combination, however, HSV1716 + AZD8055 caused a significant increase in apoptosis. This increased apoptosis could explain why the combination of HSV1716 + AZD8055 is synergistic in killing tumour cell lines. As the combination HSV1716+ AZD8055 failed to augment caspase 8 activity it

can be concluded that the increased levels of apoptosis seen in the combination treatment does not act through the extrinsic apoptotic pathway and is likely to be through the intrinsic apoptotic pathway.

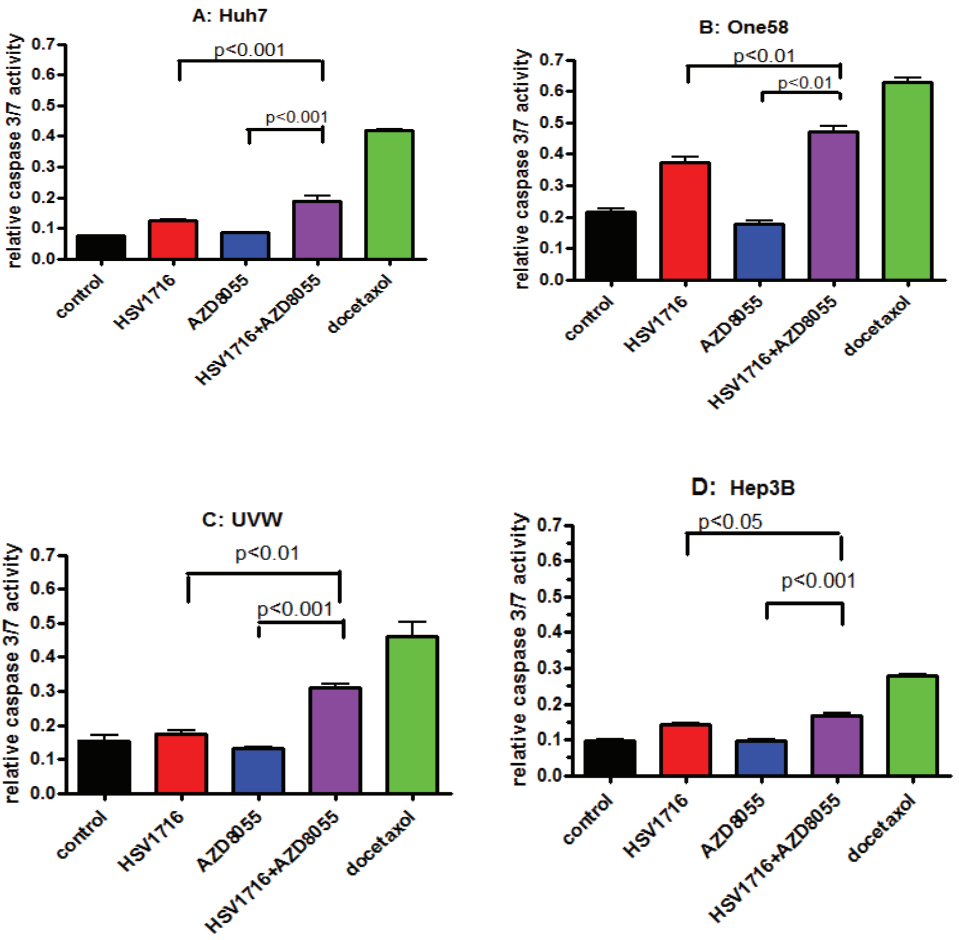

Figure 18: Caspase 3/7 assay in (A): Huh7, (B):one58, (C): UVW or (D): Hep3B cells treated with HSV1716, AZD8055 or both.

Each bar represents the average of at least four separate wells with the error bar representing the standard deviation within the data points. In all experiments docetaxel is used as a positive control. Results were analysed by ANOVA with post test Tukey's analysis which analyses the differences between each group. For each graph the p values are shown, p values < 0.05 are considered to be statistically significant.

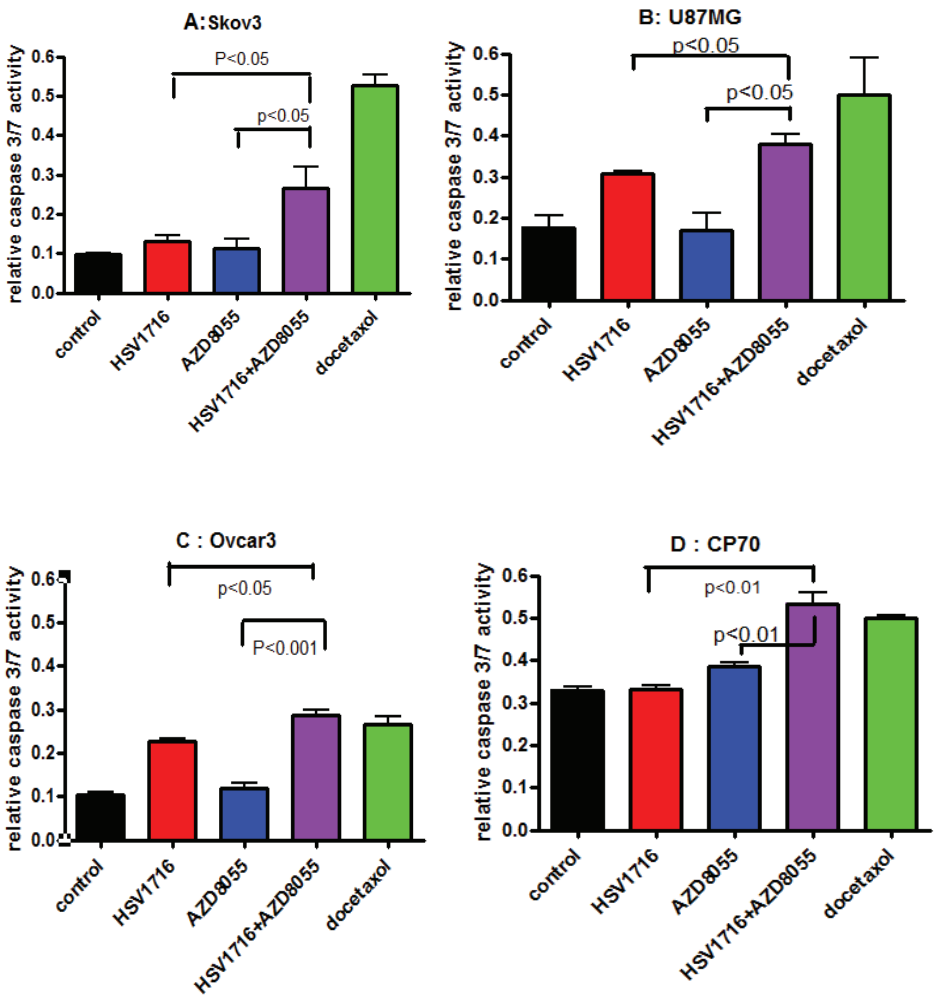

Figure 19: Caspase 3/7 assay in (A): Skov3, (B): U87MG (C):Ovcar3 & (D):CP70 cells treated HSV1716, AZD8055 or both in combination.

Docetaxel is used as a positive control. Results were analysed by ANOVA with post test Tukey's analysis which analyses the differences between each group. For each graph the p values are shown p values < 0.05 are considered to be significant.

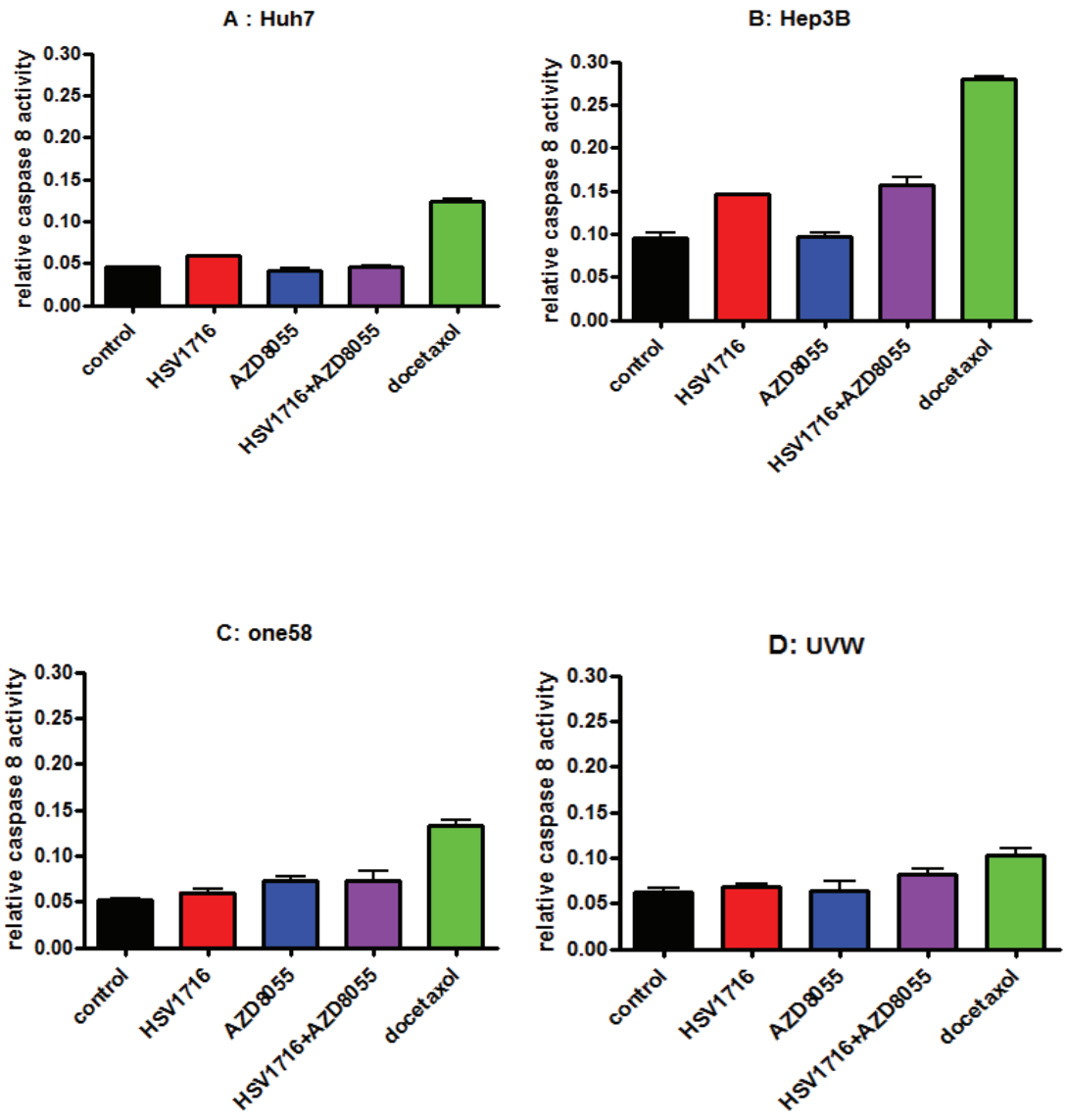

Figure 20: Caspase 8 assay in (A): Huh7, (B): Hep3B (C): one58 & (D): UVW cells treated with HSV1716, AZD8055 or both.

Docetaxel is used as a positive control. Each bar represents the average of at least four separate wells with the error bar representing the standard deviation within the data points. Results were analysed by ANOVA with post test Tukey's analysis which analyses the differences between each group. In each comparison $P<0.05$ indicating that none of the measured differences in caspase 8 activity was statistically significant.

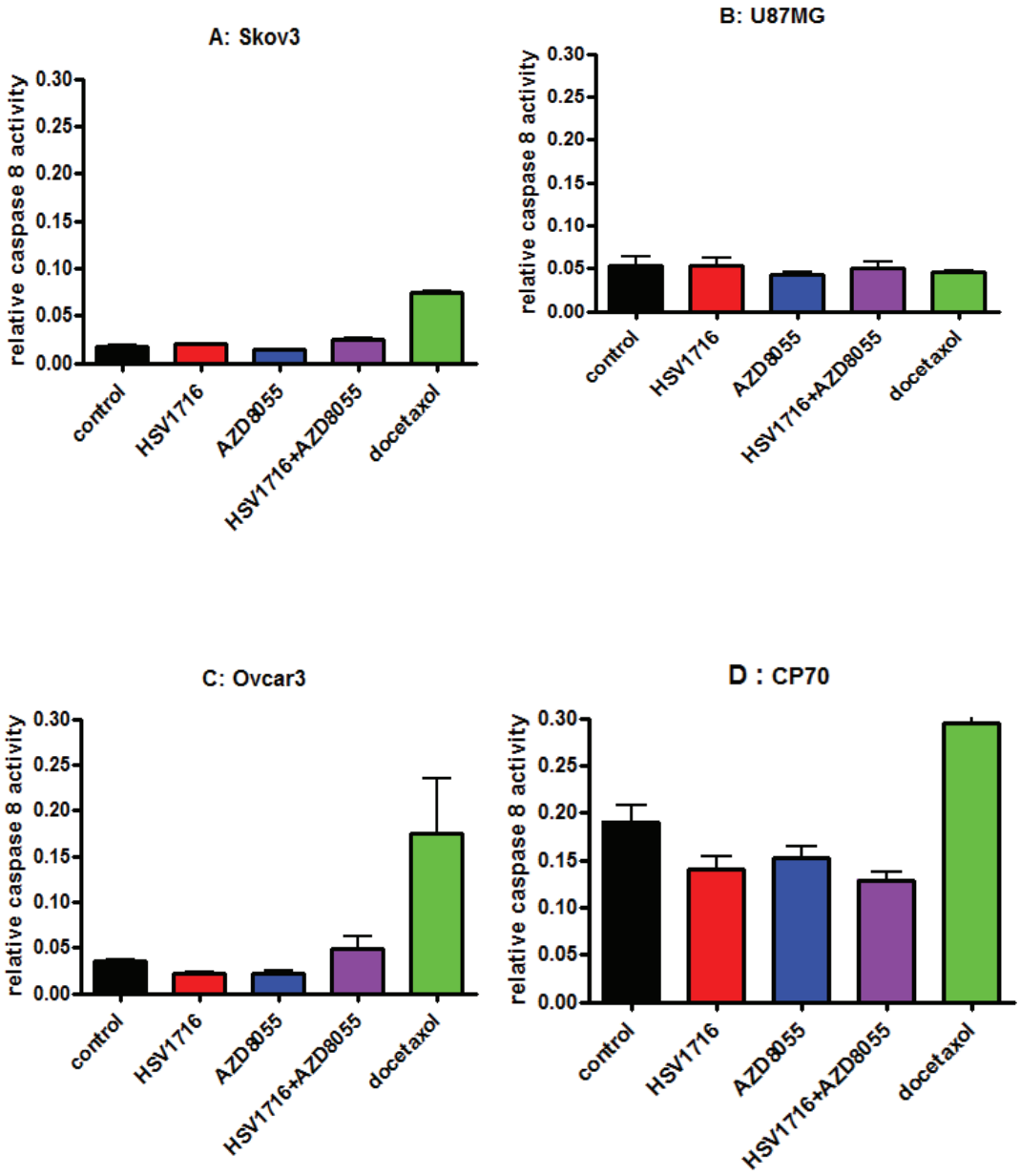

Figure 21: Caspase 8 assay in (A): Skov3, (B): U87MG (C):Ovcar3 & (D): CP70 cells-treated with HSV1716, AZD8055 or both.

Docetaxel is used as a positive control. Each bar represents the average of at least four separate wells with the error bar representing the standard deviation within the data points. Results were analysed by ANOVA with post test Tukey's analysis which analyses the differences between each group. In each case $P>0.05$ indicating that the measured differences in caspase 8 activity in all cell lines was not statistically significant.

Table 15: Summary of caspase 3/7 and caspase 8 activities in Hep3B, HuH7, CP70, Ovcar3, Skov3, U87MG, UVW and one58 cells following treatment with HSV1716 and AZD8055 in combination.

Cell line	Caspase 3/7	Caspase 8
Hep3B	+++	-
HuH7	+++	-
CP70	+++	-
Ovcar3	+++	-
SKOV3	+++	-
U87MG	+	-
UVW	+++	-
One58	+++	-

+++ = significantly increased relative to either or both HSV1716 and AZD8055 alone, + = increased relative to both HSV1716 and AZD8055 alone, - no effect.

4.2: Results – HSV1716 + Sunitinib: synergistic combinations of HSV1716 and Sunitinib correlates with enhanced caspases 3/7 activity

Sunitinib is a small-molecule, multi-targeted receptor tyrosine kinase inhibitor. Results in Table 12 show that the combination of HSV1716 + Sunitinib is highly synergistic in Ovcar3, Hep3B, one58, & U87MG cells but not in Huh7, Skov3 or UVW cells. In all cell lines tested (Table 13**Error! Reference source not found.**) HSV1716 replication was significantly decreased in the presence of Sunitinib indicating the mechanism by which the HSV1716 + Sunitinib combination is synergistically increasing cell death is not due to increased viral replication in the presence of Sunitinib.

Caspase assays were carried out to investigate if the synergistic effect is due to increased apoptosis (Described in Materials and Methods section 2.9). The caspase 3/7 assay measures changes in apoptosis levels and the caspase 9 assays allow differentiation between the intrinsic and extrinsic pathways.

The cell lines Hep3B and Ovcar3, where HSV1716 + Sunitinib were shown to be synergistic by combination analysis, also had significantly enhanced caspase 3/7 activity. Figure 22A and C respectively shows the levels of caspase 3/7 in Hep3B and Ovcar3 while Figure 22B and D shows the caspase 9 activity. Caspase 3/7 is significantly increased in the combination treatment compared to either treatment alone. Caspase 9 activity was significantly increased in both lines tested with HSV1716 + Sunitinib suggesting that the stimulated pro apoptotic response acts primarily through the intrinsic, mitochondria – dependent pathway. The other two cell lines in which HSV1716 + Sunitinib were synergistic by combination analysis (Table 11) (U87MG and one58) were also analysed by caspase 3/7 assay. The results are shown in Figure 23. Again, caspase 3/7 activity was significantly enhanced compared to either treatment alone. In the 3 cell lines in which the combination analysis revealed no synergy between HSV1716 and Sunitinib (UVW, Huh7 and Skov3) there was no increases in caspase 3/7 activity (Figure 24).

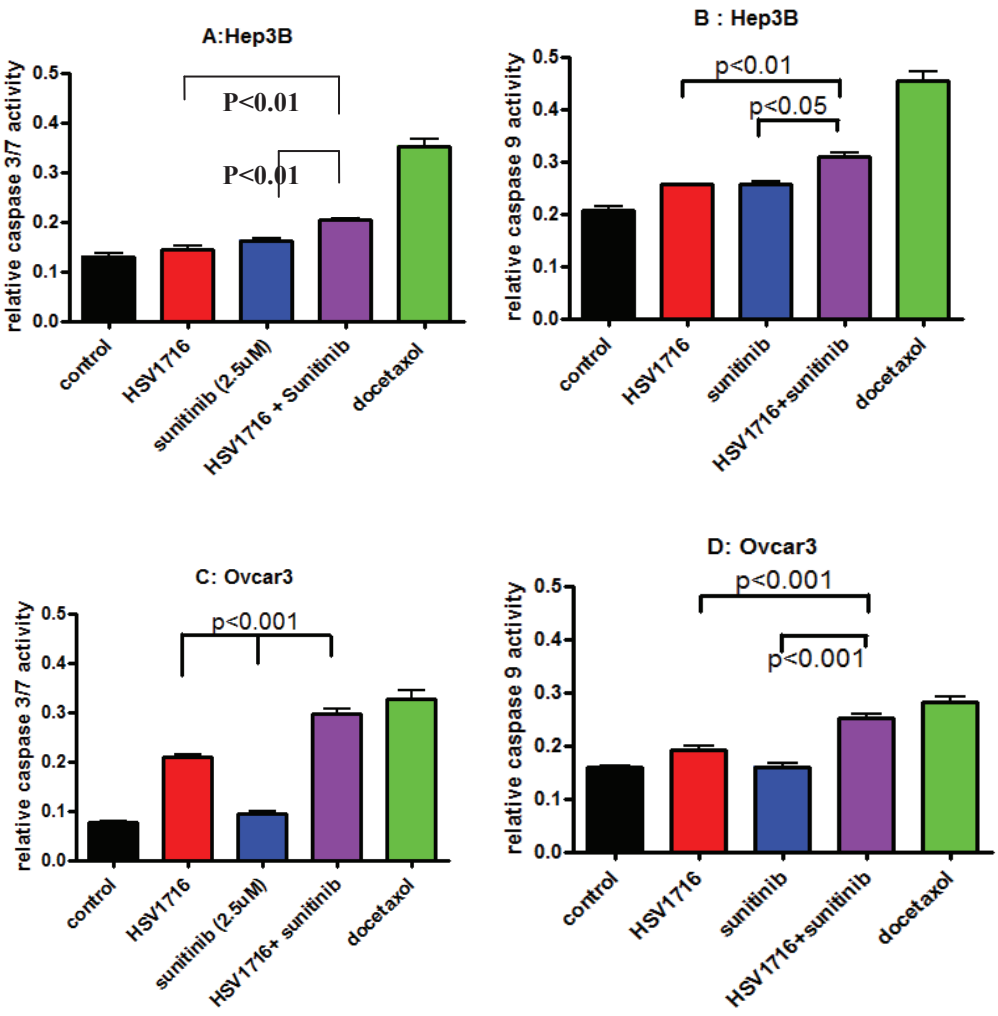

Figure 22: HSV1716 + Sunitinib significantly enhanced caspase 3/7 activity compared to HSV1716 or Sunitinib alone.

(A)Hep3b and (B) Ovcar3.Caspase 9 activity is shown in (C) Hep3b and (D) Ovcar3. Each bar represents the average of at least 4 separate data points. Error bar on graphs representing the standard deviation. Results were analysed by ANOVA with post test Tukey analysis which analyses the differences between each group with P values shown on graphs p values of <0.05 are considered to be statistically significant.

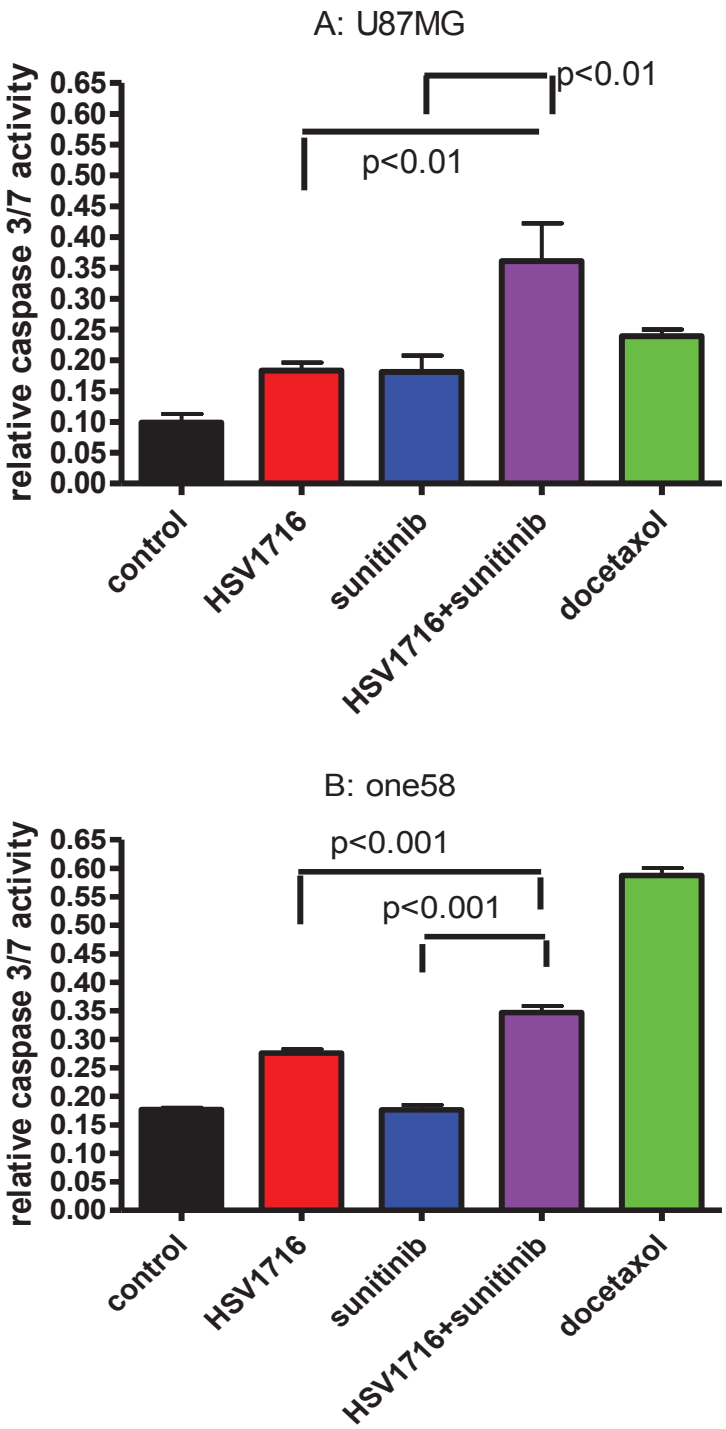

Figure 23: HSV1716 + Sunitinib significantly enhanced caspase 3/7 activity compared to HSV1716 or Sunitinib alone.

(A)U87MG, (B)one58. Each bar represents the average of at least 4 separate data points. Error bar represent the standard deviation. Results were analysed by ANOVA with post test Tukey analysis which analyses the differences between each group with P values shown on graphs p values of <0.05 are considered to be statistically significant.

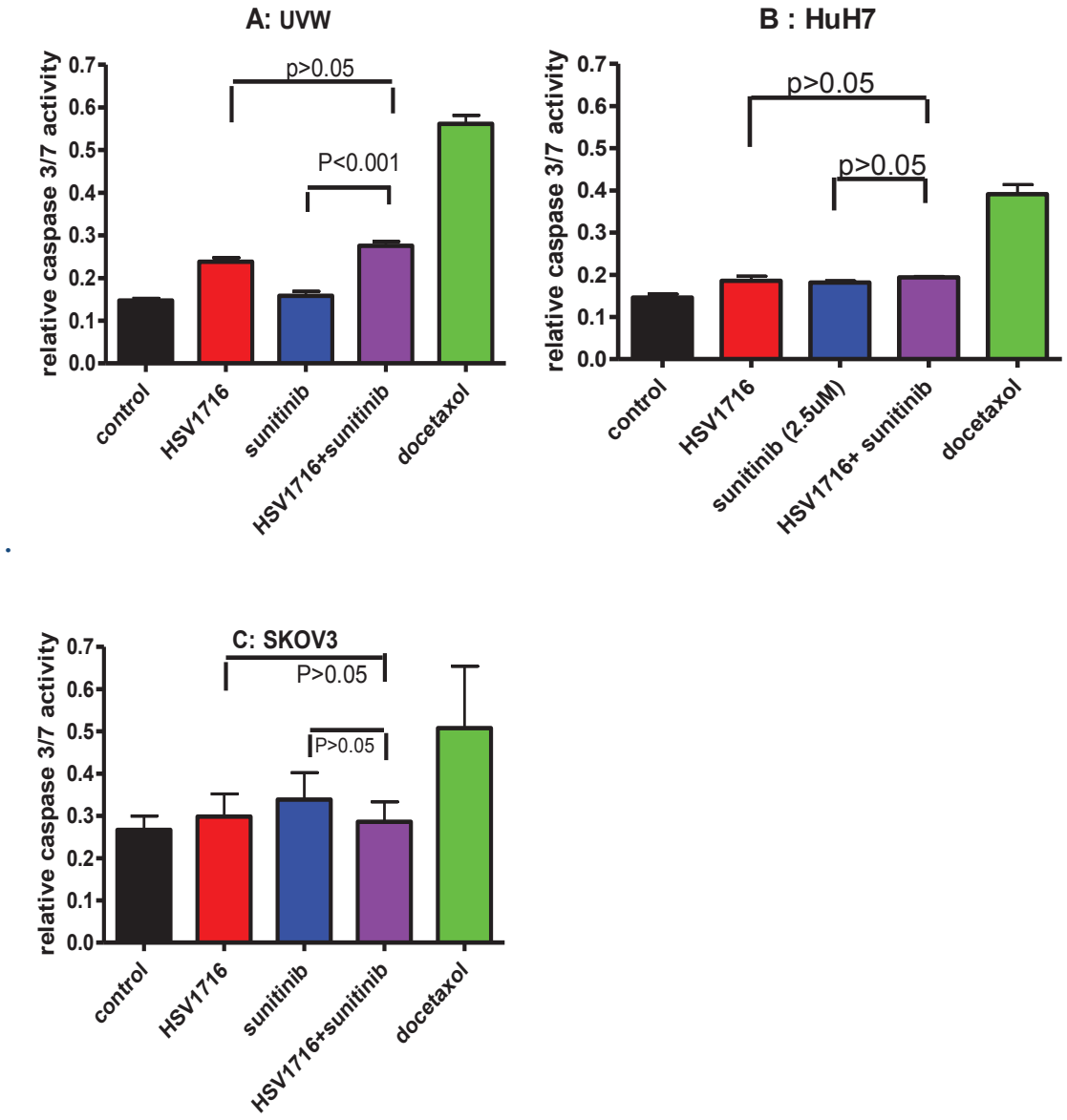

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Figure 24: HSV1716 in combination with Sunitinib does not enhance caspase 3/7 activity compared to HSV1716 or Sunitinib alone.

(A)UVW, (B)Huh7 , (C) Skov3. In all three cell lines there is little or no evidence of synergy between HSV1716 and Sunitinib (Table 11). Each bar represents the average of at least three separate data points with the error bar representing the standard deviation within the data points. Results were analysed by ANOVA with post test Tukey analysis which analyses the differences between each group with P values shown on graphs p values of <0.05 are considered to be statistically significant.

4.3: HSV1716 + Doxorubicin– synergistic combinations of HSV1716 and doxorubicin correlates with enhanced caspases 3/7 activity

Introduction

Doxorubicin is an agent that interacts with DNA by intercalation and inhibits topoisomerase II. By stabilizing the DNA topoisomerase complex after it has broken the DNA chain the DNA double helix is prevented from resealing. The accumulation of such DNA damage leads to apoptosis. In preliminary experiments (Table 11) Doxorubicin and HSV1716 show synergy or enhanced cell death in Hep3b, Huh7 & Ovcar3 cells despite HSV1716 replication being inhibited.

Figure 25 shows relative caspase 3/7 and caspase 9 activity for Hep3B & Huh7 cells treated with HSV1716 + Doxorubicin. In both cell lines there were significantly increased levels of both caspase 3/7 and 9 compared to either treatment alone. As caspase 9 is activated only via the intrinsic apoptosis pathway this suggests that the method by which HSV1716 + Doxorubicin combine to enhance cell death is mediated via this pathway.

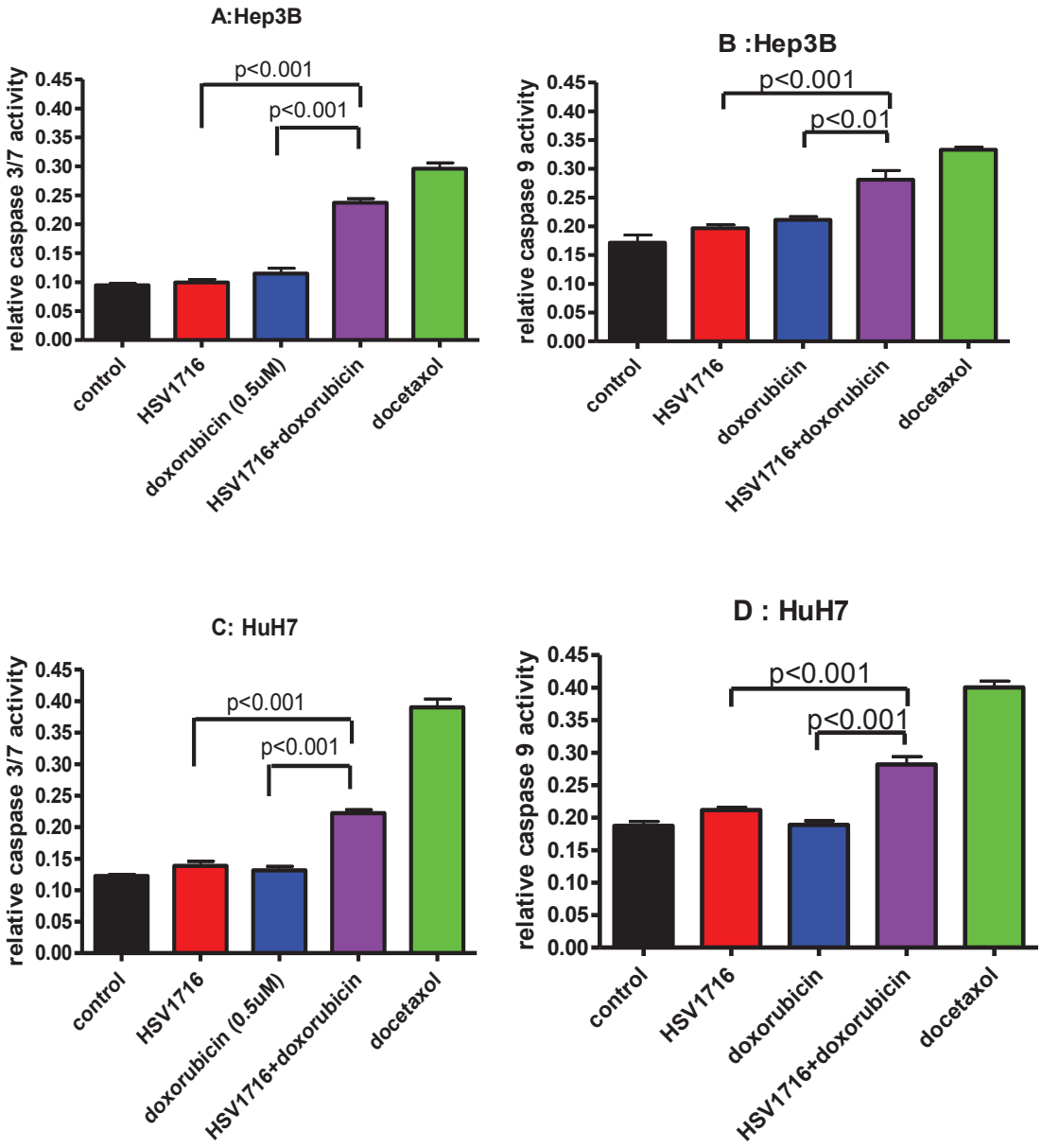


Figure 25: HSV1716 in combination with doxorubicin significantly enhances caspase 3/7 and 9 activity compared to HSV1716 or Doxorubicin alone.

Hep3B (A&B), Huh7(C&D). Each bar represents the average of at least three separate data points with the error bar representing the standard deviation within the data points. Results were analysed by ANOVA with post test Tukey analysis. P values are shown on the graphs, p values of <0.05 are considered statistically significant.

4.4: Dovitinib and Erlotinib do not combine synergistically with HSV1716 and fail to activate caspase 3/7.

The EGFR TK inhibitors Dovitinib and Erlotinib are mostly antagonistic with HSV1716 (Table 11). In order to investigate whether the increase in caspase 3/7 activity was a general result of giving HSV1716 in combination with another drug, caspase 3/7 levels were assessed in cells treated with HSV1716 in combination. Cell lines in which all combination analysis points were antagonistic were chosen. In Hep3B, Hepg2 and Huh7 cells (Figure 26) Dovitinib does not combine synergistically with HSV1716 and the combination failed to enhance caspase 3/7 activity compared to drug or virus alone. Likewise, Erlotinib (Table 11) did not combine synergistically with HSV1716 in Hep3B or Ovcar3 and only at 1 point in Huh7 cells and the combination failed to enhance caspase 3/7 activity compared to drug or virus alone (Figure 27).

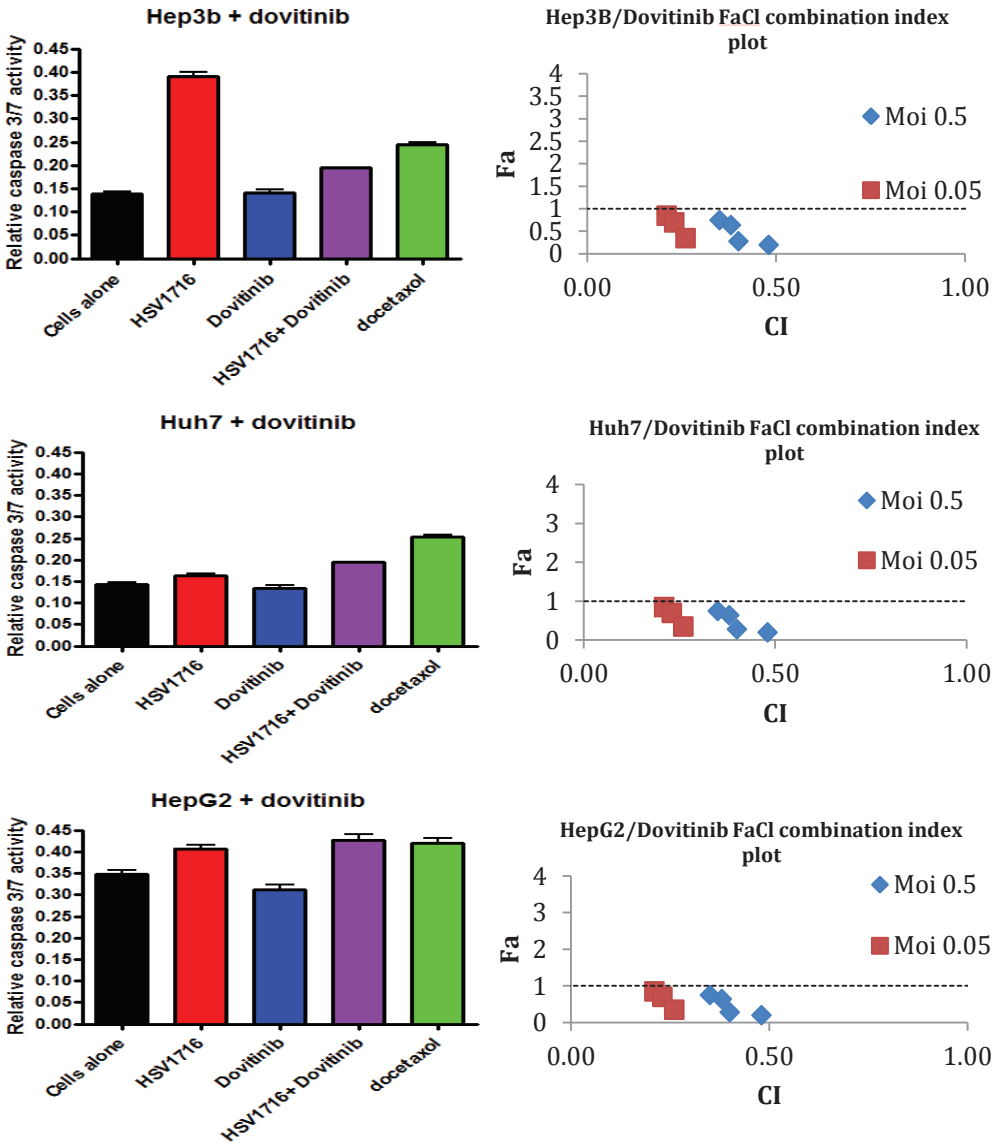


Figure 26: HSV1716 in combination with Dovitinib does not enhance caspase 3/7 activity compared to HSV1716 or Dovitinib alone.

Each bar represents the average of at least three separate data points with the error bar representing the standard deviation within the data points. Results were analysed by ANOVA with post test Tukey analysis which analyses the differences between each group with P values shown on graphs p values <0.05 are considered to be statistically significant.

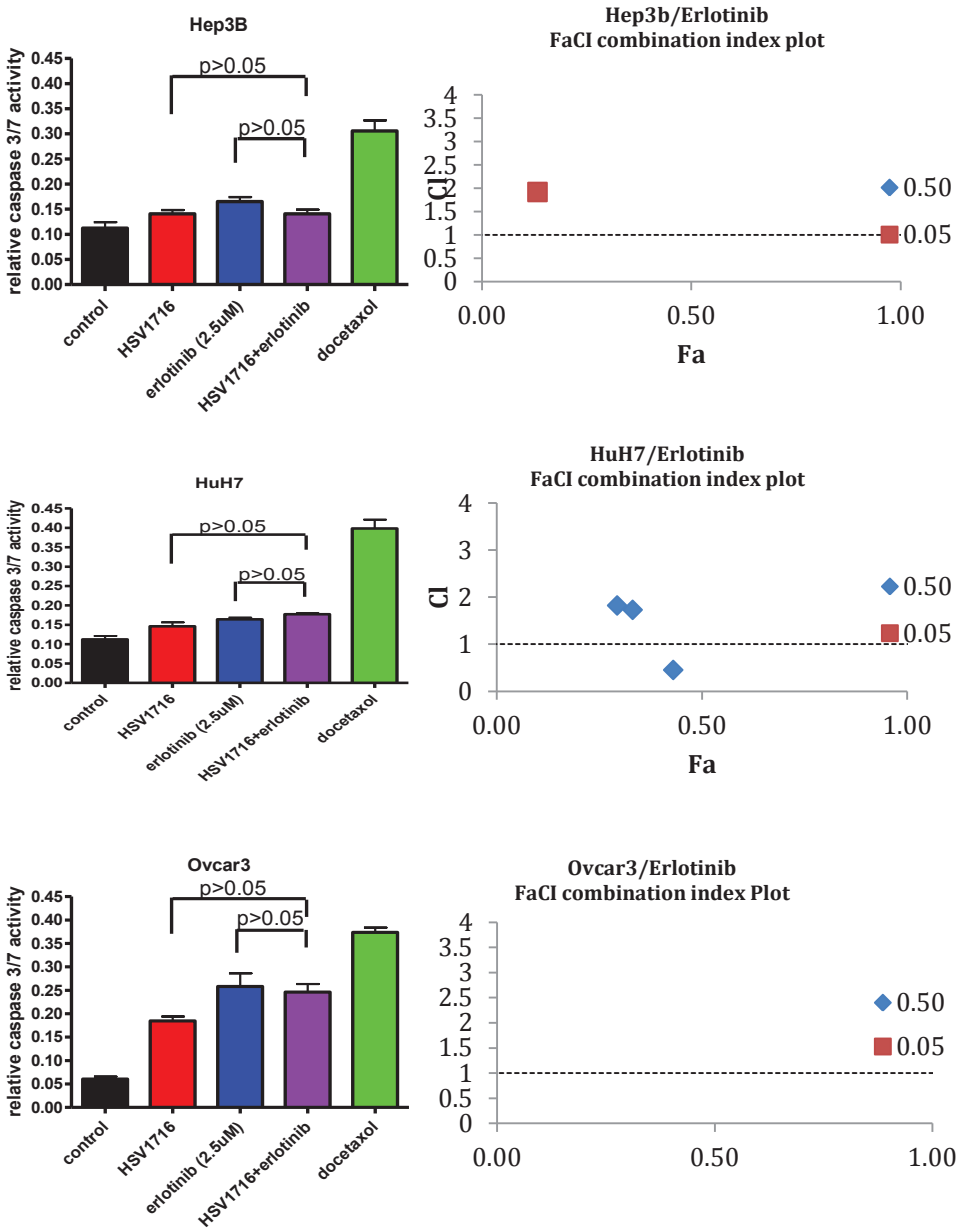


Figure 27: HSV1716 in combination with Erlotinib does not enhance caspase 3/7 activity compared to HSV1716 or Erlotinib alone.

In all 3 cell lines there is little or no evidence of synergy between HSV1716 + Erlotinib. Each bar represents the average of at least three separate data points with the error bar representing the standard deviation within the data points. Results were analysed by ANOVA with post test Tukey analysis which analyses the differences between each group with P values shown on graphs P values of less than $p<0.05$ are considered statistically significant.

4.5: HSV1716 + Sorafenib– synergistic combinations of HSV1716 and Sorafenib does not correlate with enhanced caspases 3/7 activity

Sorafenib is a multi-kinase tyrosine kinase inhibitor that is a standard systemic therapy for recurrent hepatocellular carcinoma. Like Sunitinib it targets VEGFR-2, but also targets Raf-1 and B-raf. Like Sunitinib, the combination of Sorafenib + HSV1716 synergistically enhanced cell death in a number of Virttu cell lines (data not discussed in Chapter III but results are in Table 11). Caspase assays were carried out to investigate if the synergistic effect is due to increased apoptosis (as described in Materials and Methods section 2.9). Figure 28 shows the caspase3/7 assay for Ovcar3, Hep3b and Huh7 alongside their combination analysis plots. Unlike Sunitinib the levels of caspase 3/7 activity in the HSV1716 + Sorafenib combination were not enhanced. This suggests that the mechanism for increased cell death when Sorafenib + HSV1716 are given in combination is not due to increased apoptosis.

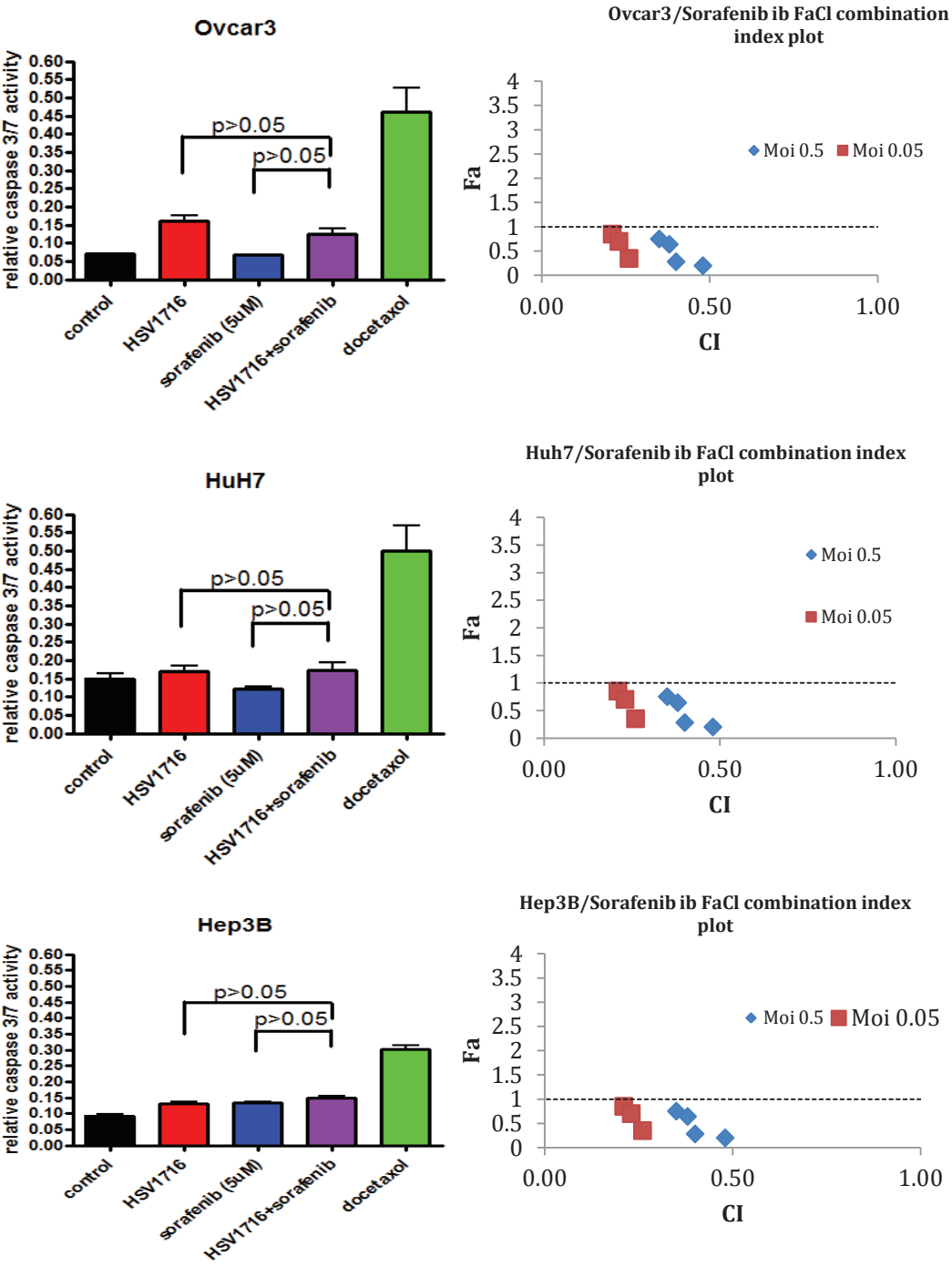


Figure 28: HSV1716 in combination with Sorafenib did not enhance caspase 3/7 activity in cell lines (Ovcar3, Hep3B & Huh7) where synergy was seen in the combination analysis. (B). Each bar represents the average of at least three separate data points with the error bar representing the standard deviation within the data points. Results were analysed by ANOVA with post test Tukey analysis which analyses the differences between each group with P values shown on graphs p values of <, 0.05 are considered to be statistically significant.

Conclusions- Chapter IV apoptosis

Combination analysis described in chapter III revealed that HSV1716 was synergistic with AZD8055, Sunitinib and Doxorubicin, in increasing cell death in a number of the Virttu cell lines despite viral replication being substantially decreased. In order to elucidate the mechanism by which such synergy was occurring apoptosis was investigated.

There is some evidence in the literature that herpes viruses can cause apoptosis of cancer cells, even when viral replication cannot proceed, in a process termed HSV dependent apoptosis (HDAP)(Nguyen and Blaho, 2009) HSV entering a host cell stimulates a host cell response, generally a pro apoptotic one. Usually viral proteins would be produced to counter this response. However, if HSV1716 replication is blocked, as in the presence of drugs, such viral proteins would not be produced and the cell would become apoptotic. This premise of upregulated apoptosis was investigated as a mechanism that could be occurring in these cells in the presence of non-replicating HSV1716 and drugs such as mTOR inhibitors, Doxorubicin and Sunitinib.

The results in this chapter (IV) indicate that, at least in some instances, increased levels of apoptosis correlated with the synergies observed in chapter III. The combination of HSV1716 + AZD8055 in almost every cell line tested was synergistic or enhanced cell death; this correlated with significantly increased levels of caspase 3/7 compared to either treatment alone. There was no such correlation with caspase 8 levels suggesting intrinsic rather than extrinsic apoptosis. Caspase 8 is activated by the extrinsic apoptotic pathway – where a signal from outside – for example a death signal such as TNFα, CD95 or Fas ligand binds to the cell surface receptor, causing cleavage of pro-caspase 8 which in turn cleaves caspase 3.As there is no increase in caspase 8 levels the upregulation of apoptosis is likely to be via the intrinsic, mitochondria pathway.

HSV1716 + Sunitinib synergy also correlated with increased levels of caspase 3/7. In cell lines where this combination was not synergistic, caspase 3/7 levels were not significantly elevated. This correlation was not restricted to targeted therapies as it was also observed with Doxorubicin, a chemotherapeutic that targets DNA. In Hep3B and Huh7 cells, both of which showed synergy with HSV1716 + Doxorubicin significantly increased levels of caspase 3/7 were also

observed. Caspase 9 activation was also examined in the Doxorubicin HSV1716 combination and was statistically significantly higher in Huh7 and Hep3B cells (Students T test $p<0.05$).

Non synergistic drugs such Dovitinib and Erlotinib were assessed in combination with HSV1716 and there was no increase in caspase 3/7 levels.

The following model is proposed in Figure 29. When HSV1716 replication is efficient, the virus is able to produce viral proteins that counteract the cells innate antiviral responses, which include induction of apoptosis. However when HSV1716 replication is blocked, e.g. by an mTOR inhibitor or a tyrosine kinase inhibitor, viral replication and the production of viral proteins to counteract the host response are blocked, therefore the cell can initiate an antiviral response which eventually leads to apoptosis of the cell. The model proposed above requires the virus to have entered the cell and will only be active at high MOIs, especially as the presence of the drug prevents virion production.

There are numerous instances in chapter 3 where the synergy observed was at low MOI in the presence of drugs that are inhibiting viral replication. This would therefore not fit with the above model since HSV1716 has to be present in the cell to initiate the apoptotic pathway. It seems likely that a secondary mechanism is active in these synergies and secreted signals, possibly produced in infected cells and ‘warning’ surrounding uninfected cells, may also be involved.

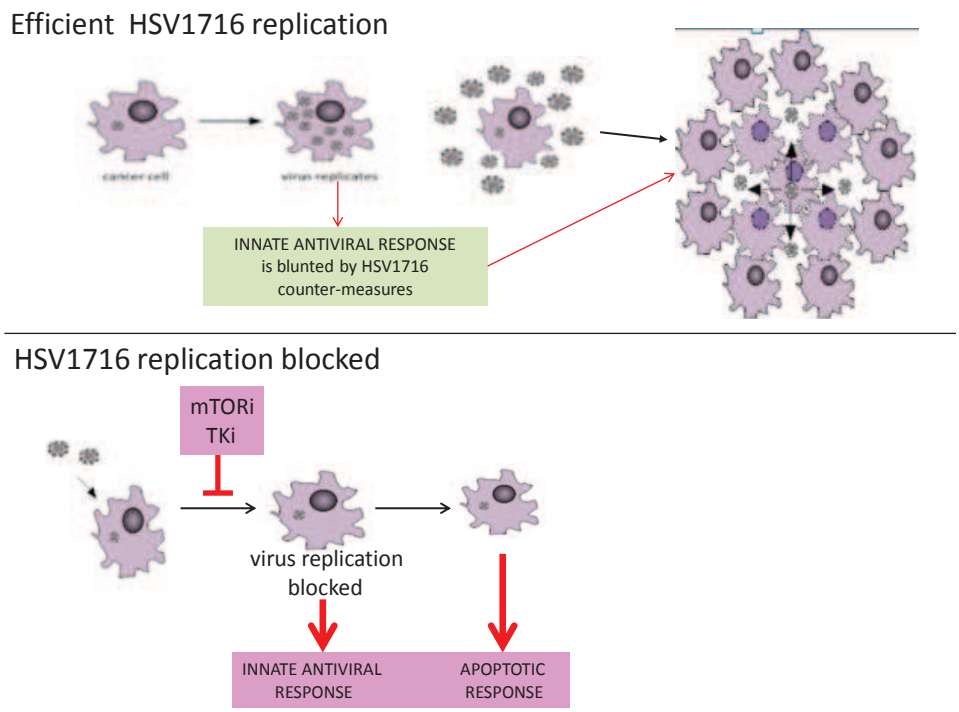


Figure 29: Route of cell death - oncolysis or innate anti-viral response

When HSV1716 replication is efficient, the virus is able to produce viral proteins that counteract the cells innate antiviral responses which includes induction of apoptosis. However when HSV1716 replication is blocked, e.g. by a mTOR inhibitor of a tyrosine kinase inhibitor, viral replication and the production of viral proteins to counteract the host response are blocked, therefore the cell can initiate an antiviral response which eventually leads to apoptosis of the cell.

Additionally, other mechanisms must be involved as Sorafenib was also synergistic with HSV1716 in combination analysis, yet there was not an increase in caspase levels. Sorafenib is a multi-kinase inhibitor that principally targets VEGFR2. It also inhibits PDGFR, Raf-1 and B-Raf. However, unlike Sunitinib, whose principal action is also thought to be through VEGFR2, its synergy with HSV1716 did not correlate with an increase in apoptosis. Reovirus type 3 RT3D in combination with B-raf inhibitors enhanced cell death in a number of cell lines and this was found to be mediated through ER stress induced apoptosis (Roulstone et al., 2015). As ER stress induced apoptosis would also result in increased caspase 3/7 measured, and this was not observed, it is unlikely that the synergy between HSV1716 and Sorafenib described in this thesis is due to increased ER stress-induced apoptosis.

A constitutively active Ras pathway has been reported to prevent the activation of type I IFN mediated anti-viral responses in human cancer cells (Battcock *et al.*, 2006), suggesting that a Ras or Raf inhibition may abrogate this blockade, leading to increases in the IFN-response. An increase in IFN response would block viral replication and it may sensitize surrounding, neighbouring cells to the effects of Sorafenib and account for the enhanced levels of cell death when HSV1716, despite not replicating (Figure 16), is given in combination with Sorafenib.

Chapter V: Mechanisms of synergy of HSV1716 with targeted agents that reduce viral replication.

Introduction

Chapter III examined the combination of HSV1716 + drugs in inducing enhanced levels of tumour cell death. In many instances HSV1716 in combination with another agent acted synergistically, or enhanced cell death in tumour cell lines, despite the drug having a negative effect on viral replication. Further examination detailed in Chapter IV (apoptosis) indicates that the increased cell death observed when some drugs, such as the mTOR inhibitors AZD8055, receptor tyrosine kinase Sunitinib, or Doxorubicin, were given in combination with HSV1716 correlated with increased levels of intrinsic apoptosis. The intrinsic apoptotic pathway, as the name suggests, is stimulated from inside the cell, therefore in order for the virus to up regulate intrinsic apoptotic pathway it must be within the cell. As viral replication is inhibited by the presence of these drugs, increased cell death via the intrinsic apoptotic pathway would be limited to those cells that the initial input dose of virus entered.

However, within the combination analysis many synergies that were observed in Chapter III with HSV1716 and a number of drugs were at low HSV1716 MOI. At an MOI 0.05 only 1 in 50 cells would be infected with the input virus and as the presence of all drugs tested substantially inhibited HSV1716 replication, the majority of the cells within the experiment are unlikely to be infected with HSV1716. Yet despite this, the combination of HSV1716 + drug is either synergistic or enhancing cell death. Therefore, it is likely that another mechanism is at play. In order to elucidate the mechanisms by which a non replicating HSV1716 at a low MOI can be combining with another agent to enhance cell death, experiments were designed to investigate the hypothesis that HSV1716 infection potentiates the anti –tumour effect of other drugs by secreting a virus-derived cell death signal into the microenvironment. This HSV1716 infection related exportable death signal (termed HIRED) could ‘warn’ neighbouring cells of the potential viral infection and coincidentally sensitise these neighbouring cells to the anti tumour effects of drugs.

5.1: Cells infected with HSV1716 produce an 'HSV1716 Infection Related Exportable Death signal (HIRED signal)

Production of Virus Free Conditioned Medium (VF-CM)

Infectious HSV1716 virus will kill cells in culture by lysis. For all cell lines in the Virttu cell line panel (Table 8), except 3T6 cells, HSV1716 replicated well and caused extensive cytopathic effect (cpe) within 72 hours in cell culture. The yields of HSV1716 (amount of progeny virions/initial input virus) produced vary from cell line to cell line, but in all cases spread and propagation of virus would mask the more subtle effect of a secreted death signal.

In order to separate out oncolysis from any 'exportable death signal' produced and secreted by HSV1716 infected cells, virus free conditioned medium (VF-CM) was produced as the basis for these experiments. Donor cells were infected with virus and the medium collected. This VF-CM was then added to non-infected cells to see if it had any effect on cell death. In order to determine if virus was fully removed by the filtration, plaque forming assays (Materials and Methods section 2.7) were performed on VF-CM. In all samples there was no detectable virus after filtration (confirming that the filtration step completely removed virus any effect seen with VF-CM was not due to oncolysis. Results are shown in Figure 11B.

Production of suitable controls

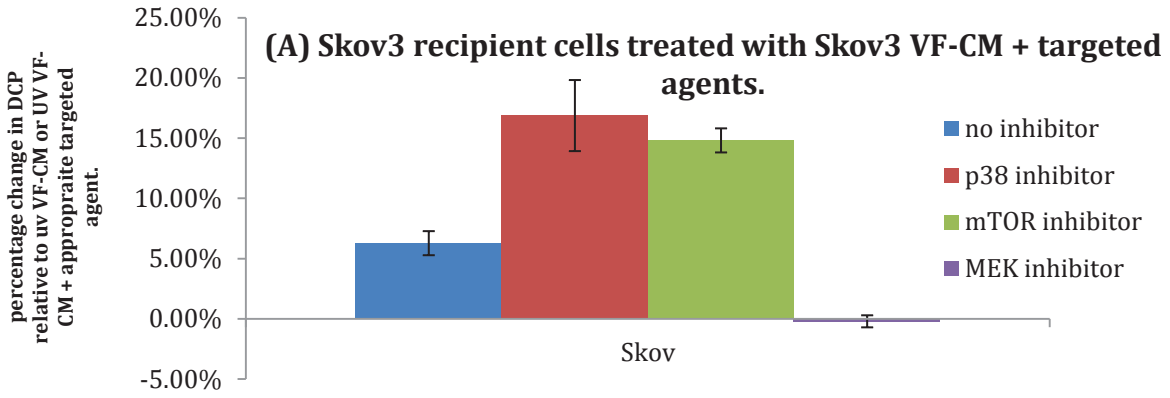
To ensure the conditioned medium transferred from donor to recipient cells did not simply cause increased levels of cell death due to the depletion of nutrients from the medium, equal volumes of fresh medium and conditioned medium were used for all groups in all experiments.

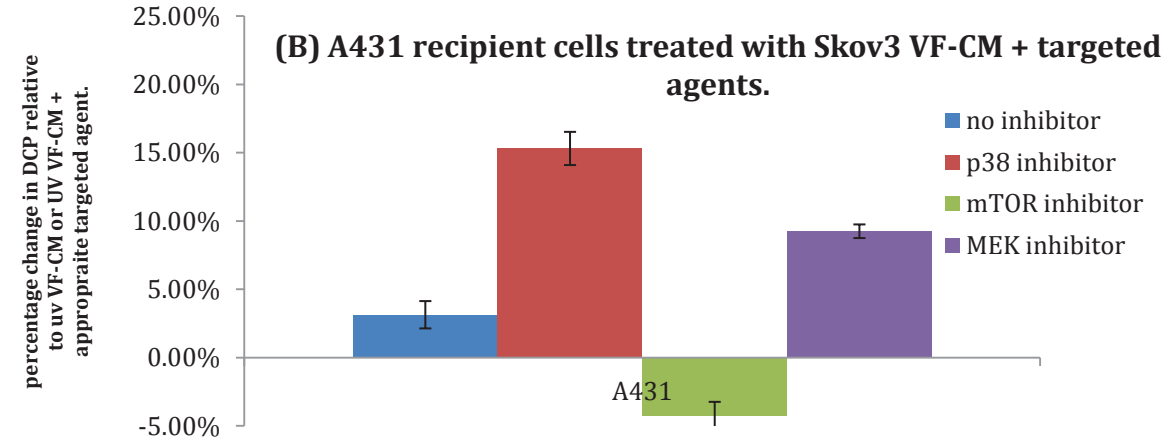
In order to ensure that any exportable death signal was being produced only by replicating HSV1716, the amount of cell death produced by a UV irradiated HSV1716 virus was compared to the amount of cell death seen in HSV1716 infected cells. Exposure to UV light for 15 minutes completely inactivates HSV1716 and UV irradiated HSV1716 had no effect on cell death as measured by DCP (data not shown).

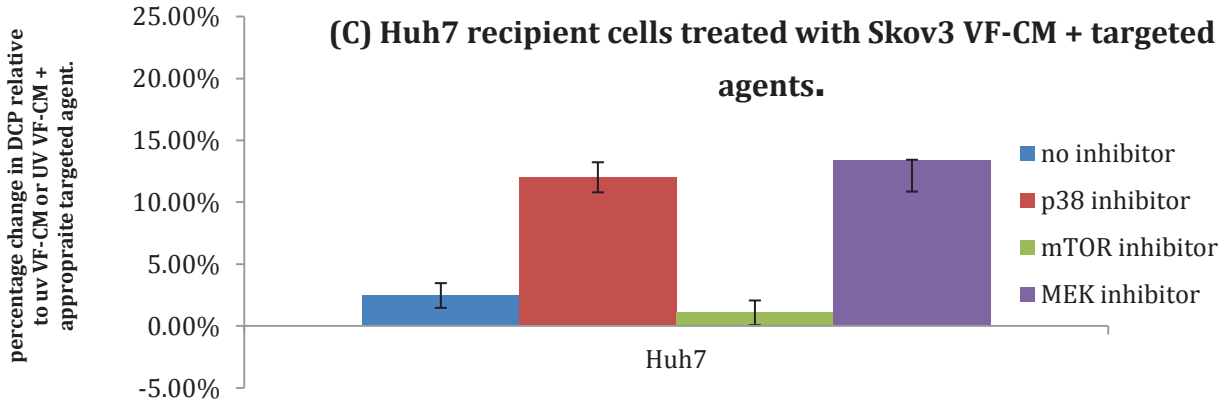
5.2: Virus Free Conditioned medium (VF-CM) from infected HSV1716 Skov3 cells exports a death signal that, in some instances, is enhanced by targeted agents.

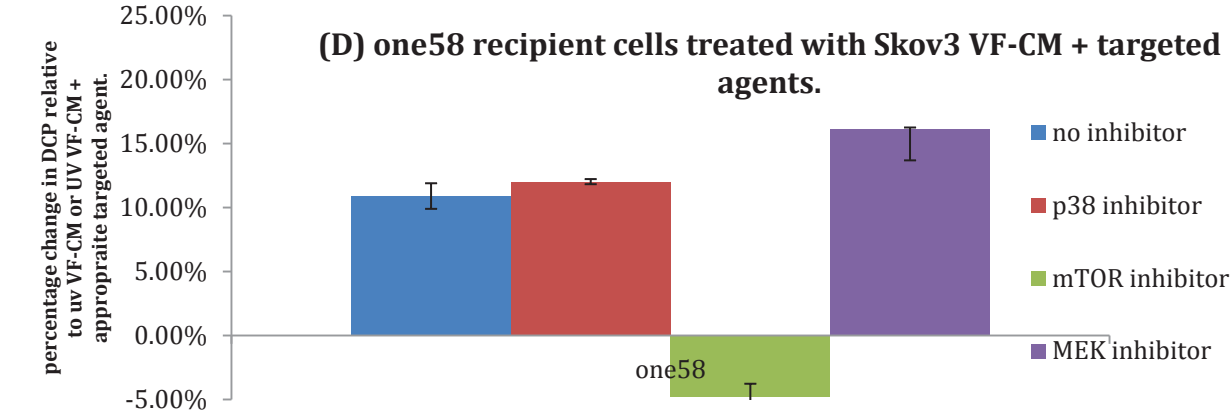
VF-CM was produced in and collected from Skov3 cells (as described in Materials and Methods section 2.10). Skov3 cells were chosen for the initial pilot experiment as they were one of the cell lines within the panel where synergy between HSV1716 and a wide variety of drugs and targeted agents was observed (see Chapter III). The effect of VF-CM alone, or in combination with compounds that specifically inhibited a commonly altered pathway within the cancer cell was measured.

P38 inhibitor LY2228820, the mTOR inhibitor AZD8055 and the MEK1 inhibitor GSK1120212 were chosen as 3 inhibitors that work in different, but key pathways that are often altered in cancer cells. Skov3, A431, one58, Hep3B Huh7 and U87MG recipient cell cultures were set up in 96 well plates and after 24 hours Skov3 VF-CM was added to recipient cells with either no inhibitor, p38 inhibitor (final concentration 1uM), mTOR inhibitor (1uM) or MEK inhibitor (1uM). DCP substrate was added 48 hours later and total luminosity measured as described in materials and methods 2.8. Figure 30 shows recipient cells treated with Skov3 VF-CM alone, or with p38 inhibitor, mTOR inhibitor or MEK inhibitor. Results are expressed as change in percentage of DCP compared to UV VF-CM alone (no inhibitor) or as a percentage increase compared to UV VF-CM + appropriate drug. UV VF-CM + drug was used as a control as this will take into account any effect of the drug on cell death. Without exception all recipient cell lines treated with VF-CM had higher levels of DCP than cells treated with UV irradiated VF-CM (Figure 30). In all cell recipient cell lines cells treated with Skov3 VF-CM + p38 inhibitor had enhanced cell death compared to cells treated with the UV VF-CM + p38 inhibitor (Figure 30A-E). In Skov3 recipient cells (Figure 30A), Hep3B(Figure 30E), U87MG(Figure 30F) cell death in the presence of Skov3 VF-CM + mTOR inhibitor enhanced cell death. In the other three recipient lines A431, one58 and Huh7 (Figure 30B, D and C respectively) mTOR inhibitor did not enhance cell death, but MEK inhibitor did. The results are tabulated in Table 16. **Error! Reference source not found.**


(A) Skov3 recipient cells treated with Skov3 VF-CM + targeted agents.
percentage change in DCP relative to uv VF-CM or UV VF-CM + appropraite targeted agent.
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
-5.00%
Skov
no inhibitor
p38 inhibitor
mTOR inhibitor
MEK inhibitor


(B) A431 recipient cells treated with Skov3 VF-CM + targeted agents.
percentage change in DCP relative to uv VF-CM or UV VF-CM + appropraite targeted agent.
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
-5.00%
A431
no inhibitor
p38 inhibitor
mTOR inhibitor
MEK inhibitor


(C) Huh7 recipient cells treated with Skov3 VF-CM + targeted agents.
percentage change in DCP relative to uv VF-CM or UV VF-CM + appropraite targeted agent.
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
-5.00%
Huh7
no inhibitor
p38 inhibitor
mTOR inhibitor
MEK inhibitor


(D) one58 recipient cells treated with Skov3 VF-CM + targeted agents.
percentage change in DCP relative to uv VF-CM or UV VF-CM + appropraite targeted agent.
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
-5.00%
one58
no inhibitor
p38 inhibitor
mTOR inhibitor
MEK inhibitor

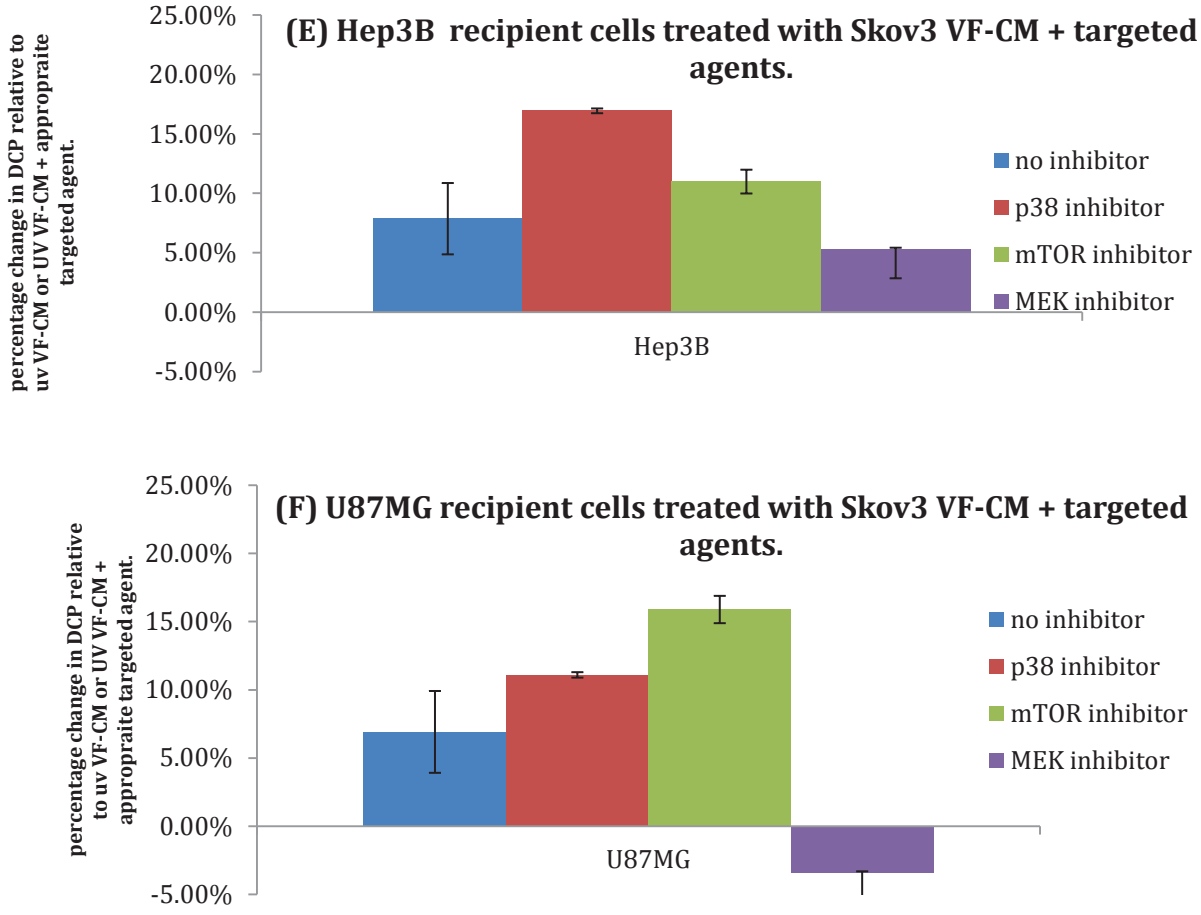


Figure 30: Recipient cell cells treated with Skov3 VF-CM + targeted agents

Results are shown as a percentage change in DCP levels compared the UV VF-CM treated cells (no inhibitor) or the percentage change from UV VF-CM + appropriate targeted agent. DCP levels for each treatment were averaged from at least three replicates. Error bars represent the standard deviation between the replicates expressed as a percentage of the average DCP reading (A): Skov3 recipient cells treated with donor Skov3 VF-CM only, or VF-CM + targeted agent. (B): A431 recipient cells treated with donor Skov3 VF-CM only or VF-CM + targeted agent. (C): Huh7 recipient cells treated with donor Skov3 VF-CM only or VF-CM + targeted agent. (D) One58 recipient cells treated with donor Skov3 VF-CM only or VF-CM + targeted agent. (E) Hep3B recipient cells treated with donor Skov3 VF-CM only or VF-CM + targeted agent. (F) U87MG recipient cells treated with donor Skov3 VF-CM only or VF-CM + targeted agent.

Table 16: Enhancement of death signal exported by Skov3 (Skov3VF-CM) by targeted agents.

	No inhibitor *	P38 inhibitor **	Mtor inhibitor **	MEK inhibitor **
Skov3	6	16	14	0
A431	3	15	-4	9
One58	11	12	-5	16
Hep3B	8	17	11	5
Huh7	2.5	12	1	13
U87MG	7	11	15	3

Enhancement of cell death is shown in light green. No effect/inhibitory effect on cell death are shown in red. * is compared to the UV VF-CM **compared to the UV VF-CM + appropriate agent.

5.3: Virus Free Conditioned medium (VF-CM) from HSV1716-infected 3T6 cells significantly enhances cell death compared to conditioned medium from untreated/UV inactivated HSV1716-treated 3T6 cells

Section 5.1 described experiments carried out in a number of recipient cell lines looking for a death signal produced by HSV1716 infected Skov3 VF-CM that can be exported to uninfected cells and increase cell death in the recipient cells. Increases in cell death when recipient cells were treated with Skov3 VF-CM were seen across a number of different recipient cell lines and in a number of individual experiments. These increases in cell death were modest and failed to reach a statistically significant level. However the experiments were repeated with similar results suggesting the effect is real, albeit the effect is too small to be reliably measured using the techniques described in this thesis.

In order to study the exportable death signal further, VF-CM was produced from a panel of cell lines to look for any donor line in which the enhancement of cell death by VF-CM was much larger and therefore changes in the effect would be more measurable using the DCP as a measure of cell death.

3T6 cells are a cell line derived from Swiss mouse embryos. Unlike all the other cell lines described in this thesis, HSV1716 is unable to productively replicate within 3T6 cells. HSV1716 enters 3T6 cells, and viral protein synthesis is not inhibited but rather there is a block in virus release or egress (Jing et al., 2004).

3T6 VF-CM was produced (as described in Materials and Methods section 2.10). The differences in DCP level between the 3T6 and UV VF-CM for 9 recipient cell lines shown in Figure 31 are highly significant (all are $p<0.001$) by Students t Test.

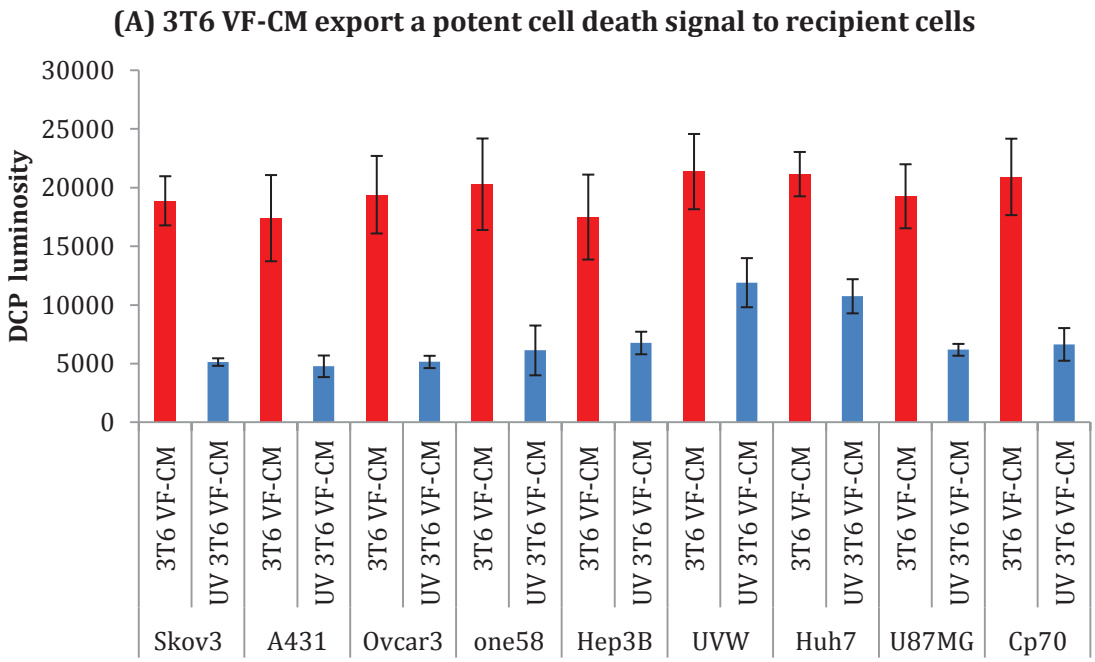


Figure 31: DCP values for VF-CM produced by infected 3T6 cell and added to recipient Skov3, A431, Ovcar3, One58, Hep3B, UVW, Huh7, U87MG and Cp70 cells.

5.4: Consistency of exportable death signal in 3T6 cells

In order to check the consistency of the 3T6 cells to produce an exportable death signal, 3T6 VF-CM was produced from 3 different cell seed stocks from the Virttu cell bank. 3T6 cells were grown up on separate occasions and infected with either UV-inactivated HSV1716 or HSV1716 at MOI of 1 to produce VF-CM. The medium was harvested and filtered. CP70, U87MG & Ovcar3 cells were set up in 96 well plates as described and DCP measured at 48 hrs.

The results for recipient cell lines Cp70, U87MG and Ovcar3 are shown in Figure 33**Error! Reference source not found.**. There is a significant increase in DCP levels when all recipient cell types were treated with 3T6 VF-CM. The changes in DCP levels between batches (as measured in the same cell line) were not significant. Cells treated with UV irradiated virus had similar DCP levels to mock-treated cells.

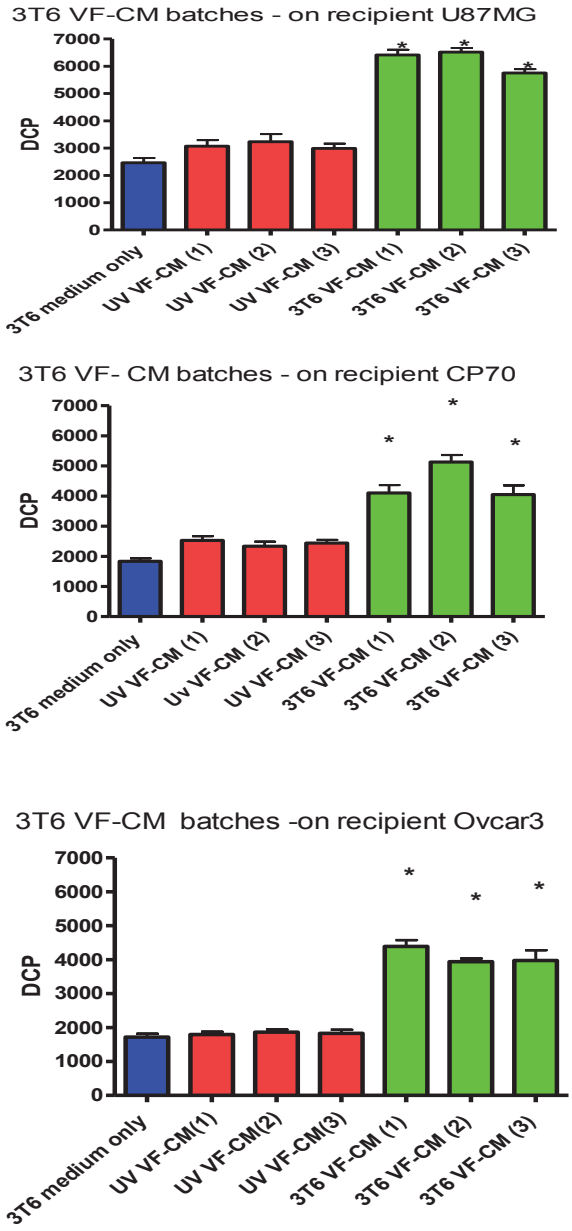


Figure 32: Consistency of exportable death signal produced by HSV1716 infected 3T6 cells and added to Cp70, U87MG or Ovcar3 cells.

3T6 medium only – medium from 3T6 cells only (no virus added), UV VF-CM: 3T6 cells treated with UV irradiated HSV1716. 3T6 VF-CM (1, 2 and 3): 3T6 cells infected with HSV1716 at MOI of 1, harvested at 48 hours and VF-CM produced. 1, 2, 3 represent the VF-CM, produced by different cell stocks and made at different times. DCP (y axis) is total luminosity. * P value < 0.05 by ANOVA (one way analysis of variance).

5.5: Cell death induced by 3T6 VF-CM is MOI dependent

Donor 3T6 cells were infected with HSV1716 MOI of 1 to produce 3T6 VF-CM (Figure 31). This VF-CM significantly increased cell death in non HSV1716 infected recipient cell lines. The results were consistent across 3 experiments, using different batches of 3T6 cells to produce the death signal, and consistently increased cell death on all recipient cell lines tested (Figure 32). The effects of infecting the donor cells with higher and lower MOI of HSV1716 were assessed. VF-CM was produced as described in Materials and Methods section 2.10 but using differing MOI (from 5 -0.1) of HSV1716 were used to make the VF-CM. The various VF-CM were plated out on recipient cells as described previously and DCP assays were preformed (Materials and Methods section 2.8). The results are shown in Figure 33. In all four recipient lines, cell death was maximal when the highest dose of HSV1716 was used to produce the VF-CM. For all 4 recipient lines, VF-CM produced with MOI of 0.5 or higher significantly enhanced cell death in non-infected recipient cell lines ($P<0.05$ vs. 3T6 UV).

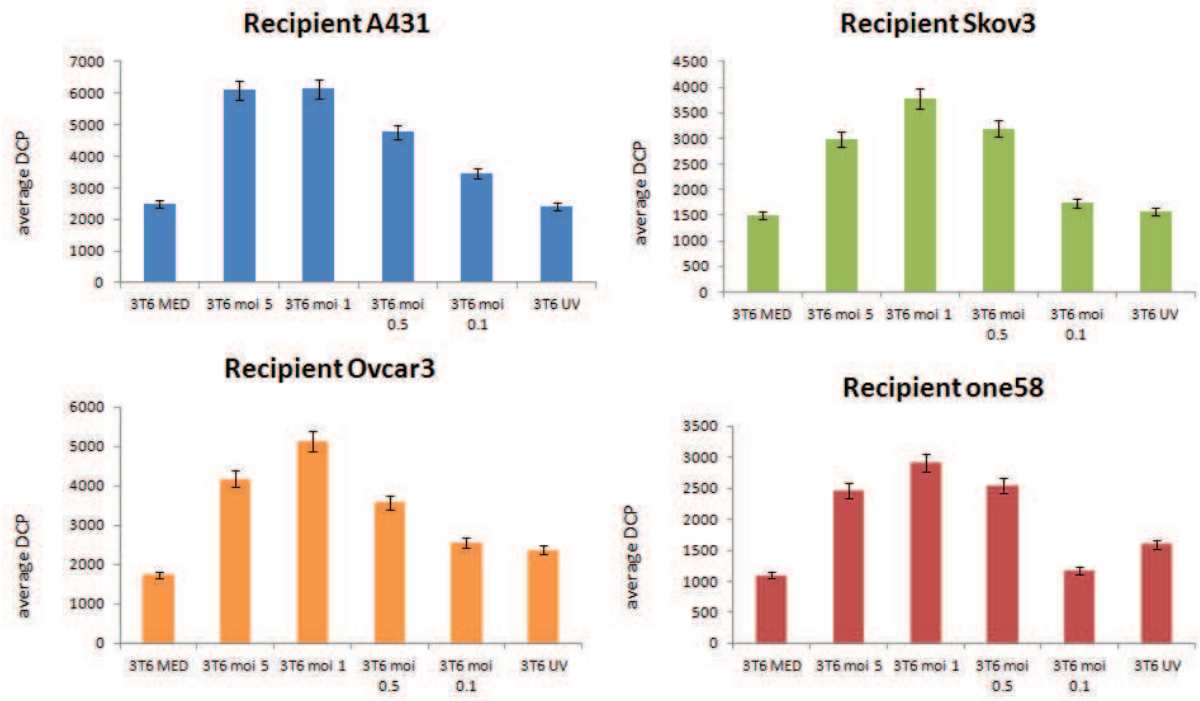


Figure 33: 3T6 VF-CM induced cell death on non infected recipient cell lines is more potent when higher MOI of HSV1716 are used to produce the 3T6 VF-CM.

3T6CM is medium collected from 3T6 cells. 3T6 UV is UV VF-CM produced in 3T6 cells.

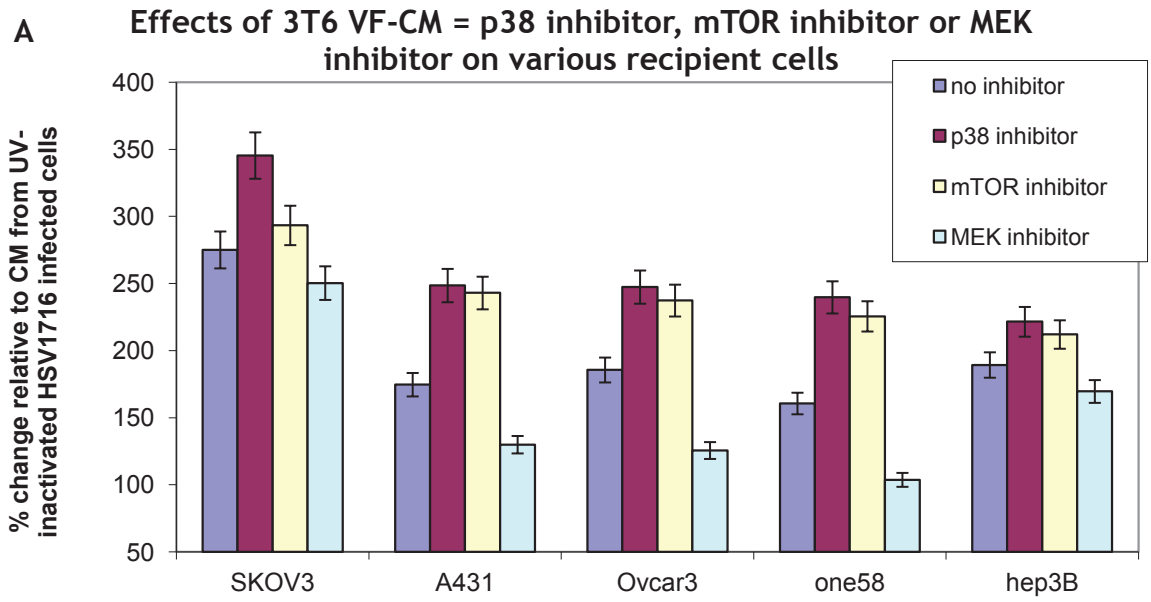
5.6: VF-CM from infected HSV1716 3T6 (3T6 VF-CM) contains a death signal that is enhanced by p38 inhibitor and mTOR but not MEK inhibitors.

3T6 VF-CM described in section 5.3 was also tested in combination with p38, mTOR and MEK inhibitors in recipient Skov3, A431, Ovcar3, one58 & Hep3b cells (Figure 34). The results show the percentage change in DCP levels compared to the DCP levels of the UV VF-CM + appropriate inhibitor. The UV VF-CM + drug was used as a control in order to account for any cell death causes by drug alone. In all 5 recipient lines, p38 inhibitor significantly increased the levels of cell death compared to 3T6 VF-CM alone (Students T test no inhibitor vs. P38 inhibitor, $P<0.01$ in all cell lines).

In all five recipient cell lines the addition of mTOR inhibitor increased cell death relative to VF-CM alone. This difference was statistically significant in A431, Ovcar3 and one58 cells ($P<0.05$: students T test no inhibitor vs. MTOR inhibitor) but the difference failed to meet significance in Skov3 and Hep3B.

The presence of MEK inhibitors in combination with 3T6VF-CM in all five recipient lines reduced the levels of cell death relative to 3T6 VF-CM alone. The results are summarised in the table in Figure 34. The death signal exported by 3T6 VF-CM is enhanced by p38 inhibitors and mTOR inhibitors in some recipient cell lines but not by MEK inhibitors. In the presence of MEK inhibitors the death signal from the 3T6 cells is reduced, suggesting that the MEK pathway is possibly required for the 3T6 exported death signal to have an effect.

A



B	No inhibitor *	P38 inhibitor **	mTOR inhibitor**	MEK inhibitor **
Skov3				
A431				
Ovcar3				
One58				
Hep3B				

Figure 34 (A): Skov3, A431, one58, Ovcar3 & Hep3B recipient cells treated with 3T6 VF- CM in combination with p38, MTOR or MEK inhibitor.

Results are shown as percentage increase compared to UV VF-CM or UV. Error bars show the standard deviation between the 3 replicate wells. (B): Comparison of the enhancement of cell death when recipient cells are treated with 3T6 VF-CM + inhibitor. Dark green = significant enhancement (by Students T test) and light green shows were enhancement was seen but failed to meet statistical significance.

5.7: Ovcar3 VF-CM produced in the presence of mTOR inhibitor (Ovcar3 VF-CM+AZD) exports a statistically significantly more potent death signal than Ovcar3 VF-CM alone in recipient Ovcar3 cells

Experiments described in earlier sections describe VF-CM produced in donor Skov3, and 3T6 cells that have been added to recipient cells in the presence of a targeted inhibitor. However, it is possible that the presence of the targeted inhibitor may influence the production of the exportable death signal in the conditioned medium. In order to investigate this, experiments was set up where donor cells used to make the conditioned medium were also treated with the mTOR inhibitor AZD8055.

The six different VF-CMs (Figure 35) were then plated on recipient Ovcar3 and Skov3 cells and DCP levels measured as described in section Materials and Methods 2.8. The results are shown in Figure 36. Recipient Ovcar3 cells treated with Ovcar3VF-CM that had been produced in the presence of mTOR inhibitor produced a significantly (by ANOVA $P<0.05$) higher level of DCP than any of the relevant controls. In Skov3 recipient cells, the levels of cell death when VF-CM was pre-treated with mTOR inhibitor was higher than VF-CM, or in CM with the mTOR inhibitor, although this difference was not significant by ANOVA.

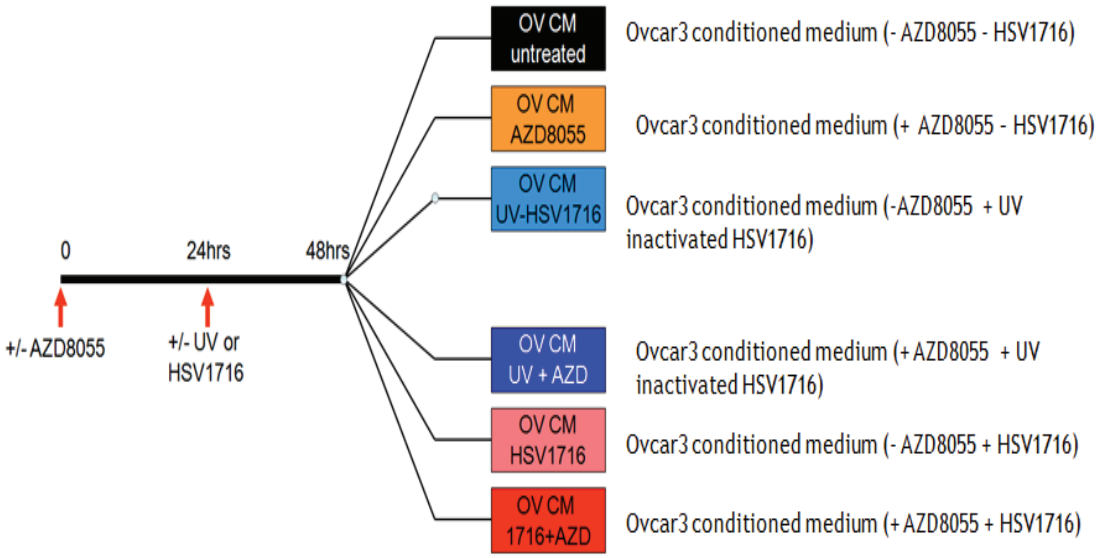


Figure 35: Schematic diagram of production of Ovcar3 VF-CM + AZD.

Ovcar3 cells were plated out at -24 hrs (not shown in the diagram). At time 0, the cells were treated with +/- 1uM AZD8055. After a subsequent 24 hrs UV inactivated virus or HSV1716 (MOI 1) was added to appropriate plates, giving 6 different versions of conditioned medium as shown. Each of these was processed as described in Materials and Methods section 2.10 to make VF-CM.

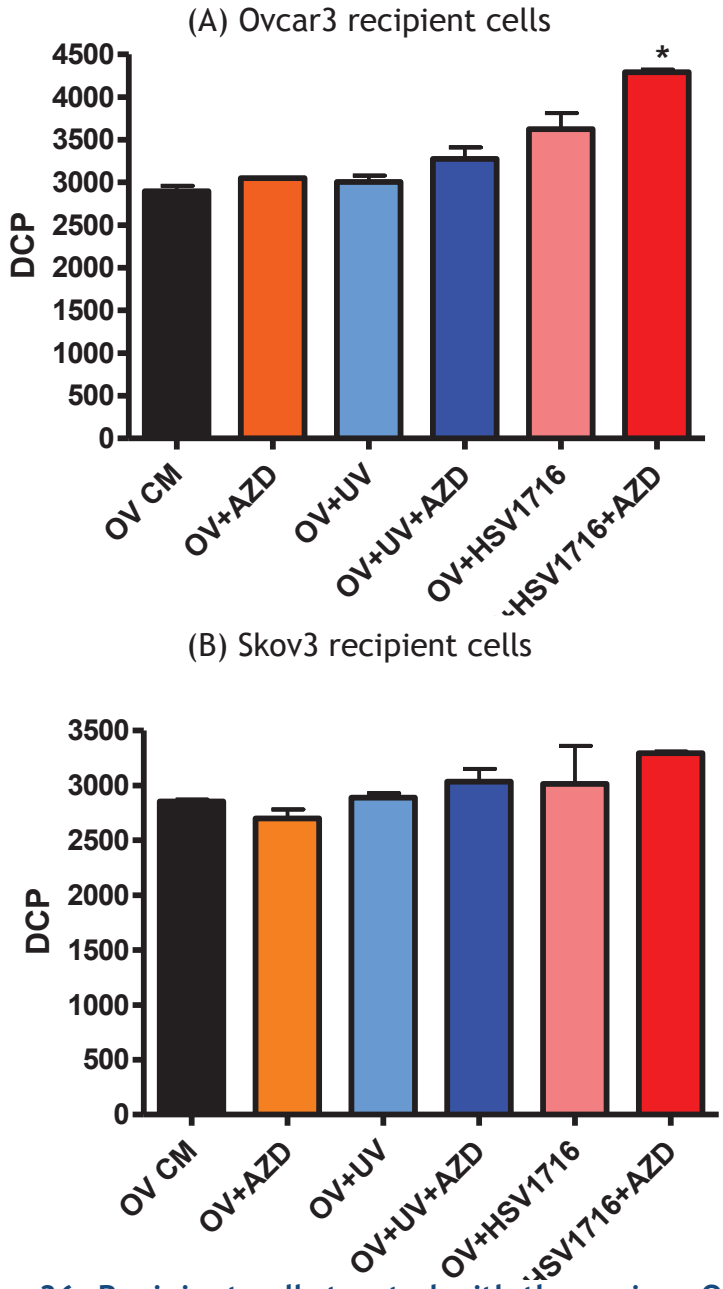


Figure36: Recipient cells treated with the various Ovcar3 VF-CM processed as described in Figure 35.

Absolute DCP levels (luminosity) are shown. (A) Ovcar3 Recipient cells treated with Ovcar3 VF-CM + AZD had significantly higher levels of DCP than recipients treated with any of the controls. $P<0.05$ vs. OV+HSV1716, $p<0.01$ vs. OV+UV+AZD, $p<0.01$ vs. OV+UV, $P<0.01$ vs. OV+AZD, $p<0.001$ vs. OV CM.

(B): Skov3 recipient cells treated with various Ovcar3 VF-CM. DCP levels in the cells treated with OV+HSV1716+AZD were higher than controls but failed to meet statistical significance level by ANOVA.

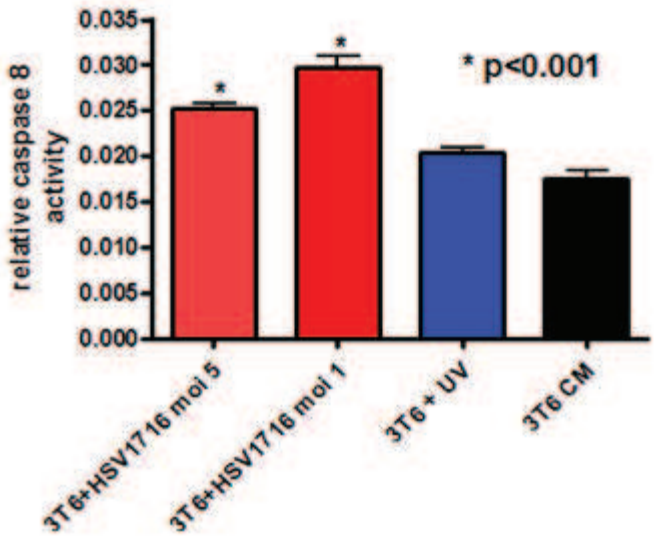
This thesis provides preliminary evidence that VF-CM from HSV1716-infected cells produces a 'death signal' that can enhance cell death when exported to non infected recipient cells. This signal was detected when VF-CM was produced in human cancer cell lines, however the levels of enhancement of cell death in recipients was small (generally <10%) and although there is a trend indicating that the VF-CM increased cell death in recipient lines, the levels of enhancement failed to meet statistical significance. However, these differences were reproducible in different experiments, done on different occasions, using different cells – suggesting that this is a real, albeit small effect that warrants further study.

In order to further investigate the mechanism by which this exportable death signal is increasing cell death in uninfected cells experiments were set up to measure apoptosis levels using the caspase activity levels assay described in Chapter 4 in cells treated with VF-CM. As the signal produced from 3T6 cells was much more potent than that produced in cancer cell lines, further experiments were carried out using 3T6VF-CM in order to maximise the chance of successfully being able to analyse the effect and mechanism behind this HIRED signal.

Results – Caspase activation by HIRED signal

Caspase assays, as described in Materials and Methods section 2.9 were set up. Ovcar3 recipient cells were plated out and exposed to 3T6 VF-CM. After 24hrs caspase 8 or 9 activity was measured (caspase 8 and 9 differentiate between the intrisinic and extrinsic apoptotic pathway, so caspase 3 was not used). Figure 37 shows the results – the HIRED signal activates caspase 8 in Ovcar3 recipient cells but does not activate caspase 9. Caspase 8 is activated through the extrinsic apoptosis pathway (Figure 36) while caspase 9 is activated by an intrinsic signal. This further suggests that an external, secreted, exportable signal is produced by HSV1716 infected cells that is stimulating apoptosis in non-infected cells.

A



B

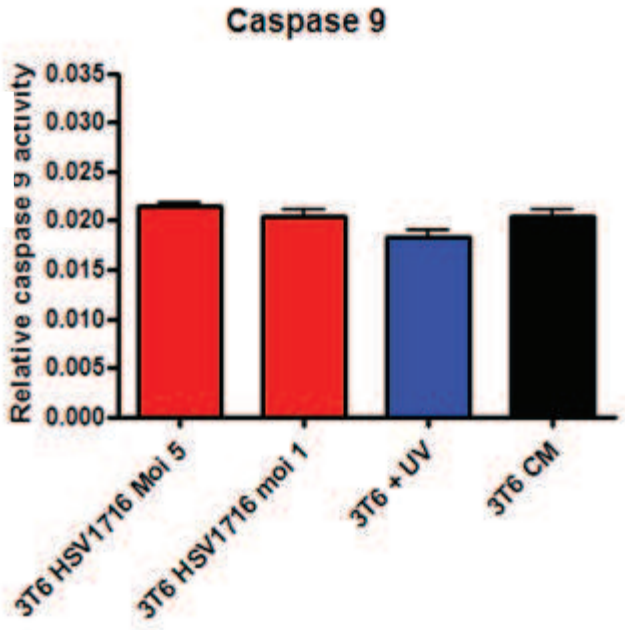


Figure 37: Caspase 8 (A) and Caspase 9 (B) activity in Ovcar3 recipient cells that have been treated with 3T6 produced VF-CM (either at HSV1716 MOI 5, 1, with UV treated virus or no virus).

Relative caspase 8 activity is significantly greater in cells treated with 3T6 VF-CM HSV1716 MOI 5 or 1 compared to control VF-CM. Caspase 9 is unchanged (bottom graph). Each bar represents the average of three replicate wells with the error bar the standard deviation. * represents $p<0.001$ (3T6 HSV1716 MOI 5 or 1 vs. 3T6 UV Students t Tests).

5. 8: Identification of the HIRED signal

The previous experiments have identified a secreted, exportable signal produced by HSV1716 infected 3T6 cells that can increase the levels of cell death in recipient cancer cell lines.

In order to identify the source of this signal, the 3T6VF-CMs were heated at 65^{o}C for 1 hr in order to denature the proteins. Surprisingly, the results shown in Figure 38 indicate that heating the VF-CM made no difference to the HIRED signal. As most proteins would be denatured by this heat treatment it suggests the source of the 'HIRED signal' is unlikely to be a protein.

5. 9: The HIRED signal correlates with levels of HSV DNA in conditioned medium.

As the HIRED signal was not affected by heat it was postulated that the signal is possibly a nucleic acid. The amount of HSV DNA in the 3T6 VF-CM was estimated using PCR. Various VF-CM produced at different time points from infected 3T6 cells were analysed using two HSV DNA PCRs and compared to the signals in VF-CM from mock-infected or infected with UV-inactivated HSV1716. PCR was performed using the primers and conditions described in Materials and Methods section 2.11 for the 2 HSV genes UL42 and gH. In order to partially quantify the results, medium spiked with known amounts of HSV1716 was analysed for comparison.

The results are shown in Figure 39. Five samples of VF-CM from HSV1716 infected 3T6 cells all had higher levels of both UL42 and gH fragments compared to the low levels in the VF-CM from 3T6 cells infected with UV-irradiated HSV1716. There was no PCR signal in the VF-CM from mock-infected 3T6 cells.

Electron microscopy (EM) and electron tomography (ET) studies of HSV-1 have revealed that virus particles have diameter ranging from 155 to 240 nms (Grunewald et al., 2003). Since the production of VF-CM involved filtering through 0.1uM filters this filtration step should remove any HSV virions. To confirm this, titration assays were performed of the VF-CM in the absence of infectious HSV virions. As there is also a weaker signal in the UV-inactivated VF-CM this suggests that viral DNA from the input dose of virus is also being detected by the PCR.

Further analysis with primers designed to amplify larger fragments were unsuccessful suggesting that these signals were derived from fragmented HSV DNA (data not shown).

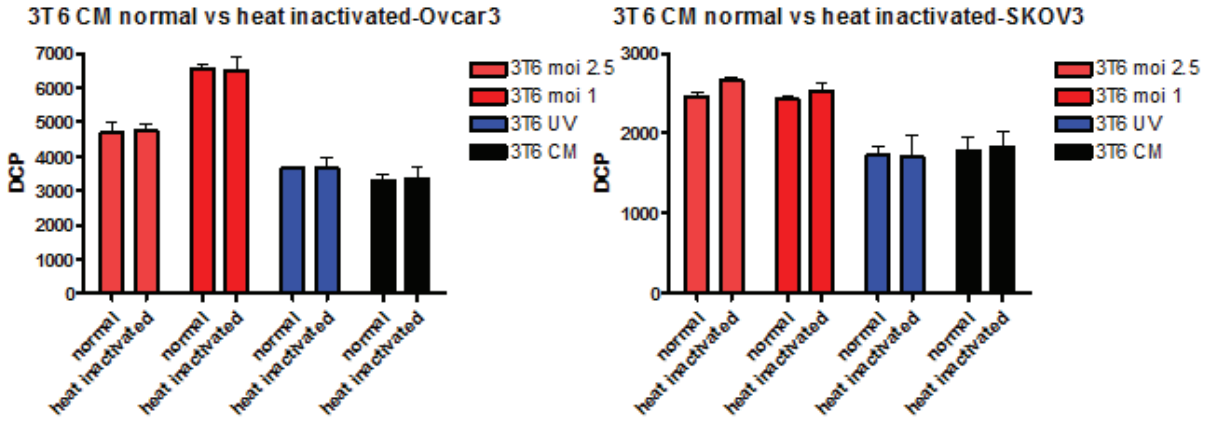


Figure 38: Comparing normal, untreated 3T6 VF-CM to heat inactivated 3T6 VF-CM.

At both MOIs there is no difference in the level of DCP after the VF-CM has been heat treated at 65°C in either recipient cell line.

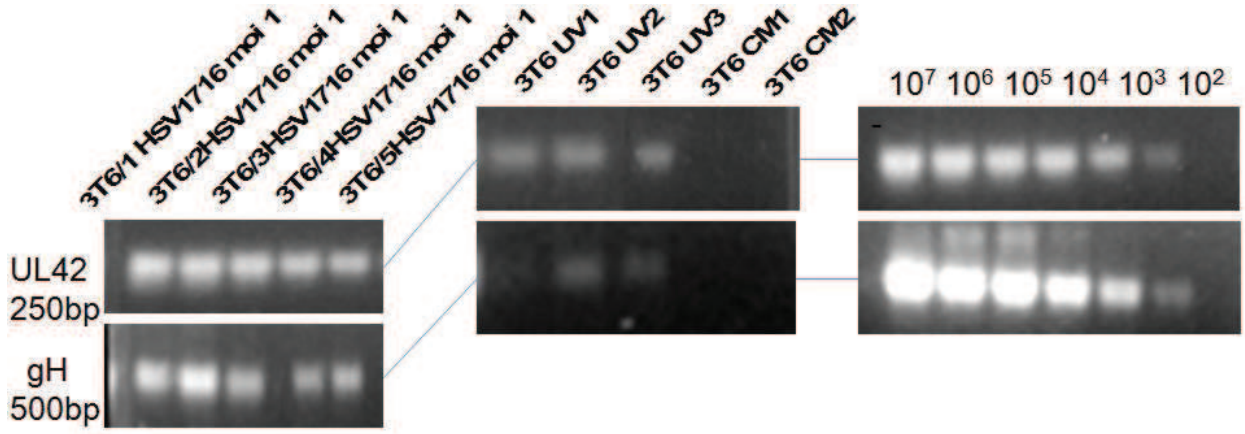


Figure 39: PCR products of UL42 & gH PCR.

Conclusions- Chapter V - HIRED signal

Cells infected with HSV1716 can produce an HIRED signal that can affect the non infected cell. All cell lines analysed in this thesis appeared to secrete some form of exportable signal, the most potent of which was generated by HSV1716-infected 3T6 cells. In these cells, the HIRED signal significantly increased cell death in recipient cell lines treated with the 3T6VF-CM, with cell death levels being approximately 4-fold higher than cells treated with UV-VF-CM (relative controls). This HIRED signal was consistently generated by different batches of 3T6 cells in three separate experiments.

3T6 cells are mouse embryo fibroblast cells that support productive wild type HSV-1 replication but not HSV1716 replication. This growth defect of HSV1716 in 3T6 cells occurs after viral protein synthesis – viral polypeptides are produced at comparable levels in 3T6 cells infected with either wild type or HSV1716; however HSV1716 viral particles appear to be trapped in the nucleus or cytoplasm and are unable to egress (Jing et al., 2004). Despite not producing progeny virus, 3T6 cells are killed by HSV1716, presumably due to the presence of viral proteins and polypeptides and not by oncolysis (Jing et al., 2004, Brown et al., 1994).

Cancer cell lines also appeared to produce this HIRED signal, although the potency was much lower, with cell death increasing by between 5 and 15%, depending on the cells used to produce VF-CM and the recipient line. Recipient cells treated with VF-CM produced in cancer cell lines showed an increased cell death compared to controls, although due to the small increases seen these failed to meet statistical significance in many instances. Similar results showing small increases in cell death in recipient cells treated with VF-CM compared to appropriate controls were reproduced across a number of experiments suggesting that although the techniques used to measure this HIRED signal were not sensitive to yield results that could be considered statistically significant, there was a definite trend that suggested this signal is real and warrants further study.

In Chapter III, numerous synergies and enhancement of cell death were seen when HSV1716 was used in combination with targeted agents such as mTOR inhibitors despite the ability of these agents to significantly abrogate HSV1716

replication. In Chapter IV, caspase assays indicate one method by which the combination of HSV1716 and mTOR inhibitors increased levels of intrinsic apoptosis. However as many of the synergies and cell death enhancements were seen at low MOI (bearing in mind these drugs block HSV1716 replication thus the amount of virus present is unlikely to increase by replication), it seems unlikely that intrinsic apoptosis is the only driver of increased levels of cell death in cells treated with HSV1716 and a targeted agent.

Figure 40 shows the proposed hypothesis. When HSV1716 enters a cell, if viral replication proceeds the efficient and productive viral infection diminishes the antiviral response and results in weakened HIRED signals. As the lytic replication results in destruction of the cell and viral spread then the oncolytic virus will be effective in reducing tumour size. If however the virus enters a cell where productive infection is diminished, be that by the presence of drugs such as mTOR inhibitors, or because viral replication is blocked, then the inefficient or incomplete viral replication strengthens the antiviral response, which enhances the HIRED signal.

An additional feature of this model is that the action of the HIRED signal in the uninfected cell could be enhanced by the targeted agent that blocks viral replication. In order to test this hypothesis, virus free conditioned medium (VF-CM) from the cancer cell lines Skov3, and the more potent 3T6 VF-CM were tested in combination with synergistic agents. The results in chapter V suggest transduction of death in the uninfected cell by a HIRED signal can be enhanced by targeted agents, especially an mTORi, which was highly synergistic with HSV1716. Interestingly, targeting specific pathways seemed important to generate the HIRED effect since the HIRED enhancement was observed using a p38, mTOR but not a MEK inhibitor; for example, the death signals exported by 3T6 cells were enhanced by p38 and mTOR inhibitor, but not by MEK inhibitor. Figure 40 shows the route cell death when HSV1716 is able to efficiently replicate and cell death when HSV1716 replication is blocked. When HSV1716 replication is blocked, the anti viral innate immune response is not blunted, and the infected cell dies by apoptosis as shown in Figure 40. This apoptosis leads to the release of factors from the HSV1716 infected cell, a secreted death signal that sensitise the surrounding cells to cell death. The potency of the exportable death signal is inversely proportional to the amount of productive viral

replication: when HSV1716 replication is productive and infectious particles are produced then the HIRED signal is blunted. When HSV1716 replication is non productive (either blocked or aborted) the viral DNA itself, or as a consequence of the presence of the viral DNA presence, the death signal is more potent at warning neighbouring cells and sensitising them to cell death.

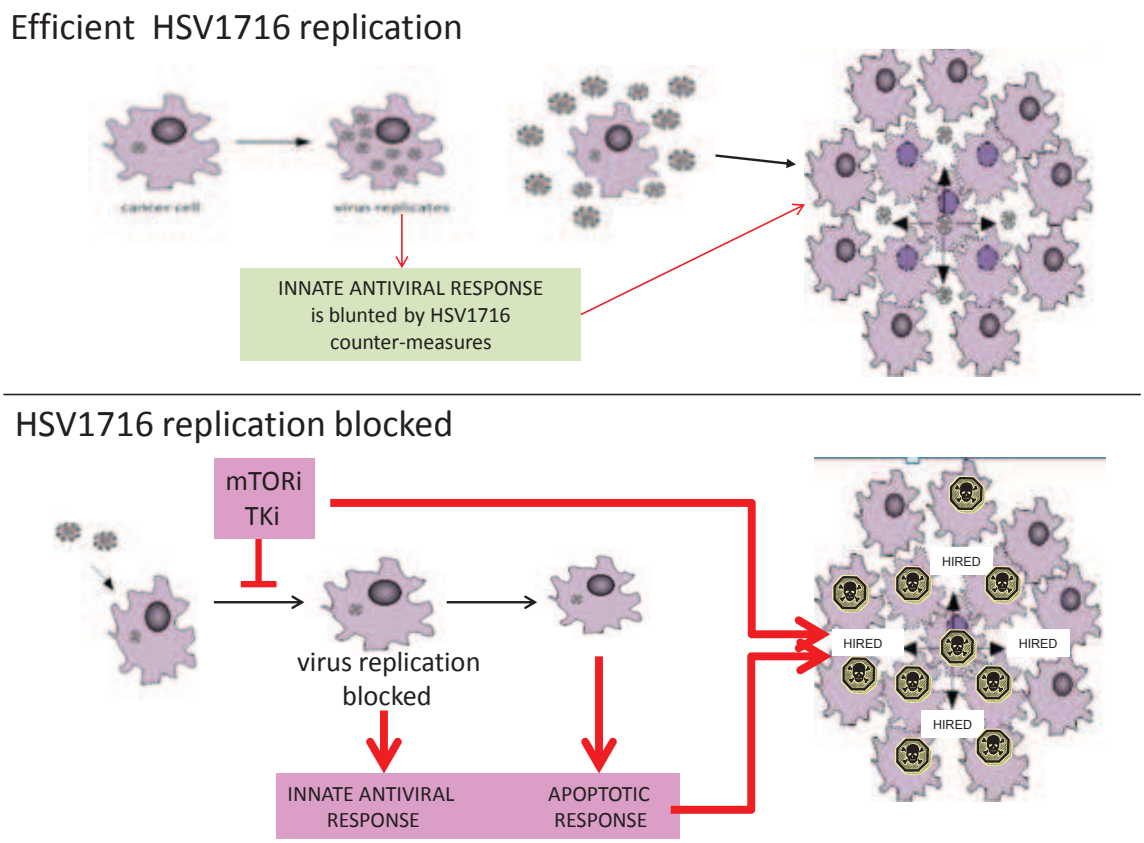


Figure 40: Route of cell death.

When HSV1716 replication is efficient, the virus is able to produce viral proteins that counteract the cells innate antiviral responses which include induction of apoptosis. However when HSV1716 replication is blocked, e.g. by a mTOR inhibitor of a tyrosine kinase inhibitor, viral replication and the production of viral proteins to counteract the host response are blocked, therefore the cell can initiate an antiviral response which eventually leads to apoptosis of the cell. During the apoptotic process a signal is released from the dying cell that causes both increased apoptosis of the uninfected cells and may also sensitise these cells to targeted agents.

If the hypothesis that cell lines in which HSV1716 replication is inefficient or aborted produce a stronger HIRED signal, then using a mTOR inhibitor to inhibit HSV1716 replication in the donor cell line during the production of VF-CM may result in a stronger HIRED signal being produced.

Ovcar3 cells were used in this experiment as HSV1716 replicates to a high yield in this line (Table 8) In addition, mTOR inhibitor AZD8055 substantially reduces the replication efficacy of HSV1716 in Ovcar3 (Table 13) Thus it was postulated that if reduced HSV1716 replication = greater HIRED signal, a difference could be measured in Ovcar3 cells. This was indeed the case, the levels of cell death seen in recipient Ovcar3 treated with the VF-CM produced in the presence of AZD8055 were significantly higher than the relative controls, indicating that using the mTOR inhibitor to block infection in the donor cell appeared to increase the potency of the exportable cell death signal.

In order to investigate the mechanism of HIRED induced cell death, caspase assays, as described in detail in Chapter IV were carried out. The HIRED signal from 3T6 cells activated caspase 8 in recipient cancer cells, but did not activate caspase 9. The caspase 9 assay detects cleaved caspase 9, a caspase cleaved by cytochrome C release, activated by the intrinsic mitochondrial apoptotic cascade. The caspase 8 assay detects cleaved caspase 8. Caspase 8 is cleaved in response to extrinsic death signals (Figure 5). By using both assays the route of the apoptotic cascade can be assessed. Increased caspase 8 and no increase in caspase 9 suggests that the increased levels of apoptosis are mediated via the extrinsic apoptotic pathway, further suggesting the presence of a secreted extrinsic factor that can be exported from HSV1716 infected cells to uninfected cells(Yu and He, 2016).

The source of this HIRED signal was at first assumed to be an IFN type response: the experiments detailed here describe a secreted signal, produced by virally infected cells that is exportable and increases apoptosis in non infected cells – all of which would indicate an IFN type response. However, when the VF-CM was heated to 65^{o}C the potency of the signal was unaffected. Although IFNs are small molecules it is unlikely they would survive such heat treatment; human IFNɣ is destroyed by denaturation at temperatures higher than 50^{o}C (Mulkerrin and Wetzel, 1989) and therefore is unlikely to be the source of the HIRED signal.

Small peptides, metabolites and nucleic acids would be unaffected by such heating – hence more likely candidates as the source of the HIRED signal.

As well as secreting molecules such as IFNs, cells also secrete extracellular vesicles. Extracellular vesicles are defined as vesicles that have the ability to transfer 'cargo' from one cell to another and can influence the recipient cell.

HSV-1 has evolved strategies that use the properties and functions of these vesicles to evade its host. As well as virions (as known as H (heavy) chain particles), other particles, L (light) particles are produced(Hogue et al., 2016). These are composed of virus envelope and tegument proteins and have been shown to facilitate HSV-1 infection (Szilagyi and Cunningham, 1991). As these vesicles contain viral proteins rather than genomes, it is unlikely these L particles are the source of the HIRED signal as these proteins would be unlikely to survive heating to 65^0C, and furthermore, the VF-CM is produced by passing the medium through a 0.1μM filter. These L particles are between 0.1-0.15μm in size(Meckes and Raab-Traub, 2011) and would be removed by such a filtration step.

There are smaller vesicles, around 50 -110 nm in size that are released from HSV-1 infected cells (Nolte-'t Hoen et al., 2016). These would be able to pass through the filtration step in the production of VF-CM. The 'cargo' of these vesicles include viral and host transcripts (mRNAs, miRNAs, and long non coding (lnc) RNAs) as well as proteins and components of innate defence against DNA viruses such as STING and markers of exosomes such as CD9, CD63 & CD81 (Kalamvoki and Deschamps, 2016).

Such exosomes were initially thought of as a way for cells to remove unwanted material from cells, but they are now recognised as important in an immune response to both viral and microbial infections as they are involved in antigen presentation. There is increasing evidence that tumour cells release excessive amounts of exosomes. In some instances, exosomes produced by cancer cells can induce proliferation and have the potential to convert non-tumourigenic cells into tumour forming cells (Zhang et al., 2014). The content of these exosomes varies between different physiological and pathological conditions and cell types. To date (July 2016) more than 9000 proteins and 5000 mRNA have been

detected and deposited in Exocarta (www.Exocarta.org) an open access database of contents identified in exosomes in multiple organisms.

Although this thesis presents no direct evidence that the HIRED signal described in this thesis is exosomal, the experiments described show that HSV1716 infected cells release a exportable signal that causes cell death in uninfected cells. The source of the signal is not infectious virions, and is under 100 nm is size (based on filtration step) and also survived being heated to 65^{o}C and exosomes seem a likely source.

The presence of the HIRED signal hypothesised in the thesis has also been observed by Prof Tim Cripe, who is working on HSV1716 in combination with the Aurora A kinase inhibitor (Alisertib). FACS analysis of cells treated with the combination revealed not only that overall cell death increased when HSV1716 was given in combination with Alisertib, but the amount of apoptosis in uninfected cells was significantly higher, suggesting a paracrine death signal being secreted by HSV1716 infected cells. The poster presenting this early work was presented at American Society of Gene and Cell Therapy 2016 (ASGCT) by Les Sprague and is shown in Appendix 4.

Final Discussion – future perspective

Oncolytic viruses, as programmable biologics that replicate in and kill cancer cells while leaving normal cells undamaged, have huge appeal as cancer treatments, and have been in development in laboratories around the world for over 20 years. In 2015 the field experienced a massive step forward, with the first oncolytic HSV, IMMLYGIC (talimogene laherparepvec, Amgen) being approved by the FDA and EMEA as a treatment for melanoma lesions in the skin and lymph nodes (Pol et al., 2016).

The aim of my project was to consider the suitability of a combination therapy using HSV1716 with a number of different classes of anti-cancer agents, especially those agents targeted to signalling pathways upregulated in cancer. The first part of this project was to develop a relatively high throughput assay suitable for use as a 'fishing' exercise, to look at a large number of agents in an *in vitro* assay system. The second aim was to investigate combinations that were synergistic and attempt to elucidate the mechanisms behind these synergies.

Both aims were successful – the work undertaken in this thesis shows:

1) A large number of positive synergies suggesting broad applicability of HSV1716 with many different current and potentially future standard of care drugs

2) The identification of mechanisms of synergy in HSV1716 infected cells and a novel mode of action in uninfected cells

Currently (as of July 2016) there are at least 2 other OVs in phase III trials, 9 in phase II trials and at least 8 in Phase I development and countless others in translational development (Pol et al., 2016).

Most of the recently published work is focused on combining oncolytic viruses with cancer immunotherapy or engineering new OVs with improved immunostimulatory functions. For example the journal Biomedicines has just produced a special Issue (July 2016) on oncolytic viruses as novel form of immunotherapy (http://www.mdpi.com/journal/biomedicines/special_issues/oncolytic_viruses_immunotherapy).

There are, however, at least four mechanisms which contribute to the efficacy of oncolytic viruses. These are (1) direct cellular lysis, (2) cytokine-induced apoptosis, (3) innate immune cell cytotoxicity and (4) antigen specific adaptive T cell killing (Figure 41). While the current trend is focusing on the immunotherapeutic effects of OVs, further understanding of all the mechanisms by which oncolytic viruses kill cells can only help design new, improved OVs and help to understand how to maximise the effect of existing OVs to provide benefit and elicit an anti tumour immune response in cancer patients. This thesis describes a 5th indirect mechanism (Figure 40) that involves induction of apoptosis in both infected and uninfected cells when an OV is combined with targeted therapeutics. The targeted therapy inhibits replication, which triggers an apoptotic response which kills the infected cell and releases an exportable death signal capable of inducing apoptosis in the uninfected cells. The HIRED effect works as an adjuvant and sensitises uninfected cells to the targeted therapy.

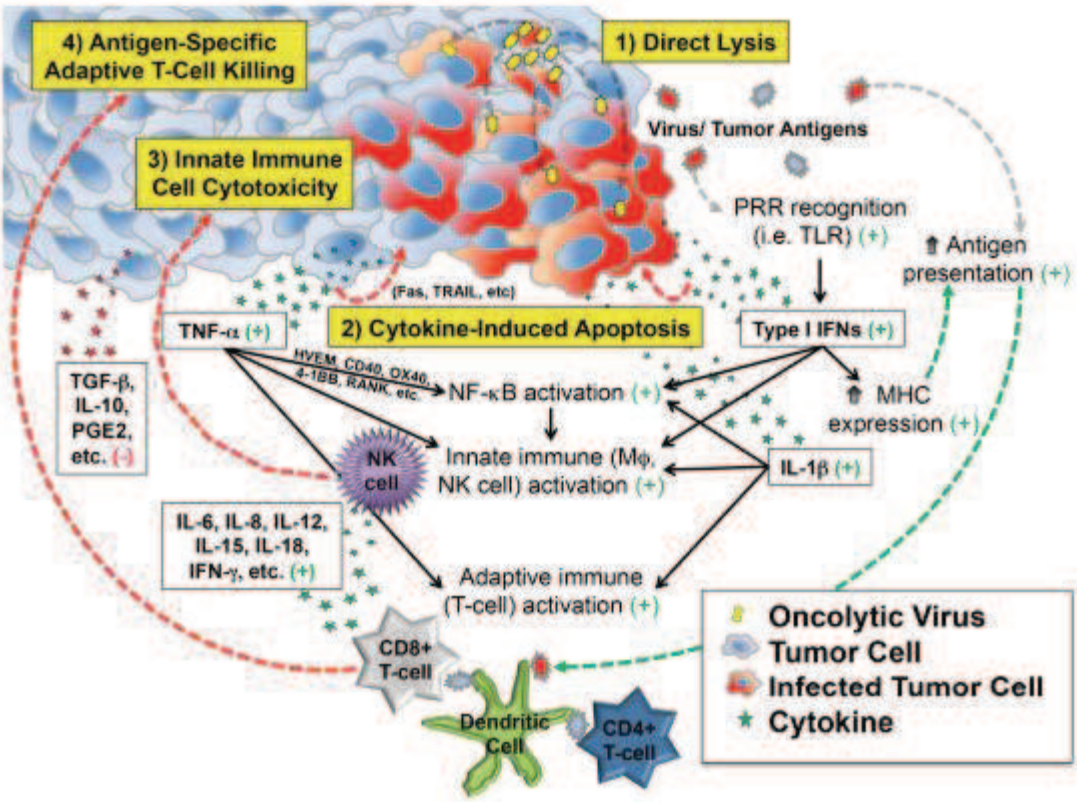


Figure 41: The current model of oncolytic virotherapeutic efficacy.

(1) Direct cellular lysis, (2) cytokine induced apoptosis (3) innate immune cell cytotoxicity & (4) antigen specific adaptive T cell killing. Each one of these distinct processes is involved in tumour regression but the extent each process plays is likely to vary from patient to patient, type of virus, presence of engineered transgenes in the virus, characteristics of the tumour cells themselves as well as the tumour microenvironment and the immunological status of the patient.

Adapted from Cassady et al., 2016.

From the results detailed in this thesis three main conclusions can be drawn.

HSV1716 used in combination with some other anti-cancer agents acts synergistically, or can enhance the amount of tumor cell killing relative to either drug or HSV1716 alone, and these synergistic effects are not due to increased oncolysis or increasing viral spread.

Increased levels of apoptosis, mediated through the intrinsic mitochondrial pathway correlates with this increase in cell death in the infected cell.

HSV1716 infected cells secrete an exportable ‘signal’ that can cause increased levels of cell death in non-infected cells, and this signal can be altered by targeted agents.

HSV1716 used in combination with some other anti cancer agents acts synergistically, or can enhance the amount of tumour cell killing relative to either drug or HSV1716 alone

It is clear from the results in chapter III that HSV1716 combines synergistically with many different targeted therapies for enhanced cell killing of cancer cells. Table 11 summarises the results of the screening.

A total of 17 agents were assessed for synergy/enhancement with HSV1716 in between 9-10 cell lines. When taken as a group, mTOR inhibitors + HSV1716 had the most 'synergistic hits'. AZD8055 + HSV1716 in combination generated 'hits' in every cell line.

In terms of least 'hits', Dovitinib, a FLT3/cKIT inhibitor and Erlotinib and Gefitinib (both EGFR inhibitors) had almost no 'hits', with only 1/8 combination points in Hep3B being synergistic with Dovitinib.

VEGFR inhibitors, Sunitinib and Sorafenib all had 'hits' in 8/10 cell lines, while Pazopanib, another similar VEGFR inhibitor had 'hits' in 9/9 lines, although Huh7 only had 1/8 points synergistic.

It should be noted that many kinase inhibitors are promiscuous and inhibit various off-target kinases and it is possible that the primary target of the inhibitor is not responsible for the effect. For example, the KIR database examines the off target effects of over 50 different kinase inhibitors (http://reactionbiology.com/webapps/largedata/).

For further analysis, the KIR database could be screened using synergistic kinases to try and identify recurring targets and comparing the results with non-synergistic kinases. More specific inhibitors could then be selected to confirm the identified targets. Furthermore, once targets are identified, RNAi could also be used to down regulate the specific pathways or kinases to determine the effects on viral replication and apoptosis.

Increase in cell killing by combination approach is attributable to an increase in apoptosis, mediated through the intrinsic mitochondrial pathway

Chou Talalay analysis described in Chapter III revealed that in a number of cell lines HSV1716 + Sunitinib, Sorafenib, AZD8055 or Doxorubicin were synergistic in terms of increasing cell death. In order to investigate the mechanism by which these synergies were occurring, caspase assays were set up to determine if the increased cell killing was due to an increase in apoptosis. There are different assays to measure different caspases. Caspase 3/7 is an executioner caspase, and all routes of apoptosis converge on it, thus if activated caspase 3/7 is observed then the total amount of apoptosis is increased. Apoptosis can occur by a number of pathways. If the extrinsic pathway (shown in figure 6) is activated then caspase 8 is cleaved which then activates caspase 3/7. If the intrinsic pathway is activated, caspase 9 is cleaved, and it then cleaves caspase 3/7. Thus by measuring both the total caspase activation (c3/7) and either caspase 8 or 9 the route of apoptosis can be determined.

The results detailed in chapter IV show that caspase 3/7 but not caspase 8 is significantly activated when HSV1716 is given in combination with synergistic drugs such as AZD8055, Sunitinib or Doxorubicin. No increase in caspase activation was seen with HSV1716 + non-synergistic drugs such as Erlotinib and Dovitinib.

Anti cancer agents, such as the mTOR inhibitor AZD8055 block tumour cell growth. Normally, HSV1716 would cause oncolysis in cancer cells. However, in the presence of drugs that block tumour growth, viral replication is also blocked- by shutting down growth of the cancer cell it also stops the virus that requires actively dividing cells for its own viral replication. However, despite this block, levels of tumour cell death are enhanced when the drug and virus are given in combination. AZD8055, Sunitinib and Doxorubicin were all highly synergistic with HSV1716 and all three increased levels of intrinsic apoptosis significantly.

It is postulated that the increase in cell death due to increased apoptosis observed when AZD8055, Sunitinib or Doxorubicin are given in combination with HSV1716 is simply due to the presence of the drug inhibiting the virus from

transcribing the necessary anti apoptotic viral proteins necessary to prevent the host cell from entering apoptosis.

Apoptosis, a critical cellular mechanism against viral infection (Blaho, 2004) is triggered early in infection through viral recognition by either TLR7, which binds ssRNA, or TLR3, which binds dsRNA. These TLRs then induce intracellular signalling, eventually resulting in the shutdown of protein synthesis within the cell and apoptosis (Kawasaki and Kawai, 2014). Membrane fusion of HSV, in the absence of viral replication, also induces a subset of interferon stimulating genes (ISGs) which may produce a pro-apoptotic signal(Noyce et al., 2011). HSV would, under normal circumstances be able to counteract this shutdown as it encodes anti-apoptotic viral proteins to subvert such apoptosis. If production of viral anti-apoptotic proteins are suppressed by a cytostatic drug, then these viral proteins will not be produced, thus the balance within the cell will be in favour of pro-apoptotic proteins, and apoptosis will proceed. The type of cell death, in cells infected with recombinant HSV lacking genes such as ICP27 and ICP4,known to have anti apoptotic function, was first referred to as HSV dependent apoptosis (HDAP) by Nguyen et al., 2007a. Cancer cells exhibit an exquisite sensitivity to HDAP (Nguyen et al., 2005, Aubert and Blaho, 2003, Nguyen et al., 2007b). In contrast, cells derived from normal cells (nontransformed) were quite resistant to this process ((Nguyen et al., 2005, Aubert and Blaho, 2003). Within the mixed population of cells within the tumour microenvironment there will be actively dividing cells sensitive to oncolysis but there will be other cell types that are not actively dividing or sensitive to oncolysis and therefore other routes to cell death such as HDAP will be important.

Thus the increase in apoptosis may be clinically relevant. Increasing the amount of apoptosis within a tumour environment will increase, for example, NF-κB activation, which will in turn activate innate immune cells, leading to an increase in adaptive immune (T –cell) activation. As a single mechanism an increase in apoptosis within the tumour microenvironment might not be enough to tip the balance in favour of an anti-tumour immune response. However, in scheduled combination of an immunotherapeutic, an oncolytic virus and possibly even targeted agent may be able to help create a ‘perfect storm’ that could lead to the ultimate goal of an anti-tumour response leading to a durable, curative treatment for cancer.

The next set of experiments within the combination of HSV1716 + drugs will require a more detailed analysis of intrinsic apoptosis by using FACs for example, to analyse the timing of apoptotic events. Furthermore, comparing mutant viruses that have defects in the anti-apoptotic genes for example, ICP27 or ICP4-deleted HSV would allow comparisons of whether these viruses have similar patterns of cell death to HSV176 that is not replicating in the presence of a drug and perhaps even stronger synergies. Further, key pro-and anti-apoptotic factors could be assessed by Western Blotting or antibody arrays and these could then be targeted by RNAi. Importantly it will be key to show that such targeting leads to better synergies.

There are other cell-type dependent routes to cell death such as necrosis, or necroptosis (the regulated form of necrosis) but these were not studied here. In follow up experiments it will be interesting to study such alternate pathway by measuring key markers such as RIPK1 and RIPK3.

Crucially experiments of HSV1716 + synergistic agents *in vivo* will be required to translate these findings into a more clinically relevant setting. It may be that with the current interest in OVs as immunotherapeutics, for example OV's in combination with immune checkpoint inhibitors, that experiments looking at the relatively small effects of increasing apoptosis in cancer cells are swamped by the potential curative potential of such immunotherapeutic combinations. That is not to say that such experiments are not worthwhile – increasing apoptosis may increase the amount of ICD. As discussed above, one of the keys to successful OV therapy is to induce an anti-tumour immune response.

The field of oncolytic viruses may be entering a phase of exponential growth due to its potential as an immunotherapeutic, but greater understanding of how viruses interact at cellular levels can only lead to further advances in the field. Furthermore, targeted agents, such as those described in this thesis are increasingly being seen as an adjunct to immunotherapy drugs. Many targeted therapies against tumour pathways affect pathways that are also crucial for immune development and function, bringing forth the possibility that targeted agents may help optimise anti-tumour responses from immunotherapies. For example Sunitinib has been shown to decrease myeloid derived suppressor cells (MSDC), a myeloid cell subtype that silences responses of cytotoxic CD8+ T Cells

and helper CD4+ T cells while promoting T_{Reg}s (Jha et al., 2011). mTOR inhibitors have also been shown to enhance CD8+ T cell activation and IFNγ production (Jiang et al., 2011). These effects could be analysed *in vivo* in combination studies with murine syngeneic models.

HSV1716 infected cells secrete an exportable 'signal' that can cause increased levels of cell death in non-infected cells, and this signal can be altered by targeted agents.

In Chapter III a number of the synergistic combinations were found when HSV1716 was at MOI of 0.1. As many of the drugs in the combination analysis significantly reduced viral replication, an HSV1716 virion is only likely to be physically present in a maximum of 10% of the cells throughout the experiment.

If the virus is not present within the cell to activate apoptosis by the intrinsic pathway then how is the synergy between the targeted agent and HSV1716 occurring?

This thesis describes a 5th indirect mechanism (Figure 40) that involves induction of apoptosis in both infected and uninfected cells when an OV is combined with targeted therapeutics. The targeted therapy inhibits replication which triggers an apoptotic response which kills the infected cell and releases an exportable death signal capable of inducing apoptosis in the uninfected cells. The HIRED effect works as an adjuvant and sensitises uninfected cell to the targeted therapy.

Further analysis of this HIRED signal is required. The next steps in this study would be to look at other cell lines with other targeted agents as the pre-treatment in order to further elucidate if drug treatment prior to infection alters, enhances or inhibits the HIRED signal produced. As with the combination of VF-CM tested when the targeted agents were added to recipient cells, responses varied according to the recipient cell line, further suggesting that there are multiple different factors at play, both in terms of the HIRED signal and the ability for certain targeted agents to enhance it. It will also be interesting to measure the HIRED signal (if any) produced by HSV mutant viruses that lack anti apoptotic genes such as ICP27 or ICP4.

Furthermore, FACS analysis of the proportion of cell death in recipient cells, rather than the DCP assay would allow greater sensitivity. Difference in DCP levels of between 5-10% were measured during these experiments however these differences failed to meet statistical significance. With FACS analysis looking at live/dead cells, much smaller differentials can be measured.

One theory is that the HIRED signal is viral DNA fragments either free or in exosomes. These fragments released from the infected and apoptotic cell could stimulate TLR and other DNA sensors in the uninfected cell via cell surface (free) or intracellular (exosome) sentinels and activate apoptotic cascades via FAS or other death ligands. Thus activation of these pathways can be analysed in cells treated with VF-CM. Recent advances in exosome research mean that now commercially magnetic bead kits have been developed for fast, reproducible isolation/characterization of exosomes and analysis of their cargo which would allow this theory to be tested (https://www.thermofisher.com/uk/en/home/life-science/cell-analysis/exosomes.html).

In conclusion, this thesis provides preliminary evidence that oncolytic viruses can exert an anti-tumour effect by inducing apoptosis in both infected and uninfected cells when combined with targeted therapeutics. The targeted therapy inhibits OV replication, but triggers an apoptotic response which kills the infected cell and releases an exportable death signal capable of inducing apoptosis in the uninfected cells.

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Appendix I –Lynne Braidwood's HSV1716 related publications

Oncolytic Virotherapy

Dovepress

REVIEW

Oncolytic herpes viruses, chemotherapeutics, and other cancer drugs

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Abstract: Oncolytic viruses are emerging as a potential new way of treating cancers. They are selectively replication-competent viruses that propagate only in actively dividing tumor cells but not in normal cells and, as a result, destroy the tumor cells by consequence of lytic infection. At least six different oncolytic herpes simplex viruses (oHSVs) have undergone clinical trials worldwide to date, and they have demonstrated an excellent safety profile and intimations of efficacy. The first pivotal Phase III trial with an oHSV, talimogene laherparepvec (T-Vec [OncoVex^{GM-CSF}]), is almost complete, with extremely positive early results reported. Intuitively, therapeutically beneficial interactions between oHSV and chemotherapeutic and targeted therapeutic drugs would be limited as the virus requires actively dividing cells for maximum replication efficiency and most anticancer agents are cytotoxic or cytostatic. However, combinations of such agents display a range of responses, with antagonistic, additive, or, perhaps most surprisingly, synergistic enhancement of antitumor activity. When synergistic interactions in cancer cell killing are observed, chemotherapy dose reductions that achieve the same overall efficacy may be possible, resulting in a valuable reduction of adverse side effects. Therefore, the combination of an oHSV with "standard-of-care" drugs makes a logical and reasonable approach to improved therapy, and the addition of a targeted oncolytic therapy with "standard-of-care" drugs merits further investigation, both preclinically and in the clinic. Numerous publications report such studies of oncolytic HSV in combination with other drugs, and we review their findings here. Viral interactions with cellular hosts are complex and frequently involve intracellular signaling networks, thus creating diverse opportunities for synergistic or additive combinations with many anticancer drugs. We discuss potential mechanisms that may lead to synergistic interactions.

Keywords: combination studies, herpes simplex virus, oncolytic virus, virotherapy

Introduction

Using viruses to treat cancer is not a new idea. For more than 100 years there have been clinical observations that cancer patients who contracted viral infections would enter periods of remission.[1] During the 1950s and 1960s, there was considerable activity using wild-type viruses as anticancer treatments, but many of these trials were limited by the toxicity of the wild-type virus (for a historical perspective see Kelly and Russell[1]). Progress has only recently been possible as advances in virology and molecular biology have allowed either the identification of naturally occurring viruses with intrinsic tumor selectivity or by genetically engineering oncolytic viruses.

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Oncolytic herpes simplex virus (oHSV)

Oncolytic herpes viruses are attenuated, replication competent, herpes simplex type 1 viruses that selectively infect, replicate within, and lyse cancer cells. One of the first reports of an oncolytic virus being used for cancer therapy was in the early 1990s when Martuza et al[3] showed that a replication competent thymidine kinase negative herpes simplex virus (HSV)-1 mutant effectively prolonged survival of nude mice bearing intracranial glioma. Since then, numerous oHSVs have been described, most of which have deletions in either *RL1*, *UL39*, or both.

ICP34.5, the protein product of the *γ34.5* gene, is a specific determinant of neurovirulence. It plays a key role by facilitating escape from a major host defense mechanism involving the protein kinase R-mediated innate immune response pathway by directly interacting with protein phosphatase 1α to dephosphorylate eIF2α (Figure 1).

In contrast, oncolytic HSV, which lacks functional ICP34.5 protein, cannot dephosphorylate eIF2α. Thus, infection with an ICP34.5 null virus causes the host cell to shut down protein synthesis, hence, preventing the virus from replicating in normal cells. Cancer cells, however, in the course of transforming to malignant cells have impaired antiviral mechanisms that permit unimpeded viral replication.[3]

UL39 is the HSV gene encoding for the large subunit of ribonucleotide reductase (RR), the main rate limiting enzyme for viral DNA synthesis and replication, controlling the nucleotide substrate pool by regulating the conversion of ribonucleotides to deoxyribonucleotides. HSV RR is required for growth in nondividing cells but not in rapidly dividing cells, in which there is ample cellular RR for the virus to utilize. Oncolytic HSV with a defective *UL39* gene exclusively replicates in and lyses rapidly dividing cancer cells, as such cells provide sufficient levels of RR activity[4] (for comprehensive review of oHSV see Cassady and Parker,[5] Manservigi et al,[6] and Varghese and Rabkin[7]).

Modified (armed and targeted) oHSV

The concept of using viral vectors to deliver therapeutic genes to tumors is well established. Many studies have evaluated both the oncolytic and antitumor activity, and the antitumor immune response of oncolytic viruses engineered to express either immunostimulatory genes or therapeutic genes, including those that can activate prodrugs.

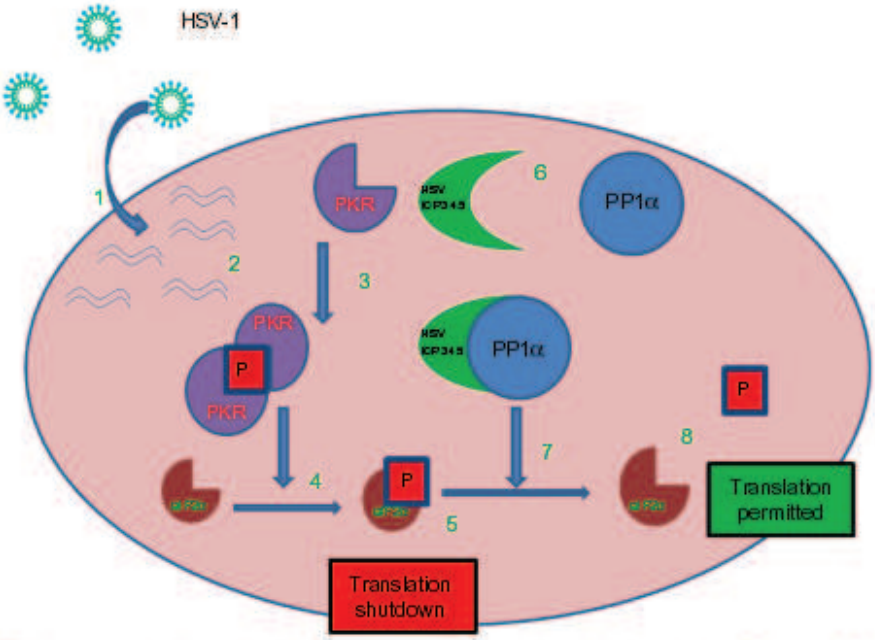

Figure 1 HSV-1 can overcome normal cells protective block in protein synthesis 1. HSV-1 enters the host cell and begins replication. 2. Complementary RNA anneal to produce dsRNA. 3. PKR binds dsRNA, dimerizes resulting in activation and autophosphorylation. 4. Phosphorylated PKR selectively phosphorylates eIF2α. 5. Phosphorylated eF2α causes the host cell to shutdown translation thereby preventing viral replication. 6. HSV produced ICP34.5 which forms a protein complex with PP1α. 7. The ICP34.5 PP1α complex dephosphorylates eIF2α so the viral replication (8) can continue unchecked.

Abbreviations: HSV, herpes simplex virus; PKR, protein kinase R; eIF2α, eukaryotic initiation factor 2; PP1α, protein phosphatase 1 alpha; ICP, infected cell polypeptide; P, phosphorylation.

The therapeutic efficacy of oncolytic HSV vectors encompasses two modes of action: direct oncolysis by the virus itself and indirect induction of an antitumor response. By arming viruses with genes that encode for immunomodulatory proteins such as IL(interleukin)-12,[8–10] IL-2,[11] soluble B7.1-Ig,[12] or granulocyte macrophage colony-stimulating factor(GM-CSF)[13–16] to help promote the antitumor immune response, the modified viruses are more efficacious.

Virus-directed enzyme prodrug therapy systems have also been utilized with oncolytic HSV. There are numerous reports of viruses that have been modified to code for enzymes that catalyze prodrugs into active substrates, such as HSV1yCD, a modified HSV coding for the yeast cytosine deaminase (CD) enzyme. HSV1yCD converts the nontoxic 5-fluorocytosine into fluorouracil (5-FU), a highly toxic chemotherapeutic agent,[17] rRp450 carrying rat cytochrome P450 (CYP2B1) (which converts cyclophosphamide into the alkylating toxin phosphoramide mustard),[18] and nitroreductase, which converts the prodrug CB1954 to an active alkylating agent.[19] The extensive field of oncolytic HSV vectors modified for enhanced efficacy is beyond the scope of this review; the major approaches are detailed here but reviewed in greater detail by Varghese and Rabkin.[7]

Table 1 lists the principal oHSV in clinical development. At least six different oHSV have undergone clinical trials worldwide to date. oHSV have demonstrated excellent safety profiles and, in numerous studies, signals of efficacy. The first Phase III trial with an oHSV, talimogene laherparepvec (T-Vec [OncoVex^{GMCSF}]) has almost been completed. Initial extremely encouraging findings of the trial have been reported, with T-Vec demonstrating a statistically significant improvement in durable response rate.[20]

Oncolytic viruses in combination with chemotherapy

The use of many chemotherapeutic agents is limited by severe dose limiting toxicities and the emergence of resistant disease.[21] In comparison, the mode of action of oncolytic viruses (lytic infection) means that cancer cells are unlikely to become resistant to them. Furthermore, oncolytic viruses have a high therapeutic index (ie, the comparison of the amount of a therapeutic agent that causes the therapeutic

Table 1 Oncolytic HSVs in clinical trials

HSV strain	Genetic modification	Stage/clinical indication	Results	References
OncoVex GM-CSF (T-Vec)	Deletion in both copies of ICP34.5+ ICP47 disruption	Phase I/II and III melanoma	Evidence of virus replication in injected and adjacent uninjected tumors (head and neck). Regression of injected and uninjected tumors in late stage melanoma.	13,20,97,98
		Head and neck cancer		
		Advanced metastatic melanoma		
			Ongoing	71
R7020 (NV1020)	Deletion of one copy of ICP34.5+ tk under ICP4 promoter control + deletion in UL24, 55, and 56	Phase I and II colorectal cancer liver metastases	In Phase II disease stabilization in 40%–45% of cases.	99–102
G207	Deletion in both copies of ICP34.5+ disruption of UL39	Phase I, IB, and II recurrent brain cancer (glioma, astrocytoma, and glioblastomas)	Well tolerated. Evidence of viral replication and radiographic and neuropathological signs of antitumor activity.	103–109
HSV1716	Deletion in both copies of ICP34.5	Glioma Phase I	Well tolerated, no toxicity. In Phase I/II (recurrent glioblastomas) three out of 12 patients showed disease stabilization. No toxicity in melanoma or HNSCC. Evidence of viral replication in tumors.	110–114,129,130
		Melanoma		
		HNSCC		
		Non-CNS solid tumors	Ongoing Phase I	
		Malignant pleural mesothelioma	Ongoing Phase I/IIa	
HF10	Spontaneous generation of HSV-1 variant	Pancreatic cancer		115–122
		Recurrent breast cancer		
		Bladder cancer		
		HNSCC		
rRp-450	ICP6 deleted and expresses prodrug enzyme for cyclophosphamide (ratCYP2B1)	Phase I liver metastases and primary liver tumors	Ongoing	131

Abbreviations: HSV, herpes simplex virus; ICP, infected cell polypeptide; tk, thymidine kinase; UL, unique long; HNSCC, head and neck squamous cell carcinoma; CNS, central nervous system.

effect, to the amount that causes toxicity) with very limited toxicities. Table 2 summarizes the potential advantages of oncolytic virotherapy.

Viral infection initiates many complex host defense pathways;[22] however, viruses have coevolved equally complex countermeasures to circumvent these activities.[23,24] Many of these countermeasures are retained by their oncolytic variants (Table 3 outlines the main cellular and viral pathways activated upon viral infection). As chemotherapeutic and targeted anticancer agents target key cellular processes that also involve complex intracellular signaling networks, there are extensive opportunities for antagonistic and synergistic interactions with oncolytic viruses, and these need to be explored and understood as the clinical acceptance of oncolytic HSV looks increasingly likely.[25]

Combining these two very different modalities in order to increase cancer cell killing is a rational approach. The clinical implications of this combination therapy are not limited to enhanced efficacy. The dose reduction index, the most relevant clinical parameter derived by Chou and Talalay analysis,[26] reveals the potential for significant dose reductions without compromising tumor cell kill. Reducing the dose of drugs such as chemotherapeutics would minimize the toxicity and may allow patients to remain on an otherwise intolerable regime, or increase their quality of life whilst still receiving treatment for their disease.

Since the initial groundbreaking studies by Toyoizumi et al[27] with HSV1716 and four standard chemotherapeutic drugs, methotrexate, cisplatin, mitomycin C, and doxorubicin, there have been many reports of the increased efficacy of oHSV in combination with a wide range of existing and potentially new anticancer drugs. Tables 4–8 present the wide variety of different combinations that have been examined, and also summarize the results. The aim of this review is not to discuss the individual results presented in these tables, but to attempt a mechanistic overview that relates to their findings. Crucially, there are a number of reasons why oncolytic virus therapy in combination with chemotherapeutic agents, or other anticancer treatments, will be beneficial. Firstly, the mode of action of oncolytic viruses is completely different from chemotherapeutics and they are not, therefore, in direct competition. Secondly, oncolytic cell killing is independent of the many genomic alterations that lead to drug-resistant tumors and so may be effective even in drug-resistant cells.

The most widely used method of studying drug/drug (or virus/drug) interactions between two modalities in vitro is using the methods of Chou and Talalay.[26,28] This type of analysis is one of the few available that identifies beneficial interactions based on an extrapolated equation. The possibility of predicting a false positive is minimized as the analysis takes into account both the potency (the IC_{50} [half the maximal inhibitory concentration] or the LD_{50} [median lethal dose]) and the slope of the dose effect curves (*m*-value) in the precise analysis of two therapeutic combinations. The method defines the expected additive effect of two (or more) agents and quantifies synergy or antagonism by way of how different the measured effect is from the expected additive effect. The equations are detailed elsewhere.[26,28,29] Interpretation of the combination index (CI) values are defined as: CI =1 indicates an additive effect; a CI of <1 indicates synergy; and a CI >1 indicates antagonism. Synergy is the working together of two agents to produce a result greater than the sum of their individual effects, while antagonism is less than that of an additive effect.

Table 2 Advantages of oncolytic virotherapy

Feature	Advantage
Replicates within tumor cells to increase viral dose	Amplification leads to oncolysis in cells beyond those initially infected Increases therapeutic index
Replicates only within tumor cells	Minimal toxicity to normal tissues
Can be used safely with other cancer treatments and may have synergistic effect	Increased efficacy of combined treatment
Can also be engineered or armed to carry a wide variety of transgenes to enhance the therapeutic effect such as prodrugs or inducers of immunological response	Dual effect of viral oncolysis and the added effect of the prodrug or immune stimulator
Some evidence that oHSV are capable of targeting and eliminating cancer stem cells	Eliminates the population of cells that are often resistant to chemotherapy and radiotherapy

Abbreviation: oHSV, oncolytic herpes simplex virus.

Table 3 Main cellular and viral pathways activated upon viral infection

Name of HSV-1 protein	Pathway
Vhs	Inhibits IRF3 and NF-kB Inhibits IFN-induced STAT 1 nuclear accumulation and phosphorylation Inhibits eIF2α phosphorylation
ICP34.5	Downregulates MHC class II cell surface expression Inhibits eIF2α phosphorylation
ICP0	Inhibits IRF3/IRF7 to repress ISG production Disrupts ND10 domains Degrades TLR adaptor proteins MyD88 and Mal
ICP27	Inhibits IRF3 and NF-kB Inhibits IFN-induced STAT 1 nuclear accumulation Inhibits eIF2α phosphorylation
US11	Prevents eIF2α activation via an interaction with PKR
US3	Controls TLR 3 RNA levels

Abbreviations: HSV-1, herpes simplex virus 1; ICP, infected cell polypeptide; IRF3, interferon regulatory factor 3; IRF7, interferon regulatory factor 7; NF-kB, nuclear factor kappa light chain enhancer of activated B cells; IFN, interferon; STAT1, signal transducer and activators of transcription 1; eIF2α, eukaryotic initiation factor 2; Vhs, virion host shutoff protein; MHC, major histocompatibility complex; ND10, nuclear domain 10; TLR, toll like receptor; MyD88, myeloid differentiation primary gene (88); Mal, myelin and lymphocyte protein; ISG, interferon stimulated gene; RNA, ribonucleic acid.

Table 4 Oncolytic viruses and chemotherapeutic agent

oHSV	Drug	Cell line	Cancer type	In vitro	In vivo	Reference
HSV1716	Cisplatin	UM_SCC	HNSCC	Additive	ND	114
		14CUM-SCC 22A	HNSCC	Additive	ND	
		UM-SCC 22B	HNSCC	Additive	ND	
HSV1716	Cisplatin, doxorubicin, mitomycin C, methotrexate	NCI-H460	NSCLC	Additive	ND	27
NV1066	Cisplatin	H-2452, H-Meso,	MPM	Synergistic	ND	41
		H-2373, H-28		Synergistic	ND	
		JMN, Meso-9		Synergistic	ND	
		MSTO-211H		Synergistic	ND	
		VAMT,		Additive	ND	
		H-2052		Additive	ND	
		Meso-10		Additive	ND	
G207	Cisplatin	SCC-25/CP	HNSCC	No effect	ND	123
		Sq20B		ND	No effect	
		UMscc-38		ND	Additive to synergistic	
G47Δ	Cisplatin	LNCaP	Prostate cancer	Antagonistic	ND	89
OncoVex-GALV/CD	Cisplatin	EJ	Bladder transitional carcinoma	Antagonistic	ND	65
		T24		Antagonistic	ND	
		TCCSUP-G		Antagonistic	ND	
rRp450 (CYP2B1)	Cyclophosphamide	Rh30	Alveolar rhabdomyosarcoma	ND	Enhanced	54
G47Δ	Doxorubicin	LNCaP	Prostate cancer	Antagonistic	ND	89
G207	Doxorubicin	KAT4	Anaplastic thyroid cancer	Additive	Enhanced	87
		DRO90-1		Additive	ND	
G47Δ	Docetaxol	LNCaP	Prostate cancer	Synergistic	Enhanced	89
		DU145		Synergistic	ND	
G207	Erlotinib	STS26T	MPNST	Additive	Not enhanced	94
G47Δ	Etoposide	LNCaP	Prostate cancer	Antagonistic	ND	89
G207	Fluorodeoxyuridine	HCT8	Colon cancer	Synergistic	ND	42
G207	5-fluorouracil	KIG8-5 (murine)	Gallbladder	Enhanced	Enhanced (Syrian hamster)	44
		MKN45 (human)	Gastric cancer	Enhanced (viral replication)	Enhanced (SCID mouse)	
NV1020	5-fluorouracil	HT29	Colon cancer	Enhanced	ND	45
		WiDr	Colon	Enhanced	ND	
		HCT116	Colon	Enhanced	ND	
		CT-26	Colon	ND	Enhanced	
NV1066	5-fluorouracil	Hs 700T	Pancreatic cancer	Synergistic	ND	39
		PANC-1 and PaCa-2	Pancreatic cancer	Synergistic	ND	
OncoVex-GALV/CD	5-fluorouracil	A549, H460	Lung cancer	Enhanced	ND	124
		CAPAN-1, MIA PACA-2, BXPC-3	Pancreatic cancer	Enhanced	ND	
		HCT-116, HT-29, SW620	Colon cancer	Enhanced	ND	
		9L LacZ (rat)	Gliosarcoma	ND	Enhanced	
NV1066	Gemcitabine	Hs 700T	Pancreatic cancer	Synergistic	ND	39
		PANC-1 and PaCa-2	Pancreatic cancer	Synergistic	ND	
R3616 hrR3	Gemcitabine	CAPAN1 and PaCa-2	Pancreatic cancer	ND	Enhanced both cell lines	64
		SW1990	Pancreatic cancer	ND	Not enhanced	
OncoVex-GALV/CD	Gemcitabine	EJ	Bladder transitional carcinoma	Antagonistic	ND	65
		T24		Synergistic	ND	
		TCCSUP-G		Antagonistic	ND	
		KU19-9		Antagonistic	ND	
HF10	Gemcitabine	CT26	Murine colorectal model	Antagonistic if given together Synergistic if GEM is pretreatment	Enhanced effect in both injected tumor and distal tumor	88

(Continued)

Table 4 (Continued)

oHSV	Drug	Cell line	Cancer type	In vitro	In vivo	Reference
NV1020	Irinotecan (SN38)	HT29 and WiDr	Colon cancer	Enhanced	ND	45
		HCT-116		Enhanced	ND	
MGH2	Irinotecan (SN38)	GI036ΔEGFR	Glioma	Enhanced	Enhanced	59
		U87ΔEGFR		Enhanced	ND	
		U251		Enhanced	ND	
		T98G		Enhanced	ND	
G207	Mitomycin C	OCUM-2MD3	Gastric cancer	Synergistic	Enhanced	36
		MKN-45-P		Synergistic	ND	
NV1066	Mitomycin C	KU19-19	Bladder transitional carcinoma	Synergistic	ND	126
		SKUB		Synergistic	ND	
OncoVex-GALV/CD	Mitomycin C	EJ	Bladder transitional carcinoma	Synergistic	ND	65
		T24		Synergistic	ND	
		TCCSUP-G		ND	ND	
		KU19-9		Synergistic	ND	
NV1020	Oxaliplatin	HT29 and WiDr	Colon cancer	Enhanced	ND	45
		HCT-116	Colon cancer	Enhanced	ND	
G207	Paclitaxel	KAT4	Anaplastic thyroid cancer	Synergistic	Enhanced	87
		DRO90-1		Synergistic	ND	
NV1023	Paclitaxel	KAT4	Anaplastic thyroid cancer	Synergistic	ND	87
		DRO90-1		Additive	ND	
G47Δ	Paclitaxel	LNCaP	Prostate cancer	Synergistic	ND	89
		DU145		Synergistic	ND	
MGH2	Paclitaxel	MDA-MB-4355	Mammary carcinoma	ND	Enhanced	127
G207	Temozolomide	U87	Malignant glioma	Synergistic	Enhanced	128
		U87-dnp53		Synergistic	ND	
		U373		Synergistic	ND	
		T98		Synergistic (with O6-benzylguanine)	ND	
		U87MGMT		Synergistic (with O6-benzylguanine)	ND	
G47Δ	Temozolomide	GBM13	Glioma stem cells (TMZ resistant/ MGMT+ve)	No synergy	ND	37
		BT74		No synergy	Not enhanced (enhanced in the presence of + O6-benzylguanine)	
		U87MG	Glioma	No synergy	ND	
		T98	Glioma	No synergy	ND	
		GBM4	Glioma stem cells (TMZ sensitive/ MGMT-ve)	Synergistic	ND	
		GBM6		Synergistic	ND	
		GBM8		Synergistic	Enhanced	
G207	Vincristine	KFR	Rhabdomyosarcoma	Enhanced	Enhanced	90
		KF-RMS-1		Enhanced	Enhanced	
NV1042	Vinblastine	CWR22	Prostate	Synergistic	Enhanced	78
		PC3		Synergistic	ND	

Abbreviations: MPM, malignant pleural mesothelioma; oHSV, oncolytic herpes simplex virus; TMZ, temozolomide; HNSCC, head and neck squamous cell carcinoma; ND, not done; MPNST, malignant peripheral nerve sheath tumor; GBM, glioblastoma; MGMT, methylguanine DNA methyltransferase.

Table 5 Oncolytic viruses and mTOR inhibitors

oHSV	Drug	Cell line	Cancer type	In vitro	In vivo	Reference
Baco-1	Rapamycin	HepG2	HCC	No effect	ND	46
		HuH-7	HCC	No effect	ND	
		MDA-MB-231	Breast cancer	No effect	ND	
		EC9706	Esophageal	Additive	Additive	
		MCF-7	Breast cancer	Additive	ND	
		HeLa	Cervical	Additive	ND	
MG18L	BEZ235	GBM4	Glioma stem cells	No effect	ND	85
		GBM8		No effect	ND	
		GBM13		Synergistic	ND	
		BT74		No effect	ND	

Abbreviations: oHSV, oncolytic herpes simplex virus; HCC, hepatocellular carcinoma; ND, not done.

Table 6 Oncolytic viruses and PI3K inhibitors

oHSV	Drug	Cell line	Cancer type	In vitro	In vivo	Reference
R7041	LY294002	U87	Glioma	Synergistic	Enhanced	86
MG18L	LY294002	GBM4	Glioma stem cells	Synergistic	ND	85
		GBM8		No effect	ND	
		GBM13		Synergistic	ND	
		BT74		Synergistic	Enhanced	
		U87	Glioma	Synergistic	ND	
		T98G		Synergistic	ND	
MG18L	GDC-0941	GBM4	Glioma stem cells	Synergistic	ND	85
		GBM8		No effect	ND	
		GBM13		No effect	ND	
		BT74		Synergistic	ND	
		U87	Glioma	Synergistic	ND	
		T98G	Glioma	Synergistic	ND	

Abbreviations: oHSV, oncolytic herpes simplex virus; ND, not done; PI3K, phosphatidylinositide 3-kinases.

Chou and Talalay[38] analysis can also be used effectively in vivo, but it is more common practice, as reported in the literature, to look for differences in tumor growth between treatment groups and to use analysis of variance or *t*-tests to determine if the differences (often either tumor volume or length of survival) between groups are significant. Information on synergy and/or enhanced efficacy of combinations will also come from clinical studies. Most patients that take part in new cancer therapy trials have already had, or are currently being treated with, the standard treatment for their particular disease, and it will be interesting to see if any group treated with oHSV and another agent respond better or worse than predicted. There are a number of different ways in which an oHSV in combination with an anticancer drug can be synergistic and these are discussed below.

Compounds that increase the replicative capacity of the virus

Oncolytic HSV have selective replication competence in cancer cells and, by increasing the replicative capacity of the virus within those cells, the number of progeny viruses produced during a cycle of infection could be increased (Figure 2).

Differentiating inducing agent hexamethylene bisacetamide (HMBA) has been shown to improve viral yield, with up to a 10,000-fold increase in vitro for an ICP34.5 null virus, R849, at low MOI (multiplicity of infection). HSV immediate early gene expression (Figure 4 shows the basic HSV replication cycle) was also increased with HMBA.[30] Mice treated with both HMBA and R849 virus had significantly smaller tumor burden and survived longer than either virus or HMBA treatment alone, with increased levels of HSV transcripts of immediate early, early, and late genes in the combination treatment group. This suggests HMBA may increase and/or activate cellular proteins such as transcription factors, which act to improve viral yield. HMBA is a drug that was thought to have some potential as a stand-alone anticancer agent; however, the level of drug required for such anticancer activity could not be achieved in patients.[31] In the study with oHSV, a much lower dose of drug was able to be used; one which could easily be achieved in patients and potentially would act as a promoting agent for oncolytic therapy.

Table 7 Oncolytic viruses and HDAC inhibitors

oHSV	Drug	Cell line	Cancer type	In vitro	In vivo	Reference
G47Δ	Trichostatin A	U87	Glioma	Synergistic	Enhanced	65
		T98		Synergistic	ND	
		SW480	Colon cancer	Synergistic	Enhanced	
		HeLa	Cervical cancer	Synergistic	ND	
		MCF-7	Breast cancer	Additive	ND	
R849	Trichostatin A	SAS	Oral SCC	Enhanced	ND	132
		Ca9-22		ND	ND	
		HSC		ND	ND	
rQNestin34.5	Valproic acid	U251	Glioma	ND	ND	133
		U87Δ EGFR		ND	Enhanced	

Abbreviations: oHSV, oncolytic herpes simplex virus; SCC, squamous cell carcinoma; ND, not done; HDAC, histone deacetylase.

Table 8 Oncolytic viruses and others

oHSV	Drug	Cell line	Cancer type	In vitro	In vivo	Reference
OncoSyn	Thalidomide	4T1	Breast	ND	Enhanced	134
R849	Hexamethylene bisacetamide	Ca9-22	Oral SCC	Enhanced	ND	30
		SAS		Enhanced	ND	
		F1		Enhanced	Enhanced	

Abbreviations: oHSV, oncolytic herpes simplex virus; SCC, squamous cell carcinoma; ND, not done.

Another mechanism for increasing viral yields may be to temporarily block apoptosis. Upon viral infection, one of the cellular host responses is to induce apoptosis in infected cells and in cells surrounding infected cells (Figure 3) in order to limit the ability of the virus to replicate and spread. Therefore, by blocking apoptosis temporarily, there is the potential for improving the propagation of viral progeny, maximizing the lateral spread of virus and increasing tumor destruction. Wood and Shillitoe[32] reported on increased viral replication in the presence of zVAD-fmk; a pan caspase inhibitor that has previously been shown to prevent HSV-1-induced apoptosis.[33] The authors showed that the inhibitor increased levels of replication in an ICP34.5 null mutant back to the levels of wild type HSV-1. Stanziale et al[34] also reported increased apoptosis in cells that neighbored NV1066-infected cells and could mitigate this effect with treatment with an inhibitor of apoptosis: N-acetylcysteine. This suggests that the increased viral yield seen with the caspase inhibitors is likely to be due to neighboring noninfected but alarmed cells being prevented from initiating apoptosis and, therefore, become lytically infected with virus.[32,34] Eisenberg et al[35] reported that hyperthermia potentiates oncolytic viral killing. After hyperthermic insult, the heat shock protein Hsp72 is upregulated, which inhibits cellular apoptosis, thereby allowing increased viral replication and, in turn, enhanced tumor kill. This finding has great potential as, in a clinical setting, the application of heat is likely to be noninvasive and relatively toxicity free.

Compounds that increase cell permissiveness to oHSV

Many chemotherapeutic drugs are DNA damaging agents and, following exposure to such agents, cells upregulate their DNA damage repair pathways. Such upregulation appears to be beneficial for oncolytic viral replication; mitomycin C,[36]

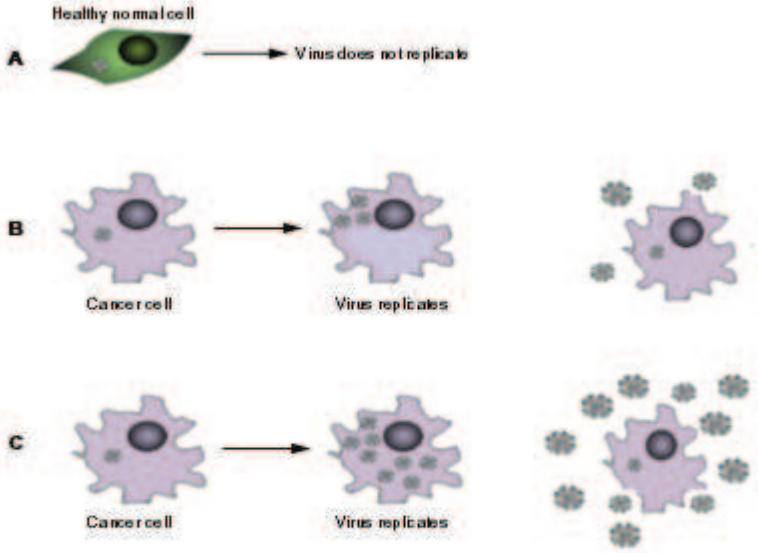

Figure 3 Increasing replicative capacity of the virus. (A) In normal cells the virus does not replicate. (B) In a cancer cell the virus replicates, lyses the cell and produces viral progeny that can go on to infect further cancer cells. (C) In the presence of certain drugs the virus can produce more viral progeny. Upon lysis more progeny virus are released – potentially increasing the number of cells that can be infected.

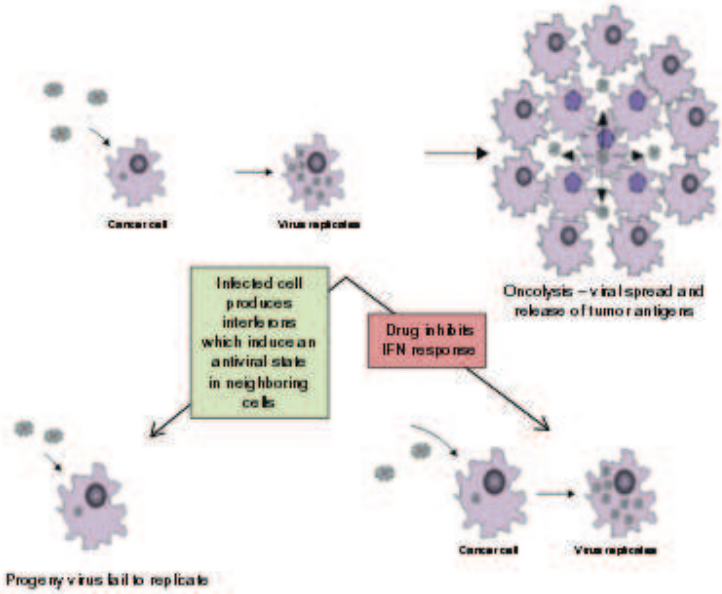

Figure 3 Anti-viral host response mediated by IFN (interferon) induces apoptosis of surrounding cells. By using drugs to block innate anti-viral defence mechanism the infected cell will not signal other nearby cells to 'warn' them about the virus, hence viral replication will occur.

temozolomide,[37,38] and 5FU[39] have all been shown to increase oncolytic HSV replication.

Growth arrest and DNA damage-inducible protein GADD34 is induced by stressful growth arrest conditions and treatment with DNA-damaging agents. The carboxyl terminal of GADD34 bears significant homology with the virulence factor ICP34.5, which is deleted in some oHSV, eg, HSV1716, NV1066, R3613, and T-Vec (Table 1). Previous studies[40] have

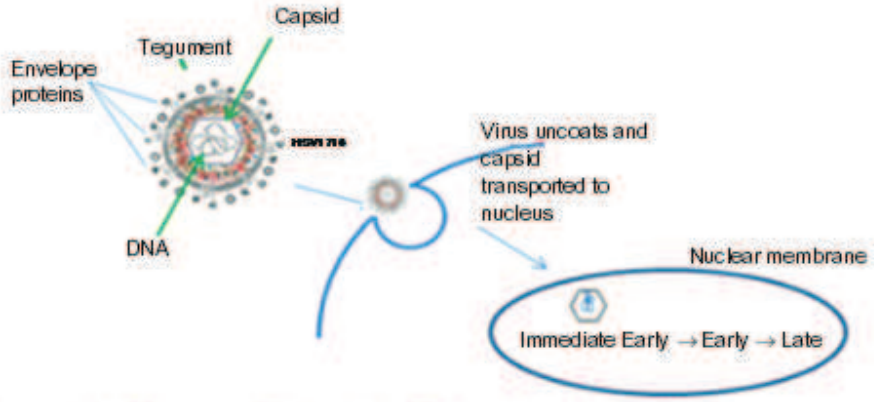

Figure 4 Herpes simplex virus (HSV) replication cycle. HSV-1 is a double stranded DNA virus which encodes for around 100 transcripts and contains three main structural components. The central capsid (or nucleocapsid) contains the viral DNA. This is surrounded by an envelope. The tegument is located between the envelope and the capsid. HSV enters the host cell at either the cell surface or via pH dependent endocytosis through a process involving envelope glycoproteins. The tegument proteins are released into the cell and the capsid is transported to the nucleus where viral DNA is released into the nucleus. There are three classes of viral genes that are transcribed and translated in a specific order: Immediate Early (IE) genes, which encode for proteins that promote expression of viral genes and also have a role in innate immune invasion. Early (E) are responsible for the replication of viral DNA and lastly Late (L) genes which include capsid, tegument and envelope proteins.

shown that the carboxyl terminus of GADD34 can substitute for ICP34.5 in preventing premature shutoff of protein synthesis, and ICP34.5 null mutants can use the host cell GADD34 protein for viral replication. Thus, the presence of GADD34 in tumor cells following treatment with a DNA damaging agent would increase the number of cells permissive to oHSV infection and increase the viral spread through the tumor. Indeed, when GADD34 small interfering RNAs (siRNAs) were added to block GADD34 expression after treatment with a DNA damaging agent (cisplatin), the previously observed synergy with the oHSV NV1066 and cisplatin was abolished.[41]

Another potential mechanism for synergy with some oHSV is upregulation of cellular RR by DNA-damaging chemotherapeutic agents.[42] High throughput screening has been reported to identify small-molecule compounds that augment the replication of HSV G47Δ,[43] and, of the 2,460 compounds screened, six compounds were identified and subsequently validated for enhanced G47Δ replication. Two of these compounds, dipyridamole and dilazep, interfered with nucleotide metabolism by potently and directly inhibiting the equilibrative nucleoside transporter-1 and were dependent on HSV mutations in ICP6, the large subunit of RR. Equilibrative nucleoside transporter-1 antagonists are thought to augment oHSV replication in tumor cells by increasing cellular RR activity.[43] As oHSV with *UL39* deletions can only replicate in cells with active cellular RR, increasing cellular RR will improve viral replication.

Nakano et al[44] reported an upregulation in RR in tumors mediated by 5FU that augmented the therapeutic effect of G207. 5FU was also found to be synergistic both in vitro and in vivo with oHSV NV1020 (an oHSV with intact ICP6),[8] suggesting the effects of 5FU are not limited to upregulation of RR. The authors speculated that the synergy was in part due to the cells being sensitized to 5FU as the virus caused the cells to arrest in S phase of the cell cycle. They further speculated that the reduction in viral progeny could be due to the immune IFN (interferon)-γ response as well as the 5FU-induced upregulation of cell death via molecules such as TRAIL (TNF [tumor necrosis factor] related apoptosis-inducing ligand) and Fas ligand.

Rapamycin markedly increased the yield and dissemination of oHSV in semipermissive tumor cells both in vitro and in vivo but had no additional effect in cell lines that are permissive to the ICP34.5 null mutant oHSV Baco1.[45] The reason behind the observation is still unclear; however, inhibitors of the mTOR (mammalian target of rapamycin) signaling pathway increase permissiveness of resistant tumor cells to oncolytic myxoma virus,[47] vesicular stomatitis virus,[48] adenovirus,[49] and cytomegalovirus,[50] suggesting that the mTOR signaling pathway has an important role to play in virotherapy.

Compounds that modulate the immune system

The immune response to oncolytic viral therapy is an essential factor determining the success of oHSV as an antitumor agent; it can be a hindrance if it causes premature viral clearance, or could be seen as a positive, with the virally infected tumor becoming a target for clearance by the immune system.

The immune response to viral infection is beyond the scope of this review, but for an excellent insight into this field see Paludan et al.[32] Briefly, the immune reaction to a viral infection (oncolytic or otherwise) is a multipronged response. Very quickly upon infection, the innate immune response recruits natural killer (NK) cells, macrophages, and neutrophils to the site of infection and mediates a nonspecific viral clearance. NK cells appear to be an important player in the response to viral infection; patients with naturally occurring NK cell deficiencies (despite there being numerous different mutations that cause such deficiencies) have severe and recurrent herpes virus infections.[51] NK cells, activated by macrophages secreting IL-12, mediate the lysis of virally infected cells by releasing cytotoxic granules containing lytic enzymes and by binding to apoptosis-inducing receptors on the infected cell. In addition, NK cells secrete IFN-γ, which activates further macrophages and, consequently, orchestrates the downstream adaptive immune response.

The oncolytic HSV rQNestin34.5 (ICP34.5 expression controlled by the nestin promoter) has been shown to induce a rapid recruitment of NK cells to orthotopic human glioblastoma xenografts with subsequent killing of the oHSV-infected xenograft cells by activated macrophages. Depletion of NK cells improved the oHSV efficacy in these glioblastoma models, further indicating the importance of the NK cells.[52] Previous studies have demonstrated that inhibition of the innate immune response using cyclophosphamide[53,56] or macrophage depletion[57] enhances oHSV replication and efficacy. An oHSV variant, rRp450, with deleted ICP6 and incorporated cytochrome P450 transgene for direct cyclophosphamide activation has been described, and the virus enhances the antitumor effects of cyclophosphamide.[18,54,58,59]

Another key event in the immune response to viral infection is the secretion of IFN-γ (for an extensive review see Roizman[60] and Bazan-Peregrino et al[61]). The cytokine IFN-γ, or type II interferon, is critical for innate and adaptive immune response to viral infection, partly from its ability to inhibit

viral replication directly, but, more importantly, also from its immunostimulatory and immunomodulatory effects. IFN-γ is produced predominantly by NK cells as part of the innate immune response, and by cluster of differentiation (CD)4+ T helper (Th)1 and CD8 cytotoxic T lymphocyte (CTL) effector cells once antigen-specific immunity develops.

Histone deacetylase inhibitors (HDIs) are a class of compounds that appear to benefit HSV oncolysis, possibly via suppression of innate immune responses. Histone deacetylases (HDACs) have pleiotropic effects on cells through deacetylation of proteins, including histones, which then alter the epigenome and transcription profiles. Numerous HDACs have been targeted for drug discovery for cancer therapies, either for use as a single agent or in combination with chemotherapeutic agents. Pretreatment with the HDI valproic acid was shown to enhance the oncolytic virus MGH2 and rQNestin34.5 replication and spread in tumors, and extended the survival of mice bearing intracerebral tumors.[59,61] The authors attributed the synergy between HDIs and oHSV to inhibition of type I interferon responses that would usually restrict viral gene expression and replication.

Drugs that cause downregulation of the innate immune response can be synergistic with oncolytic viruses but there is also evidence of the immune response enhancing tumor clearance.[62] Benencia et al[63] reported that oHSV therapy was less effective in murine metastatic melanoma models lacking NK and T cell subsets. Similarly, HSV1716-induced expression of IFN-γ inducible chemokines was accompanied by a significant increase in the number of NK and $CD8^+$ cells in the tumor microenvironment in a syngeneic ovarian carcinoma model.[59,63]

Synergy has also been reported with oHSV and compounds that increase IFN-γ production.[64] The authors found that pretreating tumor cells with gemcitabine before oHSV significantly reduced tumor growth in vivo. Pretreatment was necessary as the drug itself induces early termination of DNA synthesis, which prevents replication of oncolytic viruses.[20,64,65] Gemcitabine selectively kills myeloid-derived suppressor cells, which inhibit IFN-γ production by CD8+ cells. So, when myeloid-derived suppressor cells themselves are killed, CD8+ T cells will secrete higher levels of IFN-γ, thus directing more T cells to tumor sites, which results in an improved antitumor response. In addition, IFN-γ can change the tumor microenvironment in terms of macrophages phenotype. Macrophages are classified as m1 (classically activated) or m2 (alternatively activated). During tumor progression there is a switch from m1- to m2-like phenotype that is believed to allow the tumor cells to avoid the immune system. Higher levels of IFN-γ can change the macrophage phenotype back to m1, resulting in the cancer cells being more likely to be tagged for destruction by the immune system.[64]

Recently, a number of immunotherapeutic agents have been approved as cancer treatments. Ipilimumab, a monoclonal antibody that blocks the CTL-associated antigen 4 receptor, which would normally inhibit cytotoxic T lymphocyte, for example, is approved for use in advance metastatic melanoma.[67,68] It is by blocking the CTL-associated antigen 4 receptor that CTLs are activated and can recognize and destroy cancer cells. As the presence of an oncolytic virus within a tumor will make the tumor more antigenic, there is good reason to think that the combination of oncolytic virus and immunotherapy will be synergistic and, indeed, there are many reports of improved efficacy of oHSV engineered to express genes that make immunomodulatory proteins including IL-12, IL-24, IL-4, RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), CD80, and IFNα.[69] Granulocyte-macrophage colony-stimulating factor, which generates an antitumor response by the recruitment and differentiation of activating dendritic cells in the tumor microenvironment, has been inserted successfully into T-Vec,[69,70] and a clinical study investigating T-Vec in combination with ipilimumab is underway,[71] with primary results expected in summer 2016.

Immunomodulatory drugs highlight the complexities of potential interactions between oHSV and anticancer agents, with synergy reported with drugs that inhibit or upregulate the immune system. It is likely that drugs that inhibit the very early innate immune response will allow the virus longer to enter cells and undergo initial viral replication, increasing the spread of the virus. Drugs that act by boosting later immune responses, such as upregulating T cells, mean that the infected tumor cells and potentially uninfected neighboring tumor cells are more likely to be targeted for destruction by the immune system. It will be interesting to see if downregulating innate immunity by HDIs, for example, and upregulating T cells by gemcitabine, would result in further synergistic effects when combined with an oncolytic virus. To date, no triple combinations have been reported in the literature, probably due to the increasing complexity of such experiments.

Compounds that alter the tumor microenvironment

Angiogenesis is the formation of new blood vessels and, as tumors need blood vessels to grow and spread, inhibitors of angiogenesis, which prevent the formation of new blood vessels, could potentially prevent or slow the growth or spread

of tumors. Unlike chemotherapeutic agents, angiogenesis inhibitors will not kill cancer cells directly but instead prevent tumors from growing, so potentially, in order to completely eradicate a tumor, an antiangiogenic drug would have to be given in combination with a modality that kills cancer cells, such as an oncolytic virus.

Vascular endothelial growth factor (VEGF) is a key component in tumor angiogenesis and is overexpressed in many human tumors. It has numerous effects on tumor vasculature such as increased vasodilation and permeabilization, and inhibitors of VEGF, such as Avastin®, sorafenib, and sunitinib, appear to "normalize" tumor vasculature, potentially enhancing localization of systemic oncolytic virus. ICP34.5 null oHSV infectivity and cytotoxicity were diminished under hypoxic conditions (when the cells are deprived of oxygen) in several glioblastoma xenolines, which are cell lines maintained by xenograft passage.[69] Normalization of the blood vessels by antiangiogenic agents may reduce hypoxia within the tumor microenvironment and potentially improve oHSV replication. However, other studies have shown improved oHSV replication in hypoxic conditions.[70–73] Bevacizumab (Avastin®), a monoclonal antibody against VEGF A, had no effect on the spread or replication of oHSV in vitro. However, in vivo, in several studies using different xenograft models,[74,75] groups of mice receiving the dual therapy of both oHSV and Avastin® had tumors that were significantly smaller than tumors from either treatment alone. Results from these studies indicated that Avastin® improved replication and spread of the oHSV within the xenograft microenvironment. Although cytotoxic in vitro, in some xenograft models rRp450 had only mild antitumor effects.[76] The host inflammatory response to rRp450 therapy was found to induce an acute neutrophil infiltrate, a relative decrease of intratumoral macrophages, and a myeloid cell-dependent upregulation of host-derived VEGF. Bevacizumab and r84 (which selectively inhibit binding to VEGF receptor 2 but not VEGF receptor 1) enhanced the antitumor effects of rRp450 therapy, in part due to decreased angiogenesis. However, although neither bevacizumab nor r84 increased virus production or affected neutrophil infiltration, both partially mitigated virus-induced depletion of macrophages. Therefore, the enhancement in efficacy with the combination of oHSV therapy and anti-VEGF antibodies appears to be in part due to modulation of host inflammatory reaction to virus.

Vinblastine, a microtubule disrupting agent that has been shown to inhibit angiogenesis in humans[77] and, in combination with the oHSV NV1042, showed increased antitumor and antiangiogenic effects in vivo in prostate cancer models,[78] provides further evidence that the combination of an antiangiogenic agent and an oncolytic virus may have clinical benefit. However, to the best of our knowledge, there are no preclinical published studies of oHSV in combination with small molecule VEGF receptor inhibitors such as sorafenib or sunitinib.

HSV DNA replication occurs in discrete compartments in the nucleus that assemble as prereplicative sites with viral DNA and the HSV DNA binding protein ICP8. HSV DNA polymerase and cellular factors are then recruited to these compartments for use in viral replication. The DNA damage and repair pathways repair the damage to the cancer cell DNA caused by treatment with DNA-damaging drugs such as temozolomide (TMZ). However, in the presence of oHSV infection, key components of these pathways are sequestered into discrete compartments for use in viral replication, hence are not available to repair the damage caused by drugs. Thus, the damage, in terms of number of cancer cells killed by a specific amount of drug, is greater in the presence of oHSV.[27]

Cellular kinases play a key role in the regulation of signaling events that govern multiple pathways affecting growth, proliferation, migration, and angiogenesis. These include PI3K (phosphatidylinositide 3-kinases)-Akt-mTOR and mitogen-activated protein kinases pathways, which are often mutated in cancer cells to support unchecked cellular replication. Inhibition of these pathways could potentially reduce tumor growth, and this is reflected in the intensive drug development looking for PI3K-Akt-mTOR and mitogen-activated protein kinases inhibitors. For example, 80% of glioblastomas are having genetic alterations in the PI3K-Akt-mTOR pathways and there are at least 10 different inhibitors in development.[79] However, due to the high level of redundancy and cross regulatory feedback loops, monotherapy may be unlikely to have significant clinical efficacy;[80] for example, rapamycin only reduces mTOR activity for 12 hours before another kinase substitutes and reengages the mTOR network.[81] Furthermore, such inhibitors are likely to be cytostatic: they will stop the cancer cells from growing or dividing but will not eradicate them.

The PI3K-Akt-mTOR pathway is also important in viral replication (for a full review see Terada et al[61] and Buchkovich et al[82]). Upon infection, viruses frequently activate this pathway to benefit from the survival signaling associated with Akt activation. One of the downstream effectors of activated Akt is the mTOR kinase, a component of the mTOR complexes (mTORC) 1 and 2. Activated mTORC1 is crucial for the maintenance of cap-dependent translation which is

required by most mammalian DNA viruses and many RNA viruses. mTORC2 is less well understood, but is thought to have roles in Akt phosphorylation and the organization of the actin cytoskeleton. It would therefore seem reasonable to assume that inhibitors that block the function of mTOR or PI3K would not only block translation of cellular proteins but would drastically reduce the ability of viruses to replicate by virtue of stopping their cap-dependent translation. Theoretically, PI3K and mTOR inhibitors would be antagonistic if used in combination with oncolytic viruses. The literature, however, reveals diverse results that vary depending on the specific virus, the specific inhibitor, and the status of the cells used.

Breitbach et al[83] found that compounds such as rapamycin, which blocks the activation of mTOR, and PD098059, which blocks the activation of MAP (mitogen-activated protein) kinase, did not affect the ability of oHSV R3616 to replicate in pancreatic tumor cells. Treatment with the inhibitor LY294002, which inhibits the PI3K pathway, prevented the replication of R3616. Similarly, synergy was not observed between LY294002 and the ICP34.5 null oHSV, but was observed with oHSV mutants with a Us3 mutation.[84] The gene product of Us3 protects virus-infected cells from apoptosis; a cellular pathway that is often dysfunctional in tumors. Thus, Us3 mutants, whose replication would be inhibited by apoptosis in normal cells, would be selective for tumor cells, and the combination treatment of LY294002 and Us3-null oHSV is synergistic due to enhanced apoptosis in the combination treated cells.[85]

Compounds that affect the cell cycle

Strong synergy between oHSV and trichostatin A (an HDAC inhibitor) was observed in a wide range of cancer and proliferating endothelial cell lines but not in normal prostate or quiescent epithelial cells.[86] Unlike other HDIs, the synergy was seen regardless of the dosing sequence of the oHSV (G47Δ) or trichostatin A. The synergy was attributed to reduced cyclin D1 expression in cells that normally have a high level of cyclin D (ie, cancer cells). The combination also inhibited secretion of the angiogenic factor VEGF, which correlated with the decreased vascularity within the tumor in vivo.

Another combination that appears to affect the cell cycle occurs between the oHSV G207 and paclitaxel. Paclitaxel is an approved cancer therapy that stabilizes microtubules and, as a result, interferes with the normal breakdown of microtubules during cell division. In the presence of paclitaxel, chromosomes are unable to achieve metaphase spindle configuration. This inability to form the correct formation blocks the progression of mitosis which in turn triggers apoptosis or the cell to revert to the G phase of the cell cycle without dividing. Despite the G207/paclitaxel combination being synergistic, oncolysis or viral replication was not increased.[87] The authors concluded that they differentially affected cell cycle progression, either by the cells arresting in G1 (virus-mediated) or mitosis (paclitaxel-mediated), a combination that served to increase apoptosis further. Paclitaxel also showed synergy with other oHSV, HF10, and G47Δ, both in vitro and in vivo.[88,89] The oHSV HF10 has been studied alone and in combination with paclitaxel in colon cancer models.[88] In vivo, the combination of HF10 and paclitaxel prolonged survival of mice bearing carcinomatous dissemination of CT26 tumors compared with the control groups. G47Δ also synergized with paclitaxel and the closely related docetaxel to enhance the in vitro killing of LNCap and DU145 prostate cancer cells.[89] Docetaxel-induced accumulation of the phosphospecific mitotic markers op18/stathmin or histone H3 was significantly reduced by G47Δ, and this correlated with enhanced apoptosis and required active virus replication. Another microtubule inhibitor, vincristine, was also shown to be synergistic with oHSV in rhabdomyosarcoma xenografts.[90]

Cheema et al[91] reported synergy with etoposide, an inhibitor of topoisomerase II, and oHSV G47Δ in glioma stem cell xenografts. Gutermann et al[8] found synergy with SN38 (the active metabolite of irinotecan, a topoisomerase I inhibitor) and NV1020 in a panel of human colon carcinoma cell lines in vitro. Synergy with irinotecan and MGH2 (an oHSV with UL39 and -γ34.5 deletions) was also reported in glioma, both in vitro and in vivo.[39]

Other compounds where synergy and/or enhancement is seen but the mechanism is unclear

Although not using an oHSV, Heo et al[92] reported on the first clinical signs of positive interactions between oncolytic virotherapy and standard of care drugs with JX-594 (an oncolytic pox virus) and sorafenib, a small molecule inhibitor of the signaling oncoprotein B-raf and VEGF receptor, which is licensed as a treatment for hepatocellular carcinoma. The authors reported that a number of patients treated with JX-594 and then sorafenib up to 8 weeks later had objective tumor responses (ie, tumor shrinkage) compared to zero in 15 untreated patients matched for age, stage, and sex. Furthermore, they also reported a complete cure in one patient treated with sunitinib, another inhibitor

similar to sorafenib, 8 weeks after JX-594 treatment. As the virus is likely to be cleared from the patient by 8 weeks, the mechanism by which the oncolytic virus can sensitize tumors to these inhibitors is unclear. Interestingly, the patients who have the best responses to sorafenib are those patients who have hepatitis C related hepatocellular carcinoma,[93] suggesting that there may be a therapeutic class effect where viruses sensitize tumors to VEGF receptor inhibitors.

Erlotinib, an epidermal growth factor receptor inhibitor, combined additively with two oHSV, G207, and hrR3 in order to enhance cytotoxicity in vitro in human malignant peripheral nerve sheath tumor cells often associated with Ras/epidermal growth factor receptor hyperactivation; however, this effect did not translate into an in vivo malignant peripheral nerve sheath tumor xenograft model.[94] Thalidomide, which is now approved for use in multiple myeloma patients, was found to have significant benefit in reducing tumor burden in combination with OncdSyn (an NV1020-like oHSV) than either OncdSyn or thalidomide alone in a murine breast cancer model,[95] though the mechanism is unclear.

Conclusion

Oncolytic viruses are a new and emerging treatment for cancer. As they become an established therapy, much attention will have to be paid to the interaction between current standard of care drugs and oncolytic viruses. So far, the signs are encouraging; not only can oHSV be given alongside other cancer treatments, but can actually result in an enhancement of efficacy in reducing tumor burden and improving survival. The majority of virus–drug combinations listed in Tables 4–8 show synergistic, enhanced, or additive effects, but this may in part reflect the fact that antagonistic combinations might not be submitted for publication. Recently, Kulu et al[96] reported on the inhibition of HSV oncolysis in colon and pancreatic cancer cell lines in vitro when combined with 5-FU, irinotecan, or methotrexate. Their studies showed that replication of both ICP6 and/or ICP34.5 deleted oHSV was significantly reduced in HT29 and SW620 (colon) and Capan-2 (pancreatic) cell lines. Others have reported additive/synergistic interactions (with respect to cell killing) between 5-FU, irinotecan, and methotrexate (Table 2) with oHSV in diverse cell lines, including both colon and pancreatic lines. It is conceivable that the drugs can inhibit virus replication but the combined effects of virus and drug act in concert to enhance cell death, and seemingly conflicting results serve to illustrate our poor understanding of such interactions.

Furthermore, the sequence in which the drug and oHSV are given may impact on cell killing. For example, gemcitabine and HDIs such as valproic acid are synergistic when given as a pretreatment to the virus, thus sensitizing the tumor to virus, whereas sorafenib appeared to work better given after oncolytic virus; thus the virus is acting as the sensitizer. Similarly, when oHSV rRp450 was given before Avastin® (bevacizumab) there was a significantly prolonged survival compared to the same combination in reverse order.[74]

Many of the published combination studies examined the effects of combinations in vitro. These identify combinations that enhance cancer cell cytotoxicity. However, many of the interactions between oHSV and drugs either affect the tumor or host biology, and these interactions will only be seen in vivo. The immune system is a key player in the efficacy of any combination treatment; it appears that initial suppressing of the innate immune response in order to allow the virus to undergo replication, then an upregulation of the immune system to clear the virus and tumor, would be a rational strategy in terms of reducing tumor burdens.

The use of patient-derived tumor xenografts, where primary human tumors are transplanted into immune deficient mice within hours after the sample is collected, are increasingly being used to predict the effectiveness of chemotherapeutic drugs in patients. To our knowledge, such models have not been reported for testing combinations of oncolytic HSV together with chemotherapy or targeted drugs, but are likely to be valuable and should provide data that will improve decision making and accelerate development programs for virus/drug combinations.

As preclinical studies progress into the clinical setting, major progress in the understanding of oHSV in combination with other treatments is likely to occur. Early clinical trials usually involve patients who have already exhausted all the available standard treatment options, and even later Phase III trials will often compare standard of care versus standard of care plus oHSV. Such studies should help confirm preclinical findings on useful virus/drug combinations and hopefully bring benefit to cancer sufferers.

Disclosure

Lynne Braidwood, Joe Conner, and Alex Graham are employees of Virttu Biologics Ltd. The authors report no other conflicts of interest in this work.

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ORIGINAL RESEARCH

Potent efficacy signals from systemically administered oncolytic herpes simplex virus (HSV1716) in hepatocellular carcinoma xenograft models

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Abstract: Oncolytic herpes simplex virus (HSV1716), lacking the neurovirulence factor ICP34.5, has highly selective replication competence for cancer cells and has been used in clinical studies of glioma, melanoma, head and neck squamous cell carcinoma, pediatric non-central nervous system solid tumors, and malignant pleural mesothelioma. To date, 88 patients have received HSV1716 and the virus is well tolerated, with selective replication in tumor cells and no spread to surrounding normal tissue. We assessed the potential value of HSV1716 in preclinical studies with two human hepatocellular carcinoma cell lines, HuH7 and HepG2-luc. HSV1716 displayed excellent replication kinetics in vitro in HepG2-luc cells, a cell line engineered to express luciferase, and virus-mediated cell killing correlated with loss of light emissions from the cells. In vivo, the HepG2-luc cells readily formed light-emitting xenografts that were easily visualized by an in vivo imaging system and efficiently eliminated by HSV1716 oncolysis after intratumoral injection. HSV1716 also demonstrated strong efficacy signals in subcutaneous HuH7 xenografts in nude mice after intravenous administration of virus. In the HuH7 model, the intravenously injected virus replicated prolifically immediately after efficient tumor localization, resulting in highly significant reductions in tumor growth and enhanced survival. Our preclinical results demonstrate excellent tumor uptake of HSV1716, with prolific replication and potent oncolysis. These observations warrant a clinical study of HSV1716 in hepatocellular carcinoma.

Keywords: oncolytic herpes simplex virus, HSV1716, hepatocellular carcinoma, xenografts, efficacy

Introduction

Hepatocellular carcinoma (HCC), a leading cause of cancer-related cell deaths worldwide, is increasing in prevalence.[1–3] Early detection of the disease is limited, and many patients present with advanced, inoperable, and aggressive disease, so have an extremely poor prognosis. In addition to surgery or chemotherapy, other treatment options include local ablation by, for example, heat, radiofrequency, or transarterial chemoembolization, although no standard therapy exists for patients who are not suitable for transplantation or surgical resection, or for patients with recurrent HCC, with systemic chemotherapy often considered in these patients albeit in a palliative capacity. Sorafenib is a multikinase inhibitor drug and is the only treatment approved by the US Food and Drug Administration for patients with advanced liver cancer. However, sorafenib is not curative, and patient outcomes remain poor. Monotherapy with sorafenib in HCC reduces the risk of death in year 1 by 31%, and the median

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survival time in patients treated with sorafenib is typically 3 months longer than for patients treated with placebo.[4] New approaches to the treatment of HCC are urgently required, and recent studies with the oncolytic vaccinia virus, JX-594, suggest that oncolytic virotherapy has potential in treating advanced HCC.[5]

We have investigated the therapeutic potential of the oncolytic herpes simplex virus-1 (HSV-1) HSV1716 in HCC in preclinical studies. The HSV-1 mutant HSV1716 has deletions in both copies of the gene encoding the neurovirulence factor ICP34.5. HSV1716 effectively kills tumor cell lines in vitro and, in a wide range of in vivo cancer models, HSV1716 oncolysis has induced tumor regression and increased survival times in a wide range of solid tumors, including glioma, melanoma, medulloblastoma, mesothelioma, ovarian carcinoma, and teratocarcinoma, supporting translation into clinical studies.[6–9] In clinical trials, direct intratumoral injection of HSV1716 has been used to treat patients with recurrent glioma, metastatic melanoma, and squamous cell carcinoma of the head and neck.[10–14] A Phase I dose escalation study of intratumoral HSV1716 in pediatric/young adult patients with non-central nervous system solid tumors (http://clinicaltrials.gov/NCT00931931) and a Phase I/IIa study in malignant pleural mesothelioma (http://clinicaltrials.gov/NCT01721018) are currently ongoing. Although the efficacies of other oncolytic HSV have been assessed in various HCC cell lines in tissue culture and xenografts, to date there have been no preclinical studies of HSV1716 in HCC and no xenograft studies that have assessed the potential for its systemic administration.[15–23] In this work, we demonstrated strong efficacy signals in two xenograft HCC models, particularly when the virus was administered systemically, which support a clinical study of HSV1716 in advanced HCC.

Materials and methods

Cells

HuH7 (JCRB0403; Japanese Collection of Research Bioresources Cell Bank, Tokyo, Japan) is a well-differentiated, hepatocyte-derived carcinoma cell line that was originally taken from a liver tumor in a 57-year-old Japanese male. HepG2-luc (HT1080-*luc2*; Perkin-Elmer, Beaconsfield, UK) is a luciferase-expressing cell line stably transfected with the firefly luciferase 2 gene under the control of the human ubiquitin C promoter. The HepG2 cell line was isolated from a liver biopsy of a male Caucasian aged 15 years with a well differentiated HCC. The one58 cell line (10092313; European Collection of Cell Cultures, Salisbury, UK) was derived from the pleural fluid of a patient with malignant mesothelioma, and the U87 cell line (European Collection of Cell Cultures, 89081402) was derived from a female patient with malignant glioma. HuH7, U87, and one58 cells were cultured in Dulbecco's Modified Eagle's Medium/F12 medium with 10% newborn calf serum, and HepG2-luc cells were cultured in advanced Roswell Park Memorial Institute 1640 medium supplemented with 20% newborn calf serum (Invitrogen, Paisley, UK) at 37°C in 5% CO_2.

Viruses

Stocks of HSV1716 and wild-type HSV-1 17+ were suspended in compound sodium lactate with 10% glycerol. HSV1716gCluc was derived using a site-specific recombination system and has the firefly luciferase gene derived from pGL3 (Promega, Southampton, UK) under control of the HSV-1 gC promoter inserted in both *RL1* loci.[24,25] Stocks of HSV1716gCluc were prepared with virus suspended in Dulbecco's Modified Eagle's Medium/F12 medium. All dilutions from stock to the appropriate titers used compound sodium lactate +10% glycerol and were titrated to confirm pfu/mL prior to use.

Single and multiple step growth kinetics

HuH7 or HepG2-luc cells were plated in 60 mm dishes and after 24 hours were infected with HSV-1 17+ or HSV1716 at various multiplicities of infections (moi). The dilutions of each virus preparation used for these infections were titrated to confirm the amounts of input virus. In each single experiment, virus infection of HepG2-luc and HuH7 were performed in triplicate or quadruplicate, respectively. After 24, 48, or 72 hours of infection, cells and medium were harvested, subjected to one freeze/thaw cycle (−70°C), and titrated. The results are reported in yields of progeny/input infectious virus and the data were analyzed using GraphPad Prism version 4.02.

In vitro toxicity assays for HepG2-luc cells

HSV1716 toxicity in HepG2-luc cells was assessed using loss of light emission and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell survival assays. HepG2-luc cells were plated out in the internal 6×10 grid of a 96-well tissue culture plate (Greiner Bio-One Ltd, Stonehouse, UK) at ~5,000 cells per well. HSV1716 was added at increasing moi after 24 hours in culture in quadruplicate at least and, after a further 72 hours of incubation, the effect of the virus on light emissions and cell survival were determined. Light emission was detected after addition

of 0.05 mL luciferase substrate to each well, and after 5 minutes of incubation at 20°C, light output was measured using a 1420 multilabel counter Victor 3 (Perkin-Elmer) in luminometer mode for 0.1 sec/well. Luciferin substrate was prepared by dissolving 1 g of D-luciferin, potassium salt (OZ Biosciences, Marseilles, France) in 66 mL of phosphate-buffered saline. Cell survival was assessed by addition of 0.01 mL of MTT reagent (Promega) to each well and the absorbance at 490 nm was determined after 1 hour of incubation at 37°C in 5% CO_2. Killing curves for HSV1716 by loss of light emissions or MTT assay (GraphPad Prism version 4.02) were used to determine the ED_{50} (effective moi that kills 50% of the cells) for HSV1716.

Animals

All animal procedures were performed under license from the UK Home Office. Female athymic nude mice (6–8 weeks; Charles River Laboratories, Margate, UK) were maintained under specific pathogen-free conditions. Actively growing HuH7 or HepG2-luc cells were harvested, and after resuspension in phosphate-buffered saline, 1×10^6 cells per mouse were injected subcutaneously. When HuH7 xenografts reached approximately 5 mm in diameter or HepG2-luc xenografts emitted a stable light signal $>10^6$ radiance as determined using an in vivo imaging system (IVIS®; Perkin-Elmer), the mice were injected intratumorally or intravenously via the tail vein with phosphate-buffered saline (no virus) or HSV1716, and were inspected daily after treatment.

For IVIS analysis, the mice were subcutaneously injected in the neck with 0.2 mL of luciferin substrate and left for 15 minutes to assure peak bioluminescence. Luciferin substrate was prepared by dissolving 1 g of D-luciferin potassium salt in 66 mL of phosphate-buffered saline, which was filter-sterilized and frozen in small aliquots to avoid repeated freeze/thaw cycles. The mice were anesthetized using isoflurane gas before being moved into the IVIS machine, and each mouse was placed on a nose cone apparatus to maintain anesthesia throughout imaging. Images were acquired using the Living Image (Perkin-Elmer) software. Areas of luminescence were determined in radiance (watts per steradian per square meter). The mice were then returned to their cages and allowed to recover from the anesthetic.

When HuH7 tumor diameters reached 15 mm, the mice were sacrificed and their xenografts and organs were removed for analysis. The viral load in tumors and organs was assessed by plaque-forming assay on Vero cells. Extracted intact tumors/organs were frozen immediately at −70°C, and after thawing, the tissues were mechanically homogenized for 10 seconds in an Omni TH homogenizer in 1 mL of phosphate-buffered saline prior to titration or luciferase assay. Luciferase activity was determined in 96-well enzyme-linked immunosorbent assay plates using D-luciferin (Promega) as substrate added to 0.1 mL of extract with a 1420 multilabel counter Victor 3 in luminometer mode for 0.1 sec/well.

Results

In vitro replication in HuH7 and HepG2-luc cells

In a single experiment, HuH7 cells were plated in 60 mm dishes, and after 24 hours in culture, the cells were infected with HSV1716 or wild-type HSV-1 17+ in quadruplicate at moi of 0.001 or 0.01. After 72 hours of infection, cells were harvested into the medium and total virus was titrated. At moi 0.01, both viruses had similar levels of propagation in HuH7 cells, with 1,298±361 pfu/input virion and 1,871±412 pfu/input virion for HSV1716 and HSV-1 17+, respectively, with no significant differences between the progeny yields (Student's *t*-test, $P=0.082$). However, at moi 0.001, HSV1716 produced approximately five-fold more progeny than HSV-1 17+, with respective yields of 5,283±609 pfu/input virion for HSV1716 compared with 1,183±288 pfu for HSV-1 17+, and the difference was highly significant (Student's *t*-test, $P=0.0001$). Using the same 0.001 moi preparations but in a separate experiment, HSV-1 17+ yielded approximately 43,100±13,988 pfu and 6,850±2,087 pfu/input virion and HSV1716 yielded 8,806±2,713 pfu or 1,573±182 pfu progeny/input virus after 72 hours of infection in U87 and one58 cells, respectively. Thus, HSV1716 replication in U87 and one58 cells was impaired approximately four to five-fold compared with wild-type HSV-1 17+. As an additional control, Vero cells were infected in parallel with HSV1716 or HSV-1 17+ at the same moi, and there were no significant differences in any of the yields (data not shown).

A single multistep growth curve experiment was performed for HuH7 infected in quadruplicate with HSV-1 17+ and HSV1716 at moi 0.002 and 0.02. Cells were harvested into the medium at 24, 48, and 72 hours, total virus was titrated, and yields were determined at the three time points (Figure 1A). At moi 0.02, similar yields were obtained for both HSV-1 17+ and HSV1716 at each time point. At the lower moi 0.002, yields were similar at 24 and 48 hours, but between 48 and 72 hours there was a much larger increase in HSV1716 progeny production compared with that of HSV-1 17+. As with the single-step growth curve experiment, progeny yields for HSV1716 were again approximately five-fold higher compared with HSV-1 17+, and these differences

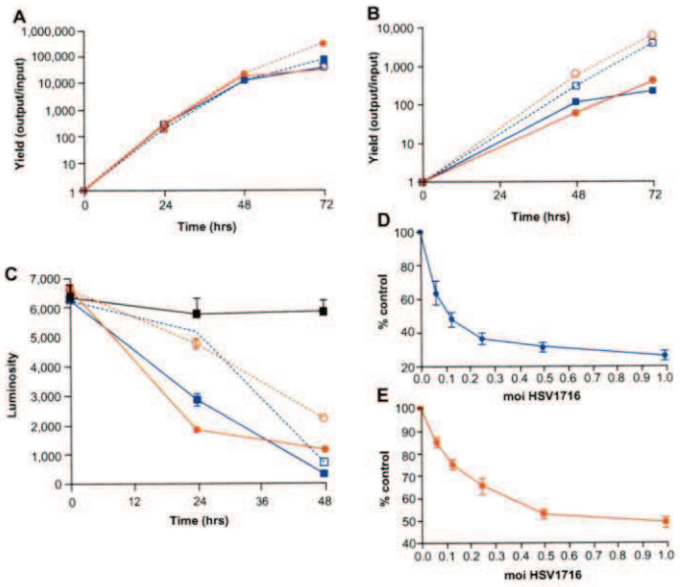

Figure 1 Replication kinetics of wild-type HSV-1 17+ and HSV1716 in two HCC cell lines, HuH7 (**A**) and HepG2-luc (**B**). (**A**) Mean (± standard deviation) yields (output pfu/input virus) for multistep growth curves from HuH7 infected with HSV-1 17+ (blue) or HSV1716 (red) at moi 0.002 (unbroken line) and 0.02 (dashed line). (**B**) Mean (± standard deviation) yields (output pfu/input virus) for HepG2-luc cells infected with HSV1716 (red) or HSV-1 17+ (blue) at 0.01 (unbroken line) and 0.001 (dashed line) moi after 48 or 72 hours of infection. (**C**) Luciferase activity (luminosity) at 24 and 48 hours in HepG2-luc cells infected with HSV1716 (red) or HSV-1 17+ (blue) at 1 (unbroken line) or 0.1 (dashed line) moi. (**D** and **E**) HSV1716 moi-dependent HepG2-luc cell killing measured by loss of light emission (**D**) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (**E**) assay. Values are presented as percent mock-infected HepG2-luc cells.
Abbreviations: moi, multiplicities of infection; HSV, herpes simplex virus; HCC, hepatocellular carcinoma.

were highly significant (Student's *t*-test, P=0.0001). Thus, in two independent experiments with HuH7 cells, HSV1716 at low moi and after 72 hours of infection, replicates with higher efficiency than wild-type HSV-1 17+, with the largest increase in replication occurring between 48 and 72 hours.

In a single experiment, HepG2-luc cells were plated in 60 mm dishes, and after 24 hours in culture, cells were infected with HSV1716 or HSV-1 17+ at 0.01 and 0.001 moi in triplicate. After 48 or 72 hours of infection, cells were harvested into the medium and total virus was titrated (Figure 1B). The infected HepG2-luc cells generated high progeny yields of both HSV-1 17+ and HSV1716 at both moi and at both time points, and the yields were similar for both viruses at 0.001 moi. However, after 72 hours of replication at moi 0.01, HSV-1 17+ yielded approximately 4,067±569 pfu progeny/input virus compared with HSV1716 yields of 6,003±987 pfu progeny/input virus, and these differences were significant (Student's *t*-test, P=0.0145). Therefore, HSV1716 replication was as at least as efficient and, in some instances more efficient, in both HuH7 and HepG2-luc cells compared with wild-type HSV-1 17+ replication.

HepG2-luc cell death during virus replication was detectable using a loss of light emission assay. Approximately 5,000 HepG2-luc cells/well were plated out in 96-well plates, and after 24 hours in culture, were infected in quadruplicate with HSV1716 or HSV-1 17+ at 1 or 0.1 moi. Luciferase activity (luminosity) was determined at 24 and 48 hours post-infection (Figure 1C). Luciferase activity was constant in uninfected control cells whereas light output decreased in both types of virus-infected cells at the two moi (Figure 1C). Similar levels of moi-dependent and progressive loss of light output were detected for HSV-1 17+ and HSV1716 at 24 and 48 hours, indicating that HSV1716 was as efficient as HSV-1 17+ at killing HepG2-luc cells. Visual inspection of the wells at 48 hours confirmed a virus-mediated cytopathic effect in most cells at this time, and this was confirmed by comparing moi-dependent cell killing for HSV1716 using loss of light emission and an MTT assay. The ED_{50} of HSV1716 in

HepG2-luc cells was equivalent to moi 0.15 as determined using loss of light emission (Figure 1D) and this was very similar to the ED_{50} of moi 0.12 as determined using the MTT cell survival assay (Figure 1E). Therefore, in HepG2-luc cells, loss of light output correlates with virus replication-mediated HepG2-luc cell death.

Intratumoral injection of nude mice with HepG2-luc xenografts

Fifty nude mice were injected subcutaneously on the flank with the HepG2-luc cells and imaged by IVIS. Luciferase expression in the HepG2-luc cells was visualized within 24–48 hrs as the xenograft formed at the injection site. At day 9 post-cell injection, light-emitting xenografts were formed, with outputs $>10^6$ radiance detected in 30 of the 50 mice. These mice were randomized into three groups that received no virus (n=12, injection of phosphate-buffered saline), intratumoral HSV1716 virus at 2×10^4 pfu (n=6), and intratumoral HSV1716 virus at 2×10^6 pfu (n=12).

Four of the six mice in the 2×10^4 pfu HSV1716 group showed a reduction in luciferase levels by day 5 post-injection (Figures 2 and 3A) and light emissions were reduced to background levels in these mice. The other two mice (mice two and three, Figures 2 and 3A) were still positive for light emission on day 32 and were given a further intratumoral injection of HSV1716 2×10^4 pfu on day 32. One mouse showed a complete loss of light emission at

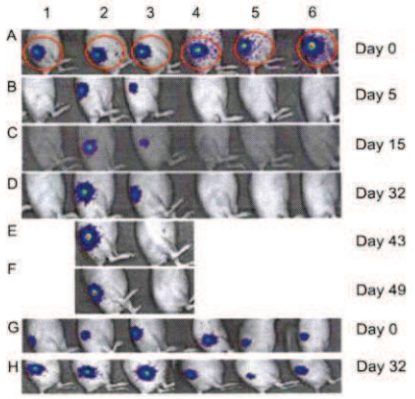

Figure 2 (**A**–**D**) IVIS images of nude mice with HepG2-luc xenografts prior to intratumoral treatment with 2×10^4 pfu HSV1716 and at 5, 15, and 32 days post-injection. The remaining two mice with an IVIS signal on day 32 (**D**) are shown 11 (**E**) and 17 (**F**) days after a second intratumoral injection of HSV1716 at 2×10^4 pfu. (**G** and **H**) Six representative control mice on days 0 and 32.
Note: Red circles indicate the region of interest for light determination.
Abbreviation: IVIS, in vivo imaging system.

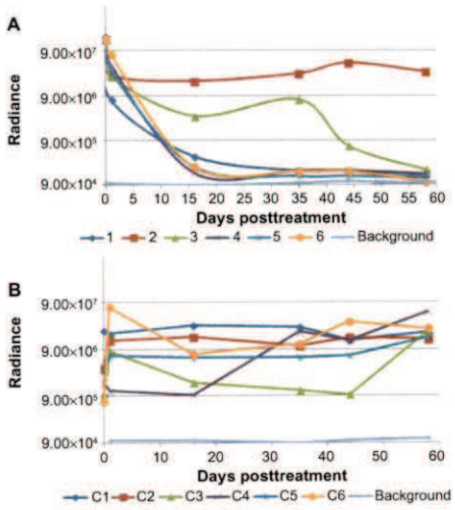

Figure 3 Individual light output from HepG2-luc xenografts as determined by IVIS imaging of nude mice treated with an intratumoral injection of 2×10^4 pfu HSV1716 (**A**) and six representative mice treated with 0.1 mL of phosphate-buffered saline (**B**). The IVIS background readings for each determination are also shown.
Abbreviation: IVIS, in vivo imaging system.

12 days after the second injection (day 43, Figures 2 and 3A). The second intratumoral injection had no effect on luciferase levels in the xenograft of the remaining mouse (mouse 2, Figures 2 and 3A) and the light-emitting xenograft was still present in this mouse up to day 58 (Figure 3A). This mouse was subsequently euthanized, the tumor was excised and analyzed for the presence of virus, but none was detected, suggesting that HSV1716 failed to establish replication after intratumoral injections in this mouse. Since there was no re-establishment of the HepG2-luc xenografts in five of the six mice receiving HSV1716 2×10^4 pfu by intratumoral injection, these were designated as cures (Table 1). All control mice maintained light-emitting xenografts throughout this time, as shown for six representative control mice in Figure 2 on the day

Table 1 Overall summary of HepG2-luc in vivo results

Treatment group	Mice (n)	Cures
2×10^4 pfu IT	6	5
2×10^6 pfu IT	12	12
1×10^6 pfu* IT	2	2
No virus control	12	0

Notes: *Mice with late-stage HepG2-luc xenografts. A cure was defined as a complete and permanent loss of light emission from HepG2-luc xenografts.
Abbreviation: IT, intratumorally.

of treatment and at day 32. Individual radiance outputs are shown in Figure 3B, with the average radiance output for the six control mice on day 0 equal to 5.47×10^6 radiance, and this had risen to 2.54×10^7 radiance by day 58, equivalent to an increase of 343,621 radiance/day.

For mice treated with HSV1716 2×10^6 pfu or with phosphate-buffered saline, there was an increase in light output between days 3 and 9 posttreatment, which returned to almost pretreatment levels by day 10 (Figure 4). The reason for this transient increase is unknown. Thereafter, all 12 of the mice treated with HSV1716 2×10^6 pfu showed a reduction in luciferase levels by day 12 post-injection, and light emissions were reduced to background levels by day 20 (Figure 4). During this time, none of the 12 mice injected with phosphate-buffered saline showed a reduction in luciferase activity levels, which increased steadily from an average of 2.44×10^6 radiance to 2.2×10^7 radiance on day 42, equivalent to an increase of 464,782 radiance/day and indicating xenograft growth (Figure 4). The radiance values of the two groups (HSV1716 2×10^6 and no virus) were significantly different (by Student's *t*-test), with *P*-values of less than 0.05 from day 16 onwards.

The ability of HSV1716 to treat long-established HepG2-luc xenografts was investigated using two mice from one of the control groups (Figure 5A and B). At day 73, these two mice had strong light-emitting xenografts and received HSV1716 1×10^6 pfu by intratumoral injection. By 17 (Figure 5A) and 11 (Figure 5B) days post-injection, all light emissions from the xenografts were completely lost. The results of the HepG2-luc studies are summarized in Table 1 and clearly demonstrate complete remission of the HepG2-luc xenografts, in most instances by HSV1716 administered via intratumoral injection. Skin from the xenograft site was collected at the time of sacrifice from a number of these cured mice, but no HSV1716 was detected by titration of tissue extracts (data not shown).

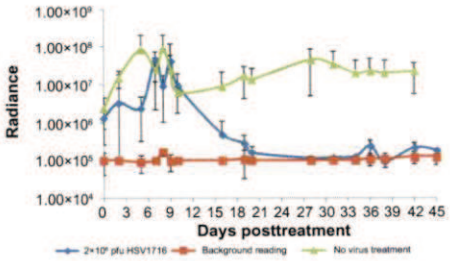

Figure 4 Average light output (± standard deviation) from HepG2-luc xenografts as determined by IVIS imaging of nude mice treated with an intratumoral injection of 2×10^6 pfu HSV1716 or phosphate-buffered saline. The IVIS background readings for each determination are also shown.
Abbreviation: IVIS, in vivo imaging system.

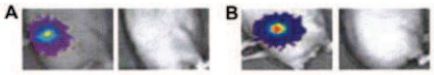

Figure 5 (**A**) IVIS image from a mouse with a 73-day-old HepG2-luc xenograft before and 17 days after receiving 1×10^6 pfu HSV1716 by intratumoral injection. (**B**) IVIS image from a mouse with a 73-day-old HepG2-luc xenograft before and 11 days after receiving 1×10^6 pfu HSV1716 by intratumoral injection.
Abbreviation: IVIS, in vivo imaging system.

Intravenous injection of nude mice with HuH7 xenografts

HuH7 cells were injected into the flanks of nude mice and subcutaneous tumors were allowed to develop. Twenty-two mice with appropriately sized tumors were divided into three groups, comprising no virus (phosphate-buffered saline control, n=7), 1×10^7 pfu (n=8), or 1×10^6 pfu (n=7) HSV1716 administered by tail vein injection on days 1 and 4. HuH7 tumor xenografts treated with HSV1716 at both 1×10^6 pfu and 1×10^7 pfu by intravenous injection on days 1 and 4 had greatly reduced rates of growth compared with controls that received intravenous phosphate-buffered saline (Figure 6A). The difference was highly significant by one-way analysis of variance ($P<0.0001$) with Tukey's multiple comparison test for each of the three groups, indicating that both doses of HSV1716 significantly reduced tumor growth compared with the no virus controls ($P<0.001$ for both). There was no significant difference between the 1×10^7 and 1×10^6 pfu doses ($P>0.05$). By day 13, all of the no virus control mice had been sacrificed due to unrestricted tumor growth (Figure 6A), and the Kaplan–Meier survival plot for each group of mice indicated highly significant improved survival (log-rank test, $P=0.0008$) in both groups compared with the no virus controls (Figure 6B). Tumor xenografts were completely eradicated by administration of HSV1716 in four of 15 long-term survivors, including one from the 1×10^7-treated group and the other three from the 1×10^6-treated group. After sacrifice of the other mice with tumors in the virus-treated groups, tumors and organs were removed and extracts were titrated. In most tumors, large amounts of virus ($>1\times10^5$ pfu/mL) were present even at 20 days after administration (Table 2), indicating excellent and persistent replication of HSV1716 in the HuH7 xenografts. No virus was detected by titration in the liver, lung, spleen, kidney, gut, skin, brain, or heart from any of

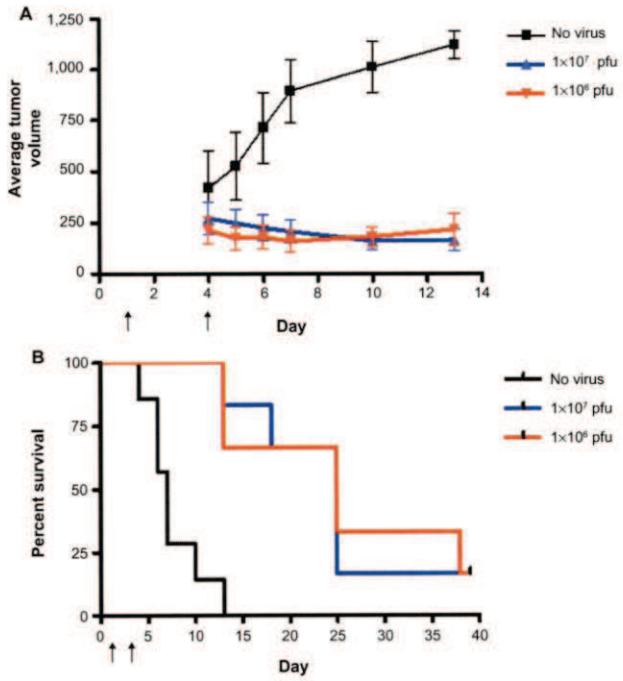

Figure 6 Growth (**A**) and survival (**B**) for mice with HuH7 xenografts treated intravenously with 1×10^6 (red line) or 1×10^7 pfu (blue line) HSV1716 on days 1 and 4.
Notes: Control mice (black line) received phosphate-buffered saline injections. Arrows indicate days of treatment.

these mice, indicating highly specific tumor localization and replication of HSV1716 (data not shown).

In a second HuH7 xenograft experiment, 17 mice were randomly assigned into three groups to receive no virus (n=5), HSV1716 1×10^5 (n=6), or HSV1716 1×10^6 pfu (n=6) on days 1, 14, and 29 by tail vein injection. Again, HSV1716 by intravenous injection had profound inhibitory effects on tumor growth (Figure 7A) and the differences between treated and control mice were highly significant by analysis of variance ($P<0.0001$). Tukey's multiple comparison test for each of the three groups indicated that both doses of HSV1716 significantly reduced tumor growth compared with no virus controls ($P<0.005$ for both); however, there was no significant difference between the 1×10^6 and 1×10^5 pfu doses ($P>0.05$). All control mice had been sacrificed by day 13 posttreatment due to their tumor burden. The Kaplan–Meier survival plots for each group of mice indicated significantly improved survival (log-rank test, $P=0.0157$) in both virus-treated groups compared with the no virus controls (Figure 7B). When the experiment was terminated on day 66, four of six mice in the 1×10^6 pfu group and two of six mice in the 1×10^5 pfu group had no visible tumor. After sacrifice, if tumors were present, they were removed, extracted, and virus-titrated. In most tumors, large amounts of virus ($>1\times10^6$ pfu/mL) were present even at 35 days after administration (Table 3). No virus was detected in a sample of skin removed from

Table 2 Titers from excised HuH7 xenografts after intravenous administration of HSV1716 on days 1 and 4

Day post-injection	Dose (pfu)	Tumor titer (pfu/mL)
10 (n=2)	1×10^7	2.0×10^6, 1.1×10^7
13	1×10^7	3.6×10^6
18	1×10^7	6.0×10^5
25 (n=3)	1×10^7	3.0×10^5, 3.0×10^6, 1.6×10^5
18 (n=3)	1×10^6	1.1×10^6, 9.0×105, 6.0×10^5
28	1×10^6	2.0×10^3

Note: Tumor was extracted in a final volume of 1 mL.

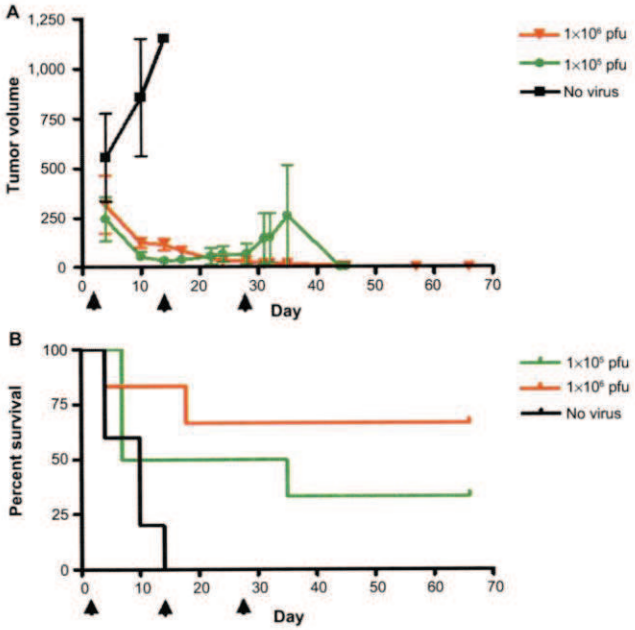

Figure 7 Growth (**A**) and survival (**B**) of mice with HuH7 xenografts treated intravenously with 1×10^5 (green line) or 1×10^6 pfu (red line) HSV1716 on days 1, 14, and 29. Control mice (black line) received phosphate-buffered saline injections. Arrows indicate days of treatment.

the xenograft sites in cured mice on day 66 after treatment. Thus, intravenously administered HSV1716 was highly effective at reducing growth and extending survival in HuH7-xenografted mice, with highly efficient replication kinetics within the tumors.

Tumor localization of intravenously injected HSV1716 in nude mice with HuH7 xenografts

The potent efficacy signals in the HuH7 xenograft studies suggested effective tumor localization of systemically administered HSV1716 followed by highly efficient replication. This was confirmed in biodistribution studies. HSV1716gCluc has the firefly luciferase gene under control of the HSV-1 gC promoter and was used in several tumor localization studies. Luciferase is expressed in the HSV1716gCluc-infected cell as a late gene approximately 8–10 hours after the lytic cycle is initiated (data not shown). Subcutaneous flank HuH7 tumors developed in ten nude mice, and HSV1716gCluc 1×10^7 pfu was administered to all mice via single tail vein injection, with tumor and organs harvested from two mice on days 1, 4, 7, 14, and 21 post-injection. Biodistribution of virus was analyzed by both titration and luciferase assay, and the average results from the two mice at each time point are presented in Figure 8. Exceptionally high titers of HSV1716, even early on after administration, were detected in the tumors of all mice at all times post-injection (Figure 8A), indicating very efficient viral replication in the HuH7 xenografts. Apart from 1,000 pfu detected transiently in the liver of one mouse on day 4 and in the lungs of another mouse on day 7, HSV1716 was absent from all other tissues at any of the time points. High levels of luciferase activity were detected

Table 3 Titers from excised HuH7 xenografts after intravenous administration of HSV1716 on days 1, 14, and 29

Day post-injection	Dose (pfu)	Tumor titer (pfu/mL)
4	1×10^6	1.21×10^8
18	1×10^6	8.0×10^7
7 (n=3)	1×10^5	1.3×10^7, 1.8×10^6, 3.9×10^7
35	1×10^5	4.0×10^6

Note: Tumor was extracted in a final volume of 1 mL.

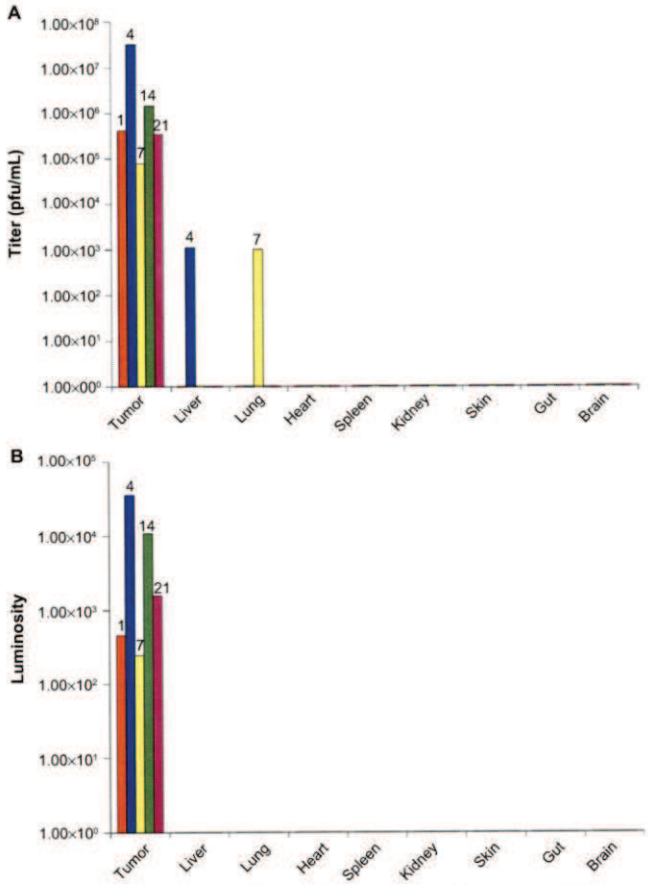

Figure 8 Biodistribution of HSV1716gCluc analyzed by both titration (**A**) and luciferase (**B**) assay in organs from nude mice with HuH7 xenografts harvested at one (red), 4 (blue), 7 (yellow), 14 (green), and 21 (pink) days after receiving a single intravenous injection of 1×10^7 pfu HSV1716gCluc. Day of sacrifice is indicated at the top of each bar.

only in tumor extracts (Figure 8B), with luciferase activity completely absent from all tissue extracts. In particular, no luciferase activity was detected in the liver extract on day 4 or the lung extract on day 7, despite the presence of titratable virus in these tissues at this time. Since luciferase will only be expressed during active HSV1716gCluc infection, this suggests non-replicating HSV1716gCluc in the liver on day 4 and in the lungs on day 7. The presence of virus in the tissues of these two mice was possibly due to leakage of virus into the circulation from the tumor, as extremely high levels of replicating virus were present in the tumor extract.

Four nude mice with HuH7 xenografts were injected via the tail vein with HSV1716gCluc 1×10^7 pfu and tumors were harvested and titrated at 16, 24, 48, and 72 hours post-injection. At 16 hours post-injection, 9×10^4 pfu/mL were titrated within the tumor indicating that, as the HSV-1 replication cycle is between 18–24 hours, tumor localization of systemically administered virus is extremely rapid and highly efficient. Titratable HSV1716 progeny increased thereafter, with 2.41×10^5 pfu/mL detected at 24 hours, 1.7×10^7 pfu/mL at 48 hours, and 8.8×10^7 pfu/mL by 72 hours (Figure 9A). Virus levels within the tumor were approximately ten-fold greater than the input dose within 72 hours, indicating rapidly

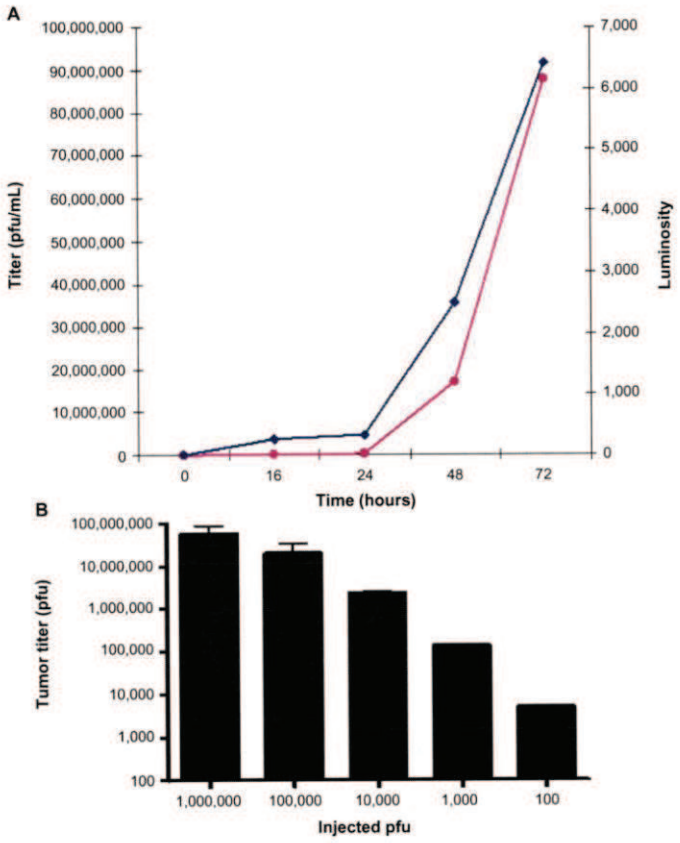

Figure 9 (**A**) Titers (dark blue) and luciferase activity (pink) in tumor extracts from nude mice with HuH7 xenografts injected via the tail vein with 1×10^7 pfu HSV1716gCluc. (**B**) Titers from HuH7 xenografts 72 hours after intravenous injection of 100, 1×10^2, 1×10^3, 1×10^4, 1×10^5, or 1×10^6 pfu HSV1716.

established highly efficient replication of HSV1716gCluc in the HuH7 xenografts. The luciferase activity in the extracted tumors was also determined and, presented alongside the titers in Figure 9A, demonstrated a good correlation between luciferase activity and virus titers in the tumor extracts.

In a further tumor localization study, ten nude mice with HuH7 xenografts received increasing amounts of HSV1716 by tail vein injection and the virus was titrated 72 hours after administration. Two mice each received HSV1716 1×10^6, 1×10^5, 1×10^4, 1×10^3, and 100 pfu, and in all cases, the tumor titers at 72 hours had exceeded the input dose by at least ten-fold and in some instances by 100-fold (Figure 9B). Systemic administration of HSV1716 as little as 100 pfu was sufficient to establish xenograft replication, and intravenously injected HSV1716 is therefore able to locate rapidly to HuH7 xenografts and establish highly efficient replication, leading to inhibition of growth, prolonged survival, and, in many instances, a cure.

Discussion

In vitro, HSV1716 demonstrated excellent replication kinetics in two well characterized HCC cell lines, ie, HuH7 and a luciferase-expressing variant of HepG2 cells. In both cell lines, HSV1716 was better at replicating and produced significantly more progeny than the wild-type virus from which it was derived, in HuH7 in two separate experiments at lower

moi and in HepG2-luc in a single experiment at higher moi. This superior replication competence in both HCC cell lines is unusual. We observed the reverse, ie, a lower replication competence of HSV1716 compared with HSV-1 17+, in U87 and one58 cells and in many different human cancer cell lines.[24] This was also observed in vivo, with infection of HuH7 xenografts rapidly generating exceptionally high titers of HSV1716 within 72 hours. It is possible that in wild-type HSV-1-infected HuH7 or HepG2-luc, ICP34.5 has a negative influence on replication efficiency and this is lost in the ICP34.5-deleted HSV1716, but more extensive studies are required to confirm this.

In vitro, HSV1716 infection reduced the light output from HepG2-luc cells in culture and was correlated with reduced cell survival; we used this to investigate HSV1716 efficacy in a HepG2-luc xenograft model. IVIS analysis was used to assess the success of the virus in eliminating the rapidly formed light-emitting HepG2-luc xenografts in the flanks of nude mice. Loss of light emission following virus treatment was permanent, and these animals were therefore scored as cures. In control animals, light emissions were consistently at least 100-fold higher than background ($>10^6$ radiance), and were often greater than 10^8 radiance, with light output increasing at a constant rate for over 100 days. Single intratumoral injections of HSV1716 were highly effective at eliminating the HepG2-luc xenografts, even at low doses of 2×10^4 pfu, with most mice cured. Even at later times after implantation of HepG2-luc cells, single intratumoral injections of HSV1716 were highly effective. Additionally, a repeat intratumoral injection was usually sufficient to eliminate xenografts that persisted after initial virus administration. A small number of mice with HepG2-luc xenografts received HSV1716 by intravenous injection, and although the xenografts were still present after the initial intravenous injection, they were eliminated in four of five mice after two subsequent intravenous injections. The xenograft persisting in one mouse despite these three injections suggests that intravenously administered HSV1716 most probably failed to localize to this tumor, and no virus was detected by titration in the xenograft of this mouse at the time of sacrifice (data not shown).

Monitoring light emission from the HepG2-luc xenografts was a useful and convenient method for monitoring the success of oncolytic virus treatment. Loss of light emission as visualized by IVIS correlated well with xenograft regression. Nonresponding mice were also readily identifiable, and further virus treatments could be administered. However, the method is limited by the availability of luciferase-expressing cell lines. We also considered the usefulness of an HSV1716 variant that expressed luciferase in a replication-dependent manner via an HSV-1 late promoter. HSV1716gCluc replication was readily visualized by IVIS in HuH7 xenografts, and light emission correlated with virus replication. Rapid and specific tumor localization of systemically administered HSV1716gCluc was readily observed by IVIS 72 hours after intravenous injection of HSV1716gCluc. These strong light emissions from the replication of HSV1716gCluc within the HuH7 xenografts persisted for at least 21 days after a single administration, indicating the continuous presence of replicating virus within the xenograft (data not shown). Further, in biodistribution studies, although some virus was detected sporadically in normal organs (liver and lung) by titration, luciferase assays demonstrated that it was nonreplicating and therefore most likely a result of tumor-derived, blood-born virus contaminating the tissues. Studies with HepG2-luc and HSV1716gCluc therefore demonstrate the utility of light-based methodologies to monitor oncolytic virus efficacy in xenograft models.

HuH7 cells formed rapidly growing aggressive xenografts, and we assessed the efficacy of intravenously administered HSV1716 in mice with HuH7 xenografts. Mice received either two injections close together or three injections at 2-weekly intervals. Although both treatment schemes were highly effective at restricting tumor growth, the three injections at 2-weekly intervals were better at effecting cures when compared with the two injections on days 1 and 4. In both experiments, all control mice had to be sacrificed by 13 days after treatment was started due to their tumor burdens. Approximately 25% of the mice treated with two doses were cured, compared with 50% of mice receiving the three doses, and interestingly, most of the mice receiving three doses of HSV1716 were sacrificed within the 14-day interval between the first and second injections (n=4), with only two mice sacrificed thereafter, one after the second injection and one after the third. These results suggest that a more effective dosing scheme may comprise two initial injections within the first 4 days followed by two subsequent injections 14 and 28 days later.

In the HuH7 xenograft model, there was extremely rapid tumor localization of intravenously administered HSV1716, which quickly established highly efficient replication, with levels of intratumoral virus exceeding the input doses. Surprisingly, uptake of HSV1716 from the circulation by HuH7 cells was highly efficient, as at least one virion from an intravenous injection of 100 pfu (ie, at least 1%) was sufficient to establish replicating

virus in the xenograft. Thus, HSV1716 is highly effective at reducing growth and extending survival in HuH7 and HepG2-luc xenografts in nude mice as a result of highly efficient uptake of virus by cells and prolific replication kinetics within the tumors.

HCC is the third most common cause of cancer-related deaths.[2] Early detection is limited, and given that the majority of patients present with advanced inoperable disease, chemotherapy is the only option. There are several internationally recognized staging systems that combine liver function and tumor extension for the prognosis and management of therapeutic options in patients with HCC, including Cancer of the Liver Italian Program, Barcelona Clinic Liver Cancer, and Japanese Integrated Staging, with the Barcelona Clinic Liver Cancer system most widely accepted in clinical practice.[3,26] Doxorubicin is routinely used as a single agent, but shows insufficient response rates of 15%–20%.[27] Sorafenib is the only systemic therapy universally approved for advanced liver cancer. Sorafenib blocks RAF protein kinase, a critical component of the RAF/MEK/ERK signaling pathway that controls cell division and proliferation. In addition, sorafenib inhibits the vascular endothelial growth factor receptor-2/platelet-derived growth factor receptor-beta signaling cascade, thereby blocking tumor angiogenesis. Phase III clinical trials have indicated that single-agent sorafenib might have a beneficial therapeutic effect, adding about 3 months to the lifespan of late-stage HCC patients with well-preserved liver function.[4,28] However, in these trials, objective tumor responses were rare, and toxicities, including rash, diarrhea, and fatigue, often led to dose reductions and/or discontinuation of treatment.[29] Recently, impressive results with the oncolytic vaccinia virus JX-594 in advanced HCC have been reported in a Phase II study.[5] JX-594 has selective replication competence for cancer cells via inactivation of the viral thymidine kinase gene, and additionally expresses immunostimulatory human granulocyte-macrophage colony-stimulating factor and beta-galactosidase.[30–32] In a randomized Phase II dose-finding study including 30 patients, JX-594 was infused intratumorally at 10^8 pfu or 10^9 pfu on days 1, 15, and 29, and demonstrated significantly improved survival between the 10^8 pfu and 10^9 pfu cohorts with median survivals of 14.1 and 6.7 months, respectively.[5] Although both oncolytic and immunostimulatory mechanisms of action were demonstrated, their relative contribution to survival could not be determined, but the authors did note that the acute peak JX-594 concentration in blood correlated with duration of overall survival, suggesting that control of tumor growth and improved survival may be achievable via high-dose intravenous administration.

In other relevant clinical studies, NV1020, which is a highly attenuated, multimutated replication competent derivative of HSV-1, has been delivered by hepatic artery infusion in patients with metastatic colorectal carcinoma to the liver in Phase I and Phase I/II studies.[33–35] Patients received four NV1020 doses via weekly hepatic artery infusion, followed by two or more cycles of conventional chemotherapy. Phase I included cohorts receiving 3×10^6, 1×10^7, 3×10^7, or 1×10^8 pfu/dose, and the 1×10^8 pfu/dose was identified as the optimal biological dose for Phase II. All of the 22 Phase I/II patients who received the optimal biological dose had previously received 5-fluorouracil, most had received oxaliplatin or irinotecan (50% had both), and many had received at least one targeted agent. After administration of NV1020, 50% showed stable disease and the best overall tumor control rate after chemotherapy was 68% (one with a partial response, 14 with stable disease). Therefore, the study suggested that NV1020 extended overall survival by stabilizing colorectal cancer liver metastases with minimal toxicity and potentially resensitizing them to salvage chemotharapy.

In our preclinical HCC studies, HSV1716 was highly efficacious by intravenous administration in an aggressive HuH7 model, and although there was some dose effect, scheduling of administration was potentially more important. Excellent uptake of HSV1716 from the circulation combined with prolific replication are the most likely key determinants of inhibition of tumor growth and enhanced survival. Our preclinical results in two HCC models therefore support clinical translation of systemic HSV1716.

Disclosure

All authors are employees of Virttu Biologics Ltd but have no other conflicts of interest in this work.

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8th International Conference on Oncolytic Virus Therapeutics

Barbara-Ann Guinn, Lynne Braidwood, Alan Parker, Kah-Whye Peng, and Leonard Seymour

Human Gene Therapy, 2014 25:1062-1084 (Dec-2014)

Abstract: The 8th International Conference on Oncolytic Virus Therapeutics meeting was held from April 10–13, 2014, in Oxford, United Kingdom. It brought together experts in the field of oncolytics from Europe, Asia, Australasia, and the Americas and provided a unique opportunity to hear the latest research findings in oncolytic virotherapy. Presentations of recent work were delivered in an informal and intimate setting afforded by a small group of attendees and an exquisitely focused conference topic. Here we describe the oral presentations and enable the reader to share in the benefits of bringing together experts to share their findings.

Appendix 2 – Posters authored/co authored by Lynne Braidwood 2013 - 2016 involving work carried out in relation to this thesis.

VIRTTUBIOLOGICS

JAK Inhibitors synergise with HSV1716 *in vitro* in human cancer cell lines

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We have investigated the effects of several well characterized JAK/STAT inhibitors (Ruxolitinib, Tofacitinib & AZD1480) combined with the oncolytic HSV-1 variant HSV1716 on cell death in a panel of human cancer cell lines reflecting 4 cancer indications of clinical interest to Virttu. Our *in-vitro* data demonstrated that HSV1716 and JAK/STAT inhibitors frequently combined to enhance cancer cell killing most probably via enhanced viral replication.

Rationale for combining JAK/STAT inhibitors with an oncolytic virus

Viral infection (both 'normal' and oncolytic) elicit a host response. Type 1 Interferon's (IFN's) are a group of antiviral cytokines that are induced during viral infection. IFN's exert their biological function by binding to specific cell surface receptors which, in turn triggers the intracellular IFN signalling pathway – mainly the JAK/STAT pathway – which eventually induces the expression of a large number of IFN-stimulated genes (ISGs) These ISG's, the workhorses of the IFN response cause the cell to enter an antiviral, anti-proliferative state. However viruses have evolved a broad spectrum of strategies to block or interfere with the JAK/STAT pathway (Figure 1).

JAK/STAT inhibitors are a group of drugs that work by inhibiting the activity of one or more of the JAK family, thereby interfering with JAK/STAT signalling.

By combining JAK/STAT inhibitors with the oncolytic virus HSV1716 the JAK/STAT signalling will be further decreased, allowing greater levels of HSV1716 replication and consequently oncolysis.

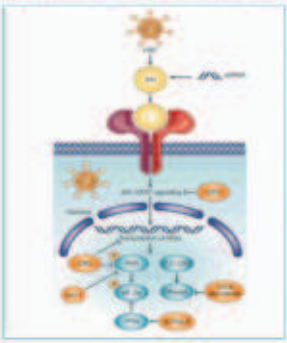
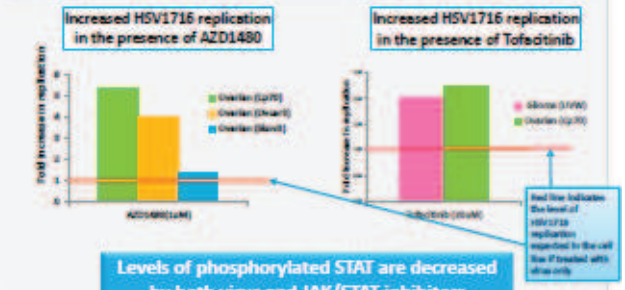
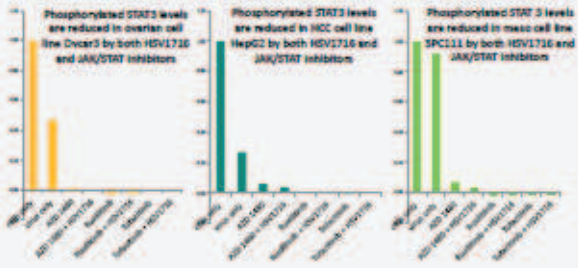


Figure 1: JAK/STAT signalling pathway. The processes of cellular exposure to HSV and viral binding or entry stimulate the production of IFN. The viral protein infected cell protein 0 (ICP0) blocks the JAK STAT pathway and directly down regulates the expression of ISG's

HSV1716 replication is increased in the presence of JAK/STAT inhibitors



Levels of phosphorylated STAT are decreased by both virus and JAK/STAT inhibitors



Combining HSV1716 with JAK/STAT inhibitors results in enhanced cell death

Indication	Cell line	AZD1480 - ATP competitive inhibitor of JAK1 and JAK2	Tofacitinib - JAK3 inhibitor, may suppress STAT1
HCC	HUH7	-	+
	HEPG2	+	+
	HEP3B	+	-
Mesothelioma	ONE58	-	+
	SPC111	+	+
Ovarian	OVCAR3	+	+
	SKOV3	+	+
	CP70	+	+
Glioma	LN229	+	+
	A172	+	+
	T98	+	+
	U373	+	+
	U138	+	-
	U87	+	+
	UVW	+	+

Table 1 : Summary of the synergies/enhancements with AZD1480 or tofacitinib combined with HSV1716.
If more than half the combinations measured were synergistic (CI value <1 from Chou/Talalay plots) the JAK/Stat inhibitor was scored as combining synergistically in that cell line (+). If more than 4/8 combinations resulted in CI values of >1 then the JAK/STAT was scored as antagonistic with HSV1716 in that cell line (-)

Conclusions:

When used in combination with HSV1716, JAK/STAT inhibitors enhance cell death in a number of cell lines representing different cancer types.
This enhanced cell death is likely to be due to an increase in viral replication

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VIRTTUBIOLOGICS

Inhibitors of mTOR combine with oncolytic HSV1716 to enhance cancer cell death.

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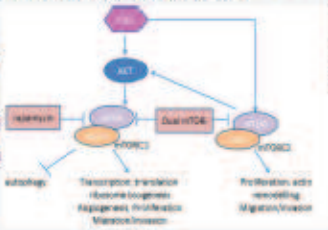
Introduction

HSV1716 (SEPREHVIR®) is an ICP34.5 null mutant of HSV-1 strain 17+ which has highly selective replication competence for cancer cells and safety trials in glioma, melanoma, H&NSCC, paediatric non-CNS solid tumours and malignant pleural mesothelioma are ongoing or have been completed. In total 79 patients have received HSV1716 and there has been no evidence of toxicity, no spread to surrounding normal tissue or no shedding in patients. The selectivity of HSV1716 for replication only in tumour cells and intimations of efficacy have been demonstrated.

The mammalian target of rapamycin (mTOR) is a protein kinase of the phosphatidylinositol 3-kinase (PI3K)/AKT signalling pathway with a central role in controlling cancer cellular growth.
Dysregulation of mTOR pathway has been found in many human tumours and implicated in the promotion of cancer cell growth and survival.
The mTOR pathway is considered an important target for anticancer drug development.



There are two classes of mTOR inhibitor: rapamycin and its closely related rapalogues which inhibit mTOR in the mTOR complex 1 only via binding to FKBP12 and dual mTOR inhibitors which bind directly to mTOR in both mTORC complexes 1 and 2. We have investigated the combination of HSV1716 with rapalogues and dual mTOR inhibitors (mTORi) in human cancer cell lines in vitro. HSV1716 combined with mTORi to enhance cancer cell death in vitro and potential mechanisms for their enhanced interactions were investigated.

Methods

Cell lines tested are relevant to Virttu's clinical development programme and comprise 3 HCC (HuH7, HepG2-luc, Hep3B), 2 HGG (U87, UVW), 2 Ovarian (CP70, SKOV3) and 2 MPM (one58, SPC111). Cell death in combination studies was measured by CytoTox-Glo Cytotoxicity Assay (Promega) and, in the same well, drug effects on virus replication was assessed using an HSV1716 variant expressing gfp

Results

Increasing mTORi concentrations between 0.0125 -25uM had limited cytotoxicity in the cell lines but frequently combined with HSV1716 to promote cell death at concentrations which had a potent inhibitory effect on viral gfp expression. In most cell types, virus-mediated cell death was constant or increased with increasing mTORi concentrations despite the potent inhibition of gfp expression between 1-10uM drug (Fig 1) suggesting that at these concentrations of mTORi, virus and drug combine to enhance cell death despite limited oncolysis.

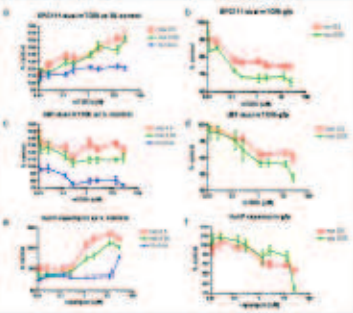


Figure 1 Levels of cell death for mTORi alone or in combination with HSV1716gfp at moi 0.5 and 0.05 in SPC111 (a,b), U87 (c,c) or HuH7 (e,f) . DCP levels are expressed as a % of the control, untreated cells (a, c, e). Levels of virus-mediated gfp expression are also shown and are expressed as a % of virus alone (b, d, f).

Analysis of HSV1716 spread/replication in the presence of 10uM mTORi confirmed their inhibitory effects on oncolysis with both reduced by the mTORi (Fig 2) and results are summarized in Table 2.

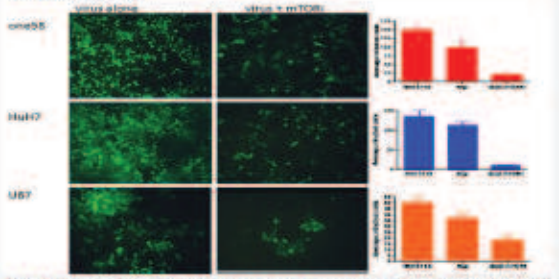


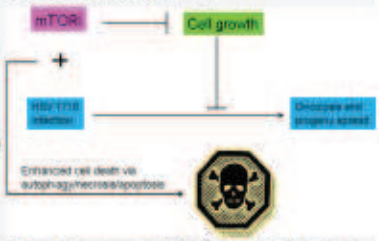
Figure 2 . Effects of mTORi on HSV1716 spread and replication. In human cancer cell line. Cells (one58, HuH7, U87) were infected with HSV1716gfp at moi 0.01 +/- mTORi at 10uM and after 72 hrs, virus spread and replication assessed by fluorescence microscopy or titration .

Table 1. Summary of effects of HSV1716 combined with rapalogues or dual mTORi
Red shading indicates inhibition and green indicates enhancement

	rapalogue		dual mTORi	
	HSV1716	death	HSV1716	death
Hep3B				
HuH7				
HepG2-luc				
CP70		No effect		
SKOV3				
U87		No effect		
UVW				
One58				
SPC111				

mTORi and HSV1716 – a conundrum?

Overall the results summarized in Table 1 indicate that, despite the ability of mTORi to inhibit HSV1716 oncolysis, levels of cell death were similar or greater than with virus alone. Thus the mTORi prevented cell growth and blocked HSV1716 replication but the presence of the non-replicating virus in the mTOR-inhibited cell was sufficient to stimulate death.



HSV infection/mTORi and the cellular autophagy response. ICP34.5 interacts with beclin 1 to block autophagy and autophagy is induced during infection with ICP34.5 null mutants (Orvedahl et al. Cell Host Microbe 2007;1:23-35) . mTORi induce autophagy and HSV1716 in combination with mTORi may induce a potent autophagic response leading to cell death.

Figure 3 Autophagy during wild-type or HSV1716 infection +/- mTORi. UVW or CP70 cells were infected with either wild type HSV-1 17+ or HSV1716 at moi 1 for 24 hrs +/- 5uM mTORi. Antibodies were to phospho-eIF2alpha, phospho-S6 (an mTOR substrate), LC3 (an autophagy marker) and actin as a loading control. 1=untreated cells, 2=wild type HSV-1 17+, 3=HSV1716, 4=5uM mTORi, 5=wild type HSV-1 17+/5uM mTORi, 6 HSV1716/5uM mTORi. ICP34.5 blocks eIF2alpha phosphorylation (lanes 2 and 3 and 5 and 6) and mTORi inhibits S6 phosphorylation (lanes 4-6). Autophagy induction results in an increase in the lower band (activated form LC3-II) relative to the upper band (LC3-I). (e.g. see UVW lanes 4 and 5 and CP70 lanes 3 and 4). Results for these and other cell lines tested are summarized in Table 2.

Table 2 Autophagy during wild-type and HSV1716 infection +/-mTORi. + = autophagy - = no effect. mTORi induces autophagy in most cell lines but no potent induction of autophagy during virus infection of mTORi-treated cells. HSV1716 and mTORi do not combine to enhance autophagy in these cell lines.

Cell Line	control	17+	1716	mTORi	17+ mTORi	1716 mTORi
Hep3B	-	-	-	-	-	-
HuH7	-	-	-	+	-	-
SKOV3	-	+	-	+	+	-
CP70	-	+	+	+	+	+
U87	-	-	-	-	-	-
UVW	-	+	+	+	+	+
One58	-	+	+	+	+	+
SPC111	-	+	+	+	-	-

Conclusions

Cell growth was blocked by mTORi and consequently reduced HSV1716 replication/spread.
Despite this mTORi combined with HSV1716 to enhance cell death
No potent induction of autophagy by mTORi/HSV1716 combination
Apoptosis or necroptosis are being investigated

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Autophagy in human cancer cell lines during infection with ICP34.5-deleted oncolytic HSV1716

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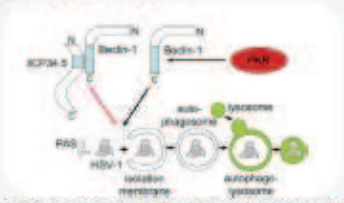
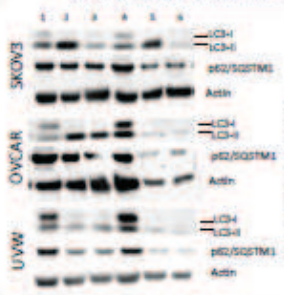
- Oncolytic HSV1716 (SEPREHVIR®), lacking the neurovirulence factor ICP34.5, has highly selective replication competence for cancer cells and has been used in clinical studies in glioma, melanoma, H&NSCC, paediatric non-CNS solid tumours and malignant pleural mesothelioma.
- In total, 84 patients have received HSV1716 and there has been no evidence of toxicity, no spread to surrounding normal tissue or no shedding in patients. The selectivity of HSV1716 for replication only in tumour cells and intimations of efficacy have been demonstrated.
- Autophagy is an adaptive, intracellular degradative, response to multiple forms of cellular stress, including reactive oxygen species, nutrient deprivation and organelle damage. The role of autophagy in cancer is unclear with conflicting evidence showing that autophagy can both promote tumor progression by helping cells survive or, in contrast, act as a tumor suppressor mechanism.
- Autophagy can have a defensive role by engulfing/degrading intracellular pathogens including viruses leading to the presentation of their intracellular antigens on the cell surface. During wild-type HSV-1 infection, ICP34.5 interferes with autophagy by binding Beclin-1 and, potentially, ICP34.5-deleted oncolytic HSV such as HSV1716 are therefore susceptible to autophagy-mediated inactivation.



- We investigated autophagy in diverse human cancer cell lines during both wild-type HSV-1 and oncolytic HSV1716 infection. Autophagy induction by HSV infection was cell type dependent with autophagy activation during both wild type and HSV1716 infections and, surprisingly, we noted that in SKOV3 cells, wild type but not HSV1716 induced a more potent autophagy response.
- In combination studies, chloroquine, an inhibitor of the autophagy-lysosomal pathway, was principally antagonistic with HSV1716, most probably by reducing the efficiency of viral replication. Therefore, our results suggest that maximal HSV1716 oncolysis may require a functional autophagy-lysosomal degradative pathway.

Autophagy response in human cancer cell lines to HSV infection is independent of ICP34.5 status



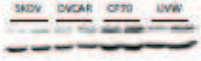
Autophagy response to 1716 in wild type HSV-1 17+ depends on host cancer cell type. There was no autophagy response to HSV infection in Hep3B, HuH7 and U87 cells whereas both HSV-1 17+ and HSV1716 induced an autophagy response in CP70, Ovcar3, UVW, one58 and SPC111 cells. Unusually, SKOV3 show greater autophagy responses following infection with HSV-1 17+.

Cell line	Cancer type	HSV-1 17+	HSV1716
Hep3B	HCC	-	-
HuH7	HCC	-	-
CP70	Ovarian	+	+
Ovcar3	Ovarian	+	+
SKOV3	Ovarian	+	-
U87	HGG	-	-
UVW	HGG	+	+
one58	MPM	+	+
SPC111	MPM	+	+

Autophagy responses are not impaired in SKOV3

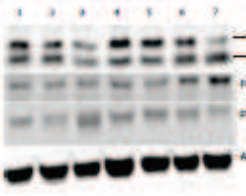
SKOV3 cells show normal nutrient-dependent autophagy signalling

Beclin-1 is present in human cancer cell lines



Differential autophagy response in SKOV3 cells does not correlate with decreased Beclin-1 levels.

HSV1716 induces an autophagy response in vivo in CP70 xenografts

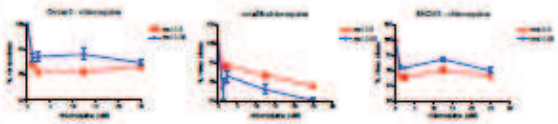


CP70 xenograft extract	HSV1716 titre (pfu/ml)
1	1.9×10^{4}
2	1.25×10^{8}
3	4.3×10^{8}
4	3.0×10^{8}
5	4.6×10^{7}
6	2.0×10^{8}
7	1.13×10^{8}

Chloroquine antagonises HSV1716 in vitro by inhibiting replication

Chou/Talalay analysis

Inhibition of GFP expression during HSV1716gfp replication



Inhibition of HSV-1 17+ and HSV1716 replication in SKOV3

Conclusions

- The autophagy response to HSV infection in human cancer cell lines is variable and independent of ICP34.5 status
- Autophagy is induced by HSV1716 in xenografts
- Inhibition of autophagy antagonises HSV1716 most probably by reducing replication efficiency
- Autophagy is beneficial to HSV1716 replication in human cancer cell lines
- Further studies with drugs that activate autophagy are ongoing

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Appendix 3: Posters authored/co authored by Lynne Braidwood 2013-2016 related to work carried out not in relation to this thesis.

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Cytokine responses following intrapleural administration of oncolytic HSV SEPREHVIR® in patients with malignant pleural mesothelioma

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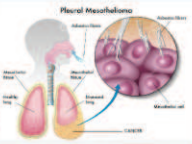
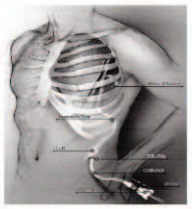
Oncolytic herpes virus therapy for mesothelioma – a phase 1/2a study of intrapleural Seprehvir (NCT01721018)

Malignant pleural mesothelioma (MPM) is an aggressive, asbestos-related tumour of the pleural and peritoneal cavities and remains a major challenge with limited therapeutic options. The disease has a dismal prognosis, a high symptom burden (pain, breathlessness) and is fatal with the median overall survival following treatment of ~1 year.

MPM incidence is increasing steadily with peak mortality expected to occur between 2020 and 2050 and current treatment options of surgery, radiotherapy, chemotherapy and palliative care have limited evidence of effectiveness in MPM. Thus, new therapeutic options are required.

Seprehvir (HSV1716) is a mutant oncolytic herpes simplex virus type 1 deleted in the RL1 gene which encodes the protein ICP34.5, a specific determinant of virulence. Seprehvir is a multi-functional oncolytic immunotherapeutic with highly selective cancer cell killing capable of initiating an anti-tumour immune response.





Study Design

The trial is currently recruiting at Weston Park Hospital, Sheffield and Queen Elizabeth University Hospital, Glasgow. It is a Phase 1/2a 3+3 dose escalating (1, 2 & 4 doses at weekly intervals) study delivering Seprehvir via existing intra-pleural catheter.

Primary objectives:
- Determine the safety and tolerability of Seprehvir given intrapleurally in patients with inoperable malignant pleural mesothelioma.

Secondary objective:
- Obtain evidence of Seprehvir replication and patient's immune responses through analysis of pleural fluid and plasma samples.

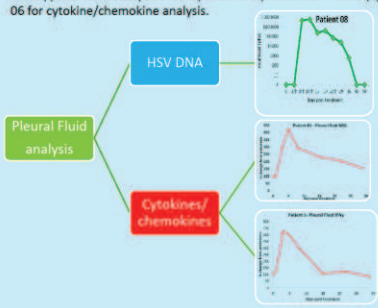
Exploratory objective:
- Assess tumour response by CT on days 29 and 57 using modified RECIST criteria.

	No. of doses	Number of patients	Status
Part A	1 dose of $1x10^7$ i.u.	Patients 1-3	Completed
Part B: Group 1	2 doses of $1x10^7$ i.u.	Patients 4-6	Completed
Part B: Group 2	4 doses of $1x10^7$ i.u.	Patients 7-9	Completed
Group 2 extension	4 doses of $1x10^7$ i.u.	3 patients	1/3

Analysis
- Pleural fluid and plasma samples are collected on treatment days and days 3, 5, 8, 15, 22 and 29 after the last virus dose.

Evidence of Seprehvir replication and patient's immune responses through analysis of pleural fluid and plasma samples.

a) Pleural fluids were analysed for HSV DNA by PCR (see Summary Table below) and for cytokine/chemokine levels by ELISA. Cytokines/chemokines status in pleural fluids before treatment is given in Table (i) and individual patient responses are presented in Table (ii). Cytokines/chemokines that showed little or no responses are shown in Table (iii). There were insufficient pleural fluids from Patient 06 for cytokine/chemokine analysis.



(i)

High (ng/ml)	Low (pg/ml)	absent
IL-6, IL-8, IL-27, MIG, VEGF	IL-2, IL-10, IL-12, IL-21, IP10	IFNγ, IFNα, IL-1α, IL-4, TNFα, GM-CSF

(ii)

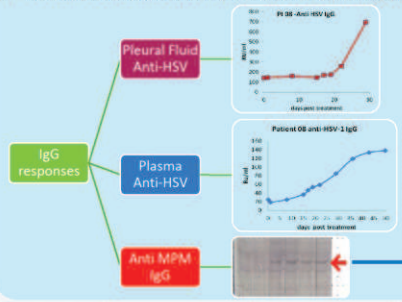
	IFNγ	IP10	MIG	VEGF	TNFα	IL2	IL6	IL8	IL10	IL12
Pt 01	++	+++	+++	↓	++	↓	+	+	+	-
Pt 02	-	↓	-	+++	+/↓	↓	↓	+/↓	+	↓
Pt 03	++	+++	+++	+++	+	↓	+	+	-/↓	++
Pt 04	+++++	+++	++++	↓	+++	↓	+	↓	+	-
Pt 05	-	-	-	++	↓	↓	↓	-	-/↓	-
Pt 07	+++++	++++	+++	-	+	++	+	+++	++	+++
Pt 08	+++++	+++	+++	↓	+	++	+++	+++	+++	+
Pt 09	++	+++	+++	+++	nd	++	+	++	+	+

(iii)

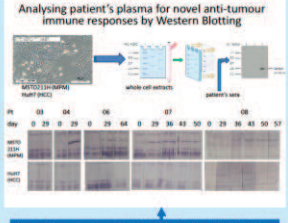
	IFNα	IL1α	IL4	IL21	IL27	GM-CSF
Pt 01	-	-	-	-	↓	-
Pt 02	-	+	-	+	↓	-
Pt 03	-	-	-	↓	↓	-
Pt 04	-	-	-	-	-	-
Pt 05	-	-	-	+	+	-
Pt 07	-	-	-	↓	++	-
Pt 08	+	-	+	+	-	-
Pt 09	nd	nd	nd	+	-	-

= cytokine/chemokine associated with aTh1 response - = unchanged, + = weak to +++++ = strong response, ↓ = decrease, nd = not done

b) Pleural fluids and plasma samples were analysed for anti-HSV IgG by ELISA and plasma samples for anti-tumour IgG responses with results summarised in Table (iv)



(iv)



Pt no	No doses	status	Plasma Anti-HSV-1 IgG response	Pleural Fluid Anti-HSV-1 IgG response	Novel anti-tumour IgG response
01	1	Seropositive	++	+++	-
02		Seropositive	-	++	++
903		Seropositive	-	↓	-
04	2	Seropositive	+++	++	+++
05		Seropositive	+++	+++	-
06		Seropositive	+++	ND (no samples)	++
07	4	Seropositive	+++	++	+++
08		Seronegative	+++	+++	++
09		Seropositive	+++	++++	+

Pt no./ Gender	HSV DNA in pleural fluids	Pleural fluid Th1 response	CT result	Status/months post treatment
01/F	-	+++	Progressive disease	Died/20
02/M	+++++	-	Stable disease	Died/13
903/M	-	++	Stable disease	Died/17
04/M	-	+++++	Stable disease	Died/18
05/M	+	-	Stable disease	Died/4
06/M	+++	no samples	Progressive disease	Alive/17
07/F	+++	+++	Progressive disease	Died/10
08/M	++++	+++++	Stable disease/ partial response	Alive/13
09/F	+	++	Stable disease	Alive/12

Summary Table and Trial Results

- **Well tolerated with few virus-related adverse events**
- **Potential signals of efficacy**
  - 9 patients evaluated, 1 PR, 5 SD, 3 PD
- **Evidence of viral replication/persistence in pleural fluid**
- **Evidence of Th1 cytokine response post Seprehvir administration**
- **Th1 response potentially indicative of extended survival (green shading in Summary Table)**
  - Median survival for 6 patients with Th1 response = 15 mths vs 9.5 mths historical median survival for all MPM patients (Beckett et al (2015) Lung Cancer 88, 344).
- **Evidence of a novel patient immune response**

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Neutralisation effects of pleural fluids do not predict the persistence of the oncolytic HSV Seprehvir following intrapleural administration in patients with malignant pleural mesothelioma.

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Abstract

Malignant pleural mesothelioma (MPM) remains a major challenge, with limited therapeutic options. Multifocal intrapleural disease can cause disabling symptoms of pain and breathlessness, in the absence of distant metastases, so an intrapleural treatment approach is attractive. Seprehvir (HSV1716) is a mutant oncolytic herpes simplex virus type 1 deleted in the RL1 gene which encodes the protein ICP34.5, a specific determinant of virulence. Mutants lacking the RL1 gene are capable of specific replication in cancer cells and inducing anti-tumour immune responses. Clinical studies with Seprehvir have been completed in adult high grade glioma (HGG), melanoma, squamous cell head and neck cancer, and studies are ongoing in both pediatric HGG and pediatric non-CNS solid tumors and adult MPM. In total, 81 patients have received Seprehvir and the virus is well-tolerated with no spread to surrounding normal tissue or co-shedding in patients. Seprehvir selectivity for replication only in tumour cells, signals of efficacy and immuno-stimulatory potential have been demonstrated. Of note, 47 patients with high-grade glioma received Seprehvir either intratumorally or injected into the resected tumor bed; more than 60% of treated HGG patients survived longer than the median normal survival period for their disease and, indeed, 3 have survived >10 years.
We are currently conducting a phase I/IIa trial to determine the safety and potential for efficacy of Seprehvir given intrapleurally to patients with MPM. Patients receive $1x10^7$fu through their pleural catheter on one, two or four occasions a week apart, in three separate patient cohorts. To date 9 patients have been treated, 3 in each cohort and Seprehvir has been well-tolerated with few adverse events in any patients. Pleural fluid samples have been collected pre- and post-treatment and analysed to assess virus neutralisation capacity in titration assays and Seprehvir persistence post administration.
Pre-clinical studies with pleural fluids demonstrated variable effects on HSV titration assays from strong to little or no neutralisation of Seprehvir plaque formation. Surprisingly, in tissue culture cells, even strongly neutralising pleural fluids did not significantly interfere with Seprehvir replication.
In patient samples from our clinical study, levels of neutralisation also varied with most demonstrating a strong inhibitory effect in titration assays. However, HSV DNA was detected in the pleural fluids of most patients and persisted in some at high levels for at least two weeks post-administration. Thus, neutralisation capacity of pleural fluids is not a determinant of oncolytic HSV replication and Seprehvir persisted and replicated in seemingly unfavorable conditions.

Pre-clinical testing

- 6 pleural fluids tested for effects on Seprehvir
- 3/5 rapidly neutralised 1x10e7 pfu in plaque assay, 1/5 had limited neutralisation effect and 1/5 had no effect (Figure 1)
- Despite neutralisation effect, pre-coating cells with pleural fluids did not significantly reduce Seprehvir output at 72 hrs (Figure 2)

Figure 1. Seprehvir (1x10e7 pfu) stability in Pleural Fluid Samples

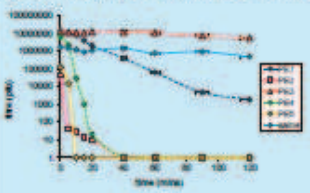
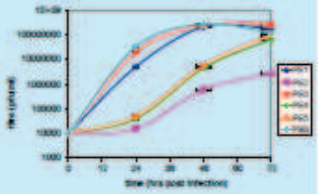
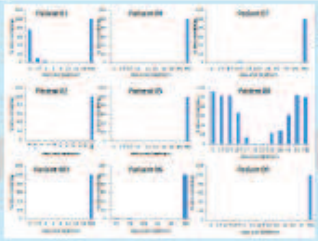


Figure 2 Seprehvir output (pfu/ml) at 24, 48 and 72hrs after infection of BHK cells pre-coated with pleural effusions or PBS

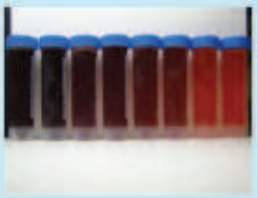


Clinical testing

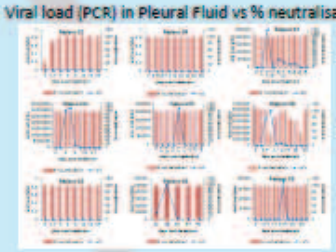
Neutralisation effect on 1x10e7 pfu Seprehvir in plaque assay



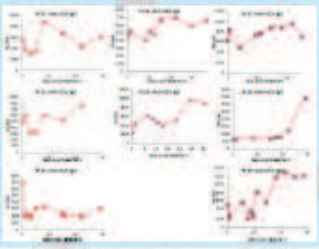
Pleural Fluid Volume

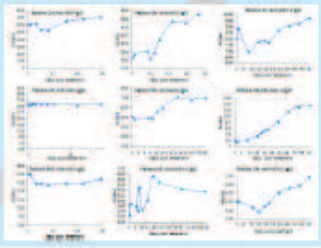


Viral load (PCR) in Pleural Fluid vs % neutralisation



Pleural Fluid Anti-HSV IgG





Serum Anti-HSV IgG

Current Status of Trial

- Purpose – safety, tolerability and potential for efficacy
- Phase 1/2a 3+3 dose escalating (1, 2 & 4 doses) study delivering Seprehvir via existing intra-pleural catheter is currently recruiting at Sheffield University Hospital
- Well tolerated with few virus-related adverse events
- Potential signals of efficacy (9 patients treated, 1 PR, 5 SD, 3 PD)

	No. of dose	Number of patients	Status
Part A	1 dose of $1x10^7$ i.u.	3 patients	Completed
Part B: Group 1	2 doses of $1x10^7$ i.u.	3 patients	Completed
Part B: Group 2	4 doses of $1x10^7$ i.u.	3 patients	Completed

Conclusions

- Pre-clinical studies suggested potent neutralisation effect in some pleural fluids did not restrict Seprehvir replication potential
- In clinical study, 8/9 pleural fluids had potent neutralisation effects on Seprehvir
- 2 and 4 intrapleural doses of Seprehvir stimulated a pronounced serum anti-HSV IgG response
- Anti-HSV IgG was detectable in all pleural fluids and increased in most post Seprehvir administration
- Despite neutralisation potential of pleural fluids and presence of anti-HSV IgG, Seprehvir persistence/replication was demonstrated in 8/9 patients
- Hostile, neutralising effects of pleural fluids did not interfere with Seprehvir activity

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Appendix 4: Poster presented at ASCGT meeting – HSV1716 oncolytic herpes virotherapy induced a paracrine death signal causing synergistic antitumour efficacy with Aurora Kinase Inhibition