Evaluation of the effects of oncolytic vaccinia virus on colorectal liver metastases in cell lines and in organotypic cultures

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Faculty of Health and Medical Sciences

Thesis submitted in accordance with the requirements of the University of Surrey for the degree of Doctor of Medicine
Declaration of Originality

This thesis and the work which it refers to are the results of my own efforts. Any ideas, data, images or text resulting from the work of others are fully identified within the work, and reference made to their originator in the text, bibliography or in footnotes. This thesis has not been submitted in whole or in part to any other academic degree or professional qualification. I agree that the University has the right to submit my work to the plagiarism detection service TurnitinUK for checks on its originality. Whether or not drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above.

Marcos Kostalas

September 2018
Abstract

Vaccinia virus is one of several live viruses currently being evaluated in clinical trials as cancer therapies. One oncolytic virus, T-VEC, is already in clinical use and is NICE approved for the treatment of melanoma.

We have investigated a genetically modified, Copenhagen strain vaccinia virus encoding the fusion suicide gene (FCU1) that is able to convert non-toxic fluorocytosine into the active compound fluorouracil. The mode of cell death attributed to vaccinia virus is variable and multiple modes have been implicated based on tumour type. Through our work with colorectal cancer cell lines we have established that this form of oncolytic vaccinia virus induces cell death through a predominant picture of apoptosis. Furthermore, assessment of its immunogenicity has found there to be an increase in markers of immunogenic cell death following infection with the virus.

We have developed an organotypic culture system in our laboratory which enables us to assess oncolytic viruses on ex vivo tissue. Treatment of organotypic cultures from colorectal liver metastases have shown that vaccinia virus successfully infects and replicates in the tumour tissue. A dose-response relationship was observed with the highest doses of virus exerting the most effect on tumour tissue. Assessment of the pro-drug activation system through the conversion of 5-fluorocytosine to 5-fluorouracil in supernatant showed a high percentage conversion, whilst immunohistochemical staining did show evidence of an improved effect with the pro-drug system compared with virus alone.

We have evaluated the combination of cavitational ultrasound and sulphur hexafluoride microbubbles to enhance infection with oncolytic virus. Our results have shown that this method enhances infection of organotypic cultures treated with oncolytic vaccinia virus. This has enabled the use of a lower dose of virus whilst maintaining the same effects in tissue as that of virus at higher doses. This finding has translational implications that may enhance the efficacy of systemically administered oncolytic vaccinia virus.
Acknowledgements

Firstly, I would like to thank Professor Nariman Karanjia and the Liver Cancer Surgery Appeal charity for providing me with the funding and the opportunity to undertake this period of research. Professor Karanjia has been a great source of knowledge and support, providing me with invaluable advice and encouragement during both my time in research and in my clinical work.

I would also like to thank my academic supervisor Professor Hardev Pandha for taking me into his laboratory and allowing me to develop the organotypic culture model. It has been an extremely challenging but rewarding experience. I am grateful to Dr Nicola Annels, Dr Guy Simpson and Michael Denyer for all of their patience, tutelage and assistance during my time in the laboratory and for providing me with the lab skills required to make the project a success.

To all of the patients that have provided us with tissue samples I express my sincere thanks. Without their consent this project would not have been possible. The GUTS charity and Mr Iain Jourdan were also helpful, assisting me towards the end of my research period and I would like to say a big thank you to them for this.

Finally, I would like to thank my family, Helen and Ella for supporting me through my studies. You both mean the world to me. I could not have done this without your love and support.
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<td>7-AAD</td>
<td>amino-actinomycin D</td>
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<tr>
<td>5-FdUMP</td>
<td>5-fluor-deoxyuridine monophosphate</td>
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<td>5-FU</td>
<td>5-fluorouracil</td>
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<td>5-FUMP</td>
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<td>A56</td>
<td>haemagglutinin</td>
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<td>ALARA</td>
<td>as low as reasonably achievable</td>
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<td>analysis of variance</td>
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<td>APAF1</td>
<td>adaptor protein apoptotic protease-activating factor-1</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<td>ATCC</td>
<td>American type culture collection</td>
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<td>adenosine triphosphate</td>
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<td>cytosine deaminase</td>
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<td>CpG island methylator phenotype</td>
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<td>3,3'-Diaminobenzidine</td>
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<td>DAF</td>
<td>decay accelerating factor</td>
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<td>DAMPS</td>
<td>damage associated molecular patterns</td>
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<td>dulbecco’s modified eagle’s medium</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>FAP</td>
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<td>first apoptosis signal receptor</td>
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<td>fusion suicide gene</td>
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<td>FOLFIRI</td>
<td>5-fluorouracil/leucovorin plus irinotecan</td>
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<td>GMCSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
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<td>high mobility group box 1 protein</td>
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<td>HPLC</td>
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<td>herpesvirus entry mediator</td>
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<td>ICD</td>
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<td>intracellular adhesion molecule 1</td>
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<td>IDO</td>
<td>indoleamine-2,3-dioxygenase</td>
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<td>IEV</td>
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<td>IFN</td>
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<td>IRF</td>
<td>interferon regulatory factor</td>
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<td>mixed lineage kinase domain-like</td>
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<td>MDSCs</td>
<td>myeloid derived suppressor cells</td>
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<td>MOI</td>
<td>multiplicity of infection</td>
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<td>MOPS</td>
<td>3-(N-morpholino) propansulfonic acid</td>
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<td>mRECIST</td>
<td>modified response evaluation criteria in solid tumours</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>MSI</td>
<td>microsatellite instability</td>
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<td>mTOR</td>
<td>mammalian target of Rapamycin</td>
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<td>MTS</td>
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<td>MTX</td>
<td>mitoxantrone</td>
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<td>mature virion</td>
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<td>MVA</td>
<td>Modified vaccinia virus Ankara</td>
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<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>NICE</td>
<td>The National Institute for Health and Care Excellence</td>
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<td>NK</td>
<td>natural killer cells</td>
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<td>NLRP1</td>
<td>nucleotide-binding domain leucine-rich repeat and pyrin domain containing protein 1</td>
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<td>OD</td>
<td>optical density</td>
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<td>OV</td>
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<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
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<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>PD-L1</td>
<td>programmed death ligand 1</td>
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<td>PDMS</td>
<td>polydimethylsiloxane</td>
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<td>phycoerythrin</td>
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<td>polyethylene glycol</td>
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<td>Pfu</td>
<td>plaque forming units</td>
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<td>PKR</td>
<td>protein kinase R</td>
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<td>pattern recognition receptors</td>
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<td>response evaluation criteria in solid tumours</td>
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<td>retinoic inducible gene 1</td>
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<td>RIP</td>
<td>receptor-interacting protein</td>
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<td>RIPA</td>
<td>radioimmunoprecipitation</td>
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<td>SABR</td>
<td>stereotactic ablative radiotherapy</td>
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<td>SAT</td>
<td>System for Acoustic Transfection</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
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<td>SIRT</td>
<td>selective internal radiation therapy</td>
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<td>STAT</td>
<td>signal transducer and activation of transcription</td>
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<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<td>thymidine kinase</td>
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<td>toll-like receptors</td>
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<td>tumour necrosis factor</td>
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<td>TRAF</td>
<td>tumour necrosis factor associated factor</td>
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<td>TMBIM</td>
<td>transmembrane Bax inhibitor-containing motif</td>
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<td>talimogene laherparepvec</td>
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<td>UPRTase</td>
<td>uracil phosphoribosyltransferase</td>
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<td>US</td>
<td>ultrasound</td>
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<td>untreated</td>
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<td>VACV</td>
<td>vaccinia virus</td>
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<td>vCCI</td>
<td>vaccinia virus CC chemokine inhibitor</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>VCP</td>
<td>vaccinia virus complement protein</td>
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<td>VEGF-A</td>
<td>vascular endothelial growth factor A</td>
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<tr>
<td>VGF</td>
<td>vaccinia growth factor</td>
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<td>vGAAP</td>
<td>viral Golgi anti-apoptotic protein</td>
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<td>VSV</td>
<td>vesicular stomatitis virus</td>
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<td>vvDD</td>
<td>double-deleted vaccinia virus</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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Chapter 1

Introduction
1.1 Hallmarks of cancer

Hanahan and Weinberg proposed six hallmarks of cancer that provide a framework for the understanding of the diversity of neoplastic diseases. As normal cells evolve towards a neoplastic state they acquire a succession of these hallmark capabilities. These hallmarks include: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. Recently two emerging hallmarks have been added to this list. These emerging hallmarks include: reprogramming of energy metabolism and evasion of immune destruction.

The role that the immune system plays in resisting or eradicating formation and progression of cancers is still unresolved. The long-standing theory of immune surveillance proposes that cells are constantly monitored by the immune system which is responsible for the recognition and elimination of early stage cancer cells. The development of solid tumours suggests that these have managed to evade immune surveillance and therefore prevent eradication.

The role of the immune system in the development of cancers appears to be validated by the increase in certain cancers in individuals that are immunocompromised. Recently there is increased evidence from genetically engineered mice and from clinical epidemiology suggesting that the immune system operates as a significant barrier to tumour formation and progression.

Exposure of immune deficient mice to carcinogens found that tumours arose more frequently and/or more rapidly in the immune deficient mice compared with their immunocompetent controls. In particular, deficiencies in the development or function of CD8+ cytotoxic T lymphocytes (CTLs), CD4+ helper T cells, or natural killer (NK) cells each led to increases in tumour incidence. Mice with immunodeficiencies of both T cells and NK cells were even more susceptible to cancer development.
The results indicated that both the innate and adaptive immune system contribute to immune surveillance and are involved in tumour eradication.

Transplantation experiments have also shown that cancer cells that have developed in immunodeficient mice are inefficient at initiating secondary tumours in synergistic immunocompetent hosts, whilst cancer cells from immunocompetent hosts are equally efficient at initiating transplanted tumours in both types of host. These results would suggest that highly immunogenic cancer cell clones are routinely eliminated from immunocompetent hosts leaving behind weakly immunogenic variants that grow and generate solid tumours. This can occur in both immunodeficient and immunocompetent hosts. In immunodeficient hosts highly immunogenic cancer cells are not depleted and these can develop into solid tumours along with the weakly immunogenic cancer cells.

Epidemiological studies also support the existence of an antitumoral immune response in some forms of cancer. In patients with colonic and ovarian cancers that have high levels of CTLs and NK cells prognosis is improved when compared to patients with tumours lacking such abundance of killer lymphocytes. Additionally, some immunosuppressed organ transplant recipients have been seen to develop donor-derived cancers that were potentially held in check by a fully functional immune system in the donor.

Tumour-host immunological interactions are complex. Highly immunological cancer cells may evade immune destruction through the disablement of immune components that have been produced to eliminate them. Cancer cells may paralyse infiltrating CTLs and NK cells by secreting transforming growth factor β (TGF-β) or other immunosuppressive factors. Other mechanisms also include the recruitment of inflammatory cells that are actively immunosuppressive such as regulatory T cells and myeloid-derived suppressor cells (MDSCs) which both suppress the action of cytotoxic lymphocytes.
1.2 Colorectal Cancer

1.2.1 Epidemiology

Colorectal cancer (CRC) is the third most common cancer in the developed world and the 4\textsuperscript{th} most common cancer in the UK. The World Health Organisation (WHO) estimates that there are approximately 1 million new cases and 500,000 deaths annually worldwide\textsuperscript{2}. The lifetime incidence of CRC is 5\% however both incidence and mortality have been found to be decreasing\textsuperscript{2-4}. At the time of diagnosis approximately 20-25\% of patients with CRC will have already developed metastatic disease of which the most common site of metastasis is the liver\textsuperscript{5}. This is likely attributable to tumour spread via the portal system\textsuperscript{6-8}. Approximately 50\% of patients will develop liver metastases during...
their disease course\textsuperscript{9}. The natural history of metastatic disease is variable however without treatment, patients will have a median survival of less than 12 months\textsuperscript{9,10}.

Of the patients that develop metastatic disease these can be classified into the following groups regarding treatment: resectable disease, unresectable disease or disease that can become resectable after initial treatment and downstaging of tumour\textsuperscript{11}. Of the 20-25\% patients that are found to have metastatic colorectal cancer at the time of diagnosis, only 20\% of patients will have liver metastases (CRLM) that are considered amenable to resection whilst the majority (80\%) of patients have disease which is initially considered unresectable\textsuperscript{12}.

Chemotherapy is a key treatment in the management of patients with CRLMs. It is used in patients with both disease that is considered resectable and unresectable. The aim of this treatment in patients with initially unresectable disease is to down-stage the tumour to enable curative resection. Standard chemotherapy regimens in the UK include 5-fluorouracil (5-FU)/leucovorin plus irinotecan (FOLFIRI) or oxaliplatin (FOLFOX). These regimens are successful in facilitating resection in 7 - 40\% of patients that initially have unresectable disease\textsuperscript{13}.

As well as chemotherapeutic agents, biological agents have also been developed based on our greater understanding of the biology of colorectal cancer (CRC). Two agents targeting two mechanisms: angiogenesis and epidermal growth factor have been developed. These are bevacizumab (angiogenesis), cetuximab and panitumumab (epidermal growth factor). The addition of these biological agents to established chemotherapy regimens improves response rates and can increase the number of candidates suitable for surgical resection\textsuperscript{14}.

Although progress has been made in the development of effective agents in the treatment of metastatic colorectal cancer, survival remains stubbornly low. Patients not eligible to undergo curative resection will have a median survival of between 16- 24 months\textsuperscript{15}. Given that surgical resection gives the greatest probability of cure, the need for the development of further agents remains.
1.2.2 Risk factors

Most CRC cases are sporadic however genetic and environmental factors are also important. There is no single risk factor that accounts for most cases of colorectal cancer, they are several that are either unmodifiable or modifiable and they often co-exist and interact. Unmodifiable risk factors include male sex, increasing age, inflammatory bowel disease as well as family history of disease. Modifiable risk factors include smoking, alcohol excess, increased dietary red meats, obesity and diabetes. The relative risk of developing colorectal cancer is higher in patients with one or more first-degree relative affected by the disease and in patients with inflammatory bowel disease.

The other risk factors are more common and are also modifiable. These represent a greater proportion of disease burden for the population despite having lower relative risks compared to those of having a first-degree relative with the disease and a diagnosis of inflammatory bowel disease.

Colorectal cancer has a significant heritable component with up to 35% risk potentially attributable to heritable factors. Hereditary forms of colorectal cancer such as familial adenomatous polyposis and hereditary non-polyposis colon cancer are determined by known genetic abnormalities but only account for less than 5% of colorectal cancers. Other than these well-known hereditary forms of colorectal cancer, genetic factors that determine the risk of disease development are poorly understood.
1.2.3 Molecular mechanisms of CRC pathogenesis

One of the key aspects of the formation of CRC is the progression of normal glandular epithelial cells into invasive adenocarcinomas, which occurs following an accumulation of acquired and epigenetic changes. Fearon and Vogelstein proposed the polyp to cancer sequence in 1988 when describing a tumour progression model including a stepwise progression from the formation of benign neoplasms to the progression to more histologically advanced neoplasms and finally transformation into invasive carcinomas.

With an increased understanding of the molecular pathogenesis of CRC this initial model has been revised numerous times. Now it is also recognised that serrated polyps have the potential for malignant transformation as well as the tubular and tubulovillous adenomas that were initially described. Pre-malignant serrated polyps occur more frequently in the proximal colon and are associated with CpG island methylator phenotype (CIMP) which is recognised by having a high frequency of aberrantly methylated CpR dinucleotides. Conventional tubular adenomas are more commonly initiated by inactivation of the biallelic inactivation of the tumour-suppressor gene APC and to display chromosomal instability (CIN) recognised by aneuploidy and gains and losses of large portions of whole chromosomes. In addition, BRAF mutations, are more commonly found in tumours arising from the serrated pathway.

1.2.4 Epidemiology

Colorectal cancer is the third most common cancer worldwide and is the fourth most common cancer cause of cancer death with an estimated 1.4 million cases and 693,900 deaths in 2012. Its incidence increases with increasing age with a median age at diagnosis of 67 years in men and 71 years in women. It is most prevalent in the western world such as in Europe, North America and Australia whilst its prevalence is less in Africa and Asia. The incidence of the disease is increasing in certain countries which have historically had a low-risk such as Kuwait and Israel in Western Asia, and the Czech Republic and Slovakia in Eastern Europe. Trends of the disease in high-risk countries have
been variable over the past 20 years with a gradual increase in incidence in countries such as Finland and Norway; a stable incidence in France and Australia, and a decreasing incidence in the USA \(^{33}\). The reduction in colorectal cancer incidence seen in the US is confined to patients under the age of 50 and is attributed to increased screening for the disease and removal of pre-cancerous lesions \(^{36}\). The increase in incidence seen in countries such as Kuwait, Israel, Czech Republic and Slovakia may reflect an increased prevalence of risk factors for the development of colorectal cancer such as an unhealthy diet, smoking and obesity, factors attributed to a western lifestyle \(^{35}\).

*Figure 2.1* Estimated age-standardised colorectal cancer incidence for men in 2008. Data from Globocan 2008 \(^{32}\)
1.2.5 Prognosis

The prognosis of colorectal cancer has improved steadily over time and in high income countries such as the USA, Canada and several countries in western Europe the 5-year survival has reached almost 65%, whilst remaining less than 50% in low income countries. When colorectal cancers are detected early at a localised stage the 5-year survival rate is 90.3%. Once it has spread to regional lymph nodes or adjacent organs the 5-year survival reduces to 70.4%. By the time colorectal cancer has metastasised to distant organs such as the liver and lungs the 5-year survival rate drops to 12.5%

1.2.5 Classification of colorectal cancer

Colorectal cancers are classified according to the local depth of invasion (T stage), involvement of lymph nodes (N stage), and presence of distant metastases (M stage) (Table 1.1). These stages are combined to give the patient and overall disease stage defined as per Table 1.2. This provides the basis for decision on treatments to be given.

Despite providing guidance on patient treatment and giving information with regards to prognosis the response of patients to treatment and their outcome is not predicted. In patients with stage III disease and stage II disease with additional risk factors for disease, adjuvant chemotherapy is recommended, however not all of these patients will benefit from this.

<table>
<thead>
<tr>
<th>T stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx</td>
<td>No information available for local tumour infiltration</td>
</tr>
<tr>
<td>Tis</td>
<td>Tumour restricted to mucosa, no infiltration of lamina muscularis mucosae</td>
</tr>
<tr>
<td>T1</td>
<td>Infiltration through lamina muscularis mucosae into submucosa, no infiltration into lamina muscularis propria</td>
</tr>
<tr>
<td>T2</td>
<td>Infiltration into but not beyond lamina muscularis propria</td>
</tr>
<tr>
<td>----</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>T3</td>
<td>Infiltration into subserosa or non-peritonealised pericolic or perirectal tissue, or both; no invasion of serosa or into neighbouring organs</td>
</tr>
<tr>
<td>T4a</td>
<td>Infiltration of serosa</td>
</tr>
<tr>
<td>T4b</td>
<td>Infiltration of neighbouring organs</td>
</tr>
</tbody>
</table>

### N Stage

<table>
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<tr>
<th>Nx</th>
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</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>No lymph node involvement</td>
</tr>
<tr>
<td>N1a</td>
<td>Cancer cells detectable in 1 regional lymph node</td>
</tr>
<tr>
<td>N1b</td>
<td>Cancer cells detectable in 2-3 regional lymph nodes</td>
</tr>
<tr>
<td>N1c</td>
<td>Tumour satellites in subserosa or pericolic or perirectal fat tissue, regional lymph nodes not involved</td>
</tr>
<tr>
<td>N2a</td>
<td>Cancer cells detected in 4-6 regional lymph nodes</td>
</tr>
<tr>
<td>N2b</td>
<td>Cancer cells detected in 7 or more regional lymph nodes</td>
</tr>
</tbody>
</table>

### M Stage

<table>
<thead>
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</tr>
</thead>
<tbody>
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<td>No distant metastases</td>
</tr>
<tr>
<td>M1a</td>
<td>Metastasis to one distant organ or distant lymph nodes</td>
</tr>
<tr>
<td>M1b</td>
<td>Metastasis to more than one distant organ or set of distant lymph nodes or peritoneal metastasis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Overall disease stage</th>
<th>T stage</th>
<th>N stage</th>
<th>M Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 1</td>
<td>T1/2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 2</td>
<td>T3/4</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>2a</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>2b</td>
<td>T4a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>2c</td>
<td>T4b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Any</td>
<td>N positive</td>
<td>M0</td>
</tr>
</tbody>
</table>

Table 1.1 Classification of colorectal cancer according to tumour depth (T stage), lymph node involvement (N stage) and presence of metastasis (M stage). Adapted from the TNM Classification of malignant tumors, 7th edition.
1.2.6 Pathogenesis of colorectal cancer

The pathogenesis of colorectal cancer is heterogeneous. The molecular mechanisms are clinically important as they are related to patient prognosis and patient response to treatment.40.

1.2.6.1 The adenoma carcinoma sequence

Colorectal cancer develops over several years and often arises from pre-malignant lesions, most commonly from pre-malignant dysplastic adenomas. Mutations of the tumour suppressor antigen presenting cell (APC) gene occur early in the process of cancer formation and occur in the majority of adenomas.25. The adenoma-carcinoma sequence (Figure 1.2) is further developed through mutations activating the KRAS oncogene and mutations inactivating the tumour suppressor gene, p53.28. These typical mutations are often accompanied by chromosomal instability through changes in chromosome number and structural changes.41.
Sporadic cancers however do not all have these characteristic gene mutations and develop through different pathways and events. Cancers like these include those that have developed from serrated precursor lesions. These are often characterised by the CIMP phenotype and activating mutations of the BRAF oncogene.\(^4^2\)

1.2.6.2 Hereditary colorectal cancer

Hereditary colorectal cancer accounts for up to 5% of all colorectal cancers.\(^2^7\) The two most common forms are familial adenomatous polyposis coli (FAP) and hereditary non-polyposis colon cancer (HNPCC). Both of these are inherited in an autosomal dominant fashion. HNPCC associated cancers show evidence of mismatch repair deficiency and have a high level of microsatellite instability.\(^2^7\) FAP on the other hand follow the typical adenoma-carcinoma sequence.\(^2^7\)
1.2.7 Markers of prognosis

1.2.7.1 Microsatellite instability

Microsatellites are a type of simple DNA sequence repeat. Microsatellite instability (MSI) is a unique molecular alteration induced by deficiencies in the DNA mismatch repair system characterised by unstable microsatellites that have variable lengths \(^43\). The MSI phenotype is one of the main molecular subtypes of colorectal cancers and accounts for 12-20% of colorectal cancers in Western countries and 6-13% colorectal cancers in Eastern countries \(^43\). Hereditary colorectal cancers such as hereditary non-polyposis coli that are caused by germline mutations in mismatch repair genes have a high level of microsatellite instability (MSI-H) \(^43\). These cancers account for up to 5% of all colorectal cancers. Patients with sporadic colorectal cancers with an MSI-H phenotype account for up to 15% of all colorectal cancers \(^43\). Patients with the MSI-H phenotype have a better prognosis compared to patients with microsatellite stability \(^25\). The MSI-H phenotype also gives an indication of patient response to treatment with chemotherapy as these patients have been shown not to benefit from adjuvant chemotherapy treatment with 5-fluorouracil but have an improved response to chemotherapy that is irinotecan based \(^44\).

1.2.7.2 Immune response in colorectal cancer

Patients with a high level of microsatellite instability have an associated high level of tumour-infiltrating lymphocytes. This is likely to occur due to the deficiency of mismatch repair genes and the production of antigens that are subsequently induced, leading to activation of the host's immune response \(^44\). Patients with tumour infiltration with CD45R0-positive and CD3-positive lymphocytes were found to have a better prognosis regardless of cancer stage compared to those with low lymphocyte infiltration, who had a poorer prognosis \(^45\).
1.2.7.3 KRAS mutation

KRAS is the human homolog of the Kirsten rat sarcoma 2 virus oncogene. This encodes a small GTP-binding protein that acts as a self-inactivating signal transducer by cycling from GDP to GTP bound states in response to the cell surface receptor EGFR. Activation of EGFR stimulates the RAS/RAF/MAPK, STAT and PI3K/AKT pathways. Together these pathways are involved in the regulation of cellular proliferation, adhesion, angiogenesis and survival\(^46\). Anti-EGFR targeted treatments include cetuximab and panitumumab. The KRAS oncogene can harbour oncogenic mutations in between 30% and 50% of colorectal cancers and is a marker of poor prognosis when present as well as a sign of resistance to treatment with EGFR inhibitors\(^46\).

1.2.8 Diagnosis of colorectal cancer

Patients with symptoms concerning for colorectal cancer are referred on a ‘two-week’ basis in the UK. Symptoms concerning for this diagnosis include an unexplained change in bowel habit; rectal bleeding, a palpable mass and anaemia as well as taking into consideration patient age and comorbidities. Patients with a suspected diagnosis of colorectal cancer are offered either a CT colonography or an optical colonoscopy to confirm the diagnosis. Although histological diagnosis is desirable, if a lesion that is highly suspicious for malignancy is seen on imaging from CT colonography then histology is not essential prior to surgery. Only in cases of rectal tumours when surgery may result in the formation of a permanent stoma or an ultra-low anterior resection, or if neo-adjuvant treatment is being planned\(^47\).

1.2.9 Staging of disease

Assessment of local extent of colon cancer is assessed using computed tomography (CT). This imaging allows clinicians to assess the extent of spread in relation to the bowel wall and adjacent organs. This imaging also is key to planning surgical resection and can guide neo-adjuvant treatment in patients.
that have very locally advanced disease or if their disease is initially not considered to be resectable.

1.2.10 Metastatic colorectal cancer

At the time of diagnosis approximately 20% of patients will present with distant metastatic disease. Colorectal cancers most commonly metastasize to the liver, as well as to the lungs and peritoneum. It is therefore important to perform imaging of the liver and routine imaging with a CT scan incorporating the chest, abdomen and pelvis is usually sufficient, however further characterisation can be performed with magnetic resonance imaging for liver lesions that are equivocal on CT scanning. Patients that present with metastatic colorectal cancer can be divided into three groups: patients with surgically resectable disease, patients that can become resectable following initial treatment and those are not resectable. Only 10-20% of patients with metastatic colorectal liver metastases are candidates for surgical resection, with the vast majority presenting with liver metastases that are not amenable to resection. Patients treated with chemotherapy that are subsequently able to undergo surgical resection have long-term survival rates that are comparable to those patients that are considered resectable initially. This has led to an increased use of chemotherapy and biological agents in the treatment of metastatic colorectal cancer with a subsequent increase in the numbers of patients now considered suitable to undergo surgical resection.

1.2.11 Management of patients with metastatic colorectal cancer

Patients with a diagnosis of colorectal cancer are managed by multi-disciplinary team that includes surgeons, oncologists, gastroenterologists, radiologists, pathologists as well as specialist nurses. In patients without metastatic disease the multi-disciplinary team assess in the first instance whether the tumour is able to be surgically resected and subsequently an assessment is made as to whether there they should receive chemotherapy post-operatively.
1.2.11.1 Surgery

Surgery can be performed to cure patients with colorectal cancers. This can be performed in open surgery or can be undertaken in a minimally-invasive way using laparoscopy (key-hole surgery). The benefits of laparoscopic surgery include a faster return of bowel function, shorter stay in hospital as well as a reduced requirement for a blood transfusion. It does however take longer to perform surgery and is more expensive than traditional open surgery. Despite these differences both approaches achieve the same long-term results \(^{49}\). During surgery to remove colon cancer the tumour as well as surrounding lymph nodes are taken. The extent of surgery is determined by the location of the tumour in the colon and the associated blood vessels that supply the area of colon in which it is situated.

Difficulties in deciding who is suitable to undergo surgical resection remain in clinical practice. The criteria for resectability are not standardised and are related to the experience of the surgeon and the multi-disciplinary team. Current guidelines state that resection should be considered for solitary metastases and metastases confined to the liver parenchyma \(^{13}\). The remaining liver needs to be healthy and there must be a sufficient volume remaining post-resection to avoid post-hepatectomy liver failure (approximately 25\%) \(^{50}\). Extra-hepatic disease is no longer an absolute contra-indication for resection over colorectal liver metastases. It is also possible to perform multiple hepatic resections, providing that there is sufficient remaining healthy liver with evidence of a similar survival benefit to that of primary hepatic resection \(^{51,52}\). Contraindications to surgery include unresectable extra-hepatic disease, extensive hepatic tumour burden or if a patient is not fit enough to undergo the procedure \(^{14}\).

1.2.11.2 Adjuvant treatment

The risk of disease recurrence in patients with stage III colorectal cancers is between 15-50\% \(^{25}\). It is therefore recommended that all these patients receive chemotherapy following their surgery (adjuvant chemotherapy), providing they do not have any contra-indications to this. Fluorouracil is the mainstay of chemotherapy treatment and has been shown to reduce recurrence rates by 17\% and to
improve overall survival by 13-15% \(^{53}\). In combination with oxaliplatin, the 5-year disease free survival and overall survival improved by 5.9% and 2.5% respectively \(^{54}\).

1.2.11.3 Chemotherapy

Most patients that present with colorectal liver metastases may present with extensive disease which may initially not be considered resectable. This group of patients will receive systemic chemotherapy, either given with palliative intent or as a neo-adjuvant treatment with the aim of downsizing tumour burden and converting patients to operable disease. Standard chemotherapy regimens for patients with colorectal liver metastases are FOLFOX or FOLFIRI. These chemotherapy regimens can help facilitate resection in up to 40% of patients \(^{13}\). FOLFOX has been shown to reduce the size of liver metastases by more than half in 59% of patients treated whilst facilitating curative resection in 38% of patients \(^{55}\). A recent randomised trial has found an increased conversion rate to resectability and increased overall survival following treatment with FOLFIRINOX (5-fluorouracil/leucovorin, oxaliplatin and irinotecan) chemotherapy compared with other regimes in patients with initially unresectable metastatic colorectal cancer \(^{56}\).

In patients with resectable metastatic disease, chemotherapy is helpful as it can reduce the incidence of disease relapse through the eradication of occult disease \(^{14}\). The recent EORTC 40983 trial that compared patients treated with both neo-adjuvant and adjuvant chemotherapy with FOLFOX4 chemotherapy to those undergoing surgery alone, found that there was an improvement in progression free survival in those treated with chemotherapy but ultimately no improvement in long-term survivorship between the two groups \(^{57}\).

1.2.11.4 Biological treatment

An increased understanding of the biology of colorectal cancer has led to the development of biological therapies targeting angiogenesis and epidermal growth factor receptors. The addition of
these agents along with standard chemotherapy regimens aim to increase the number of patients that are eligible for surgery.

1.2.11.5 Anti-angiogenesis agents

The addition of bevacizumab to first and second line chemotherapy in the treatment of metastatic colorectal cancer has been shown to improve both progression-free and overall survival. Due to concerns with regards to wound healing complications, data on its peri-operative use is limited.

1.2.11.6 Anti-epidermal growth factor receptor agents

Cetuximab and panitumumab are active in patients with metastatic colorectal cancer as single-line agents as well as in combination with chemotherapy. These agents’ activity is confined to patients with RAS wild-type tumours. Several randomised trials have evaluated the effects of cetuximab in patients with unresectable colorectal liver metastases. These found, to varying degrees, an increase in resection rates with an overall resection rate across these trials of 60-79%.

1.2.11.7 Other treatments for colorectal liver metastases

Radiofrequency ablation (RFA), selective internal radiation therapy (SIRT) and stereotactic ablative radiotherapy (SABR) are other modalities that can be utilised in the treatment of unresectable liver metastases. RFA is a localised thermal treatment technique that is designed to induce tumour destruction by heating the tumour tissue to temperatures exceeding 60°C. This is a well-accepted and documented treatment for patients with inoperable colorectal liver metastases. SIRT involves embolising radiolabelled spheres (SIR-Spheres) into the arterial supply of the liver. SABR is a non-invasive, ablative treatment approach for patients with liver metastases that are ineligible for resection or thermal ablation. It delivers extremely high biological doses of radiation to limited liver volumes with high precision and in few fractions which minimises normal tissue toxicity and maximises local control.
1.2.11.8 Multi-disciplinary team approach to the management of colorectal liver metastases

The management of patients with cancer is addressed by multiple specialists given their complex needs. The management of patients with metastatic colorectal cancer is undertaken by colorectal multi-disciplinary teams including a specialist hepatobiliary team that can provide the additional expertise for patients with liver metastases. The team comprises colorectal and hepatobiliary surgeons, oncologists, diagnostic and interventional radiologists, histopathologists and nurse specialists. Regular MDT discussions take place to ensure optimal treatment choices are made over their disease course and to review opportunities for potentially curative resection of their disease during their treatment. Throughout the patient journey, nurse specialists are key to providing them with support, advice and information.

1.3 Oncolytic virotherapy

1.3.1 Introduction

Oncolytic virus (OV) immunotherapy utilises viruses that replicate within tumour cells naturally or are modified to selectively replicate within tumour cells. It has long been recognised that viruses have the ability to kill cancer cells and recently clinical trials have shown evidence of therapeutic benefit in patients with multiple tumour types. The increase in interest in oncolytic viruses is a consequence of increased understanding of tumour and viral biology, tumour immunology and molecular genetics. Following a successful phase III clinical trial the first oncolytic virus was approved for use in clinical practice in the United States in the treatment of patients with advanced melanoma with further development of oncolytic virotherapy anticipated based on the success of this.

Viruses share several properties including a genetic element consisting of single- or double-stranded DNA or RNA and are able to infect host cells and replicate. The result of viral infection varies depending on the interaction between the virus and host immune system, the pathogenicity of virally encoded
genes and the ability of the virus to replicate. An increased understanding of cell entry, viral replication, immune response and lytic versus latent infections has increased the interest in using these viruses in the treatment of human diseases. OVs differ from traditional vaccines in that they directly infect and lyse tumour cells. They do not require defined antigens to be included in the vector as dying tumour cells may release tumour-associated antigens (proteins derived from tumour cells that can be recognised by the immune system) with a resultant induction of the host immune response. OVs also provide additional danger signals (nuclear and cytosolic proteins) that are released by cells during injury or necrosis and which stimulate both the innate and adaptive immune system, promoting an efficient anti-tumour immune response.

The mechanism by which anti-tumour activity is mediated by OVs is not completely understood. Two distinct mechanisms are thought to contribute to this; these are, selective replication within and lysis of tumour cells in situ and activation of systemic anti-tumour immunity.

Oncolytic viruses have the ability to enter both normal and cancer cells, however abnormalities in cancer cell response to stress, cell signalling and homeostasis allow the virus to preferentially replicate within them. Mechanisms by which viruses are detected and cleared from cells can also be aberrant in cancer cells, resulting in increased viral replication. An example of one such mechanism is protein kinase R (PKR); this is a critical factor involved in clearing intracellular viral infections. The absence of this protein in some cancer cells results in increased viral replication following infection. There is variation between cancer types and this difference has an effect on the activity of oncolytic viruses.

The response of the immune system to oncolytic virus also plays a role in its efficacy however there is a balance between the promotion of an immune response versus the neutralising effect of host antiviral responses following infection. Viruses are able to help promote an immune response against cancer cells by allowing tumour antigen presentation during active viral infection; conversely, neutralising host antiviral responses can dampen the viral effect and block replication with a resultant reduction of viral infection in tumour cells. The outcome of infection is determined by the balance of
these pathways. The activation of systemic immunity towards cancer cells results in effects at both the primary tumour as well as distant sites of metastatic disease.

Many viruses have been proposed as vectors for oncolytic virus immunotherapy. However, two viruses, T-VEC (modified herpes simplex virus) and H101 (modified adenovirus) have been approved for clinical use in the United States and China respectively.

1.3.2 Mechanism of oncolytic virus activity

The majority of OVs directly lyse and kill cancer cells. Cell entry of virus, replication and cell response to viruses determine the efficiency of this. The ability to lyse cancer cells is also dependent on the type of virus, virus concentration, tropism and susceptibility of cancer cells to the different modes of cell death.

Normal cells have various signalling pathways that detect and clear viral particles. These pathways can be stimulated by release of interferons, or through the activation of toll-like receptors (TLRs). These are present on the cell surface as well as within cells and are pattern recognition receptors that become activated in response to pathogen-associated molecular patterns (PAMPs) that are common to viruses. Stimulation of TLRs leads to the activation of the host cell antiviral response as well as the systemic innate immunity. Host factors involved in clearance of oncolytic virus include tumour necrosis factor (TNF) associated factor 3 (TRAF), interferon (IFN) related factors 3 and 7 and retinoic inducible gene 1 (RIG1). These stimulate the JAK (Janus Kinase) – STAT (signal transducer and activation of transcription) pathway that coordinates antiviral activity in cells that are infected. There is a resultant reinforcement of interferon release and this activates PKR activity. PKR is an intracellular protein that when activated stops protein synthesis within the cell, promotes cell death and viral clearance. As cancer cells are abnormal they may have aberrant interferon pathway signalling and PKR activity which prevents viral clearance.
Various viruses are also able to block apoptosis through the manipulation of aberrant signalling pathways within tumour cells (Figure 1.3). Replication of most oncolytic viruses results in cell death with a subsequent elimination of cancer cells and initiation of a systemic immune response.

*Figure 1.3 Promotion of viral spread and immune evasion by oncolytic virus in cancer cells.* Once a cell detects viral presence a signalling cascade through several interferons (IFN) elements, janus kinase (JAK), signal transducer and activation of transcription (STAT), and interferon regulatory factor 9 (IRF9) leads to a programmed transcriptional pathway that leads to a reduction in viral spread and targets infected cells for apoptosis or necrosis. Local interferon produced by the innate immune response can also result in antiviral activity through the interferon receptor (IFN receptor). Type I IFNs signal through the JAK-STAT pathway which results in the upregulation of cell cycle regulators protein kinase R (PKR) and interferon regulatory factor 7 (IRF7). These limit viral spread through binding to viral particles and triggering type 1 IFN pathways that lead to abortive apoptosis and alerting the immune system to the presence of viral infection. Cancer cells may downregulate key parts of the type 1 IFN signalling pathway with a resultant limitation in the pro-apoptotic effects. The figure demonstrates the areas that vaccinia virus affects these pathways to prevent elimination and to promote viral spread. Figure adapted from Kaufmann et al. 

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1.3.2.1 Initiation of systemic anti-tumour immunity by oncolytic virotherapy

Following oncolysis, tumour cells release tumour associated antigens. These are then able to promote an adaptive immune response that mediates an anti-tumour effect at distant tumour sites. Following oncolytic cell death tumour cells also release viral PAMPs as well as danger associated molecular patterns (DAMPs) such as heat shock proteins (HSPs), high mobility group box 1 proteins (HMGB1), calreticulin, adenosine triphosphate (ATP), and uric acid. They also release cytokines that promote the maturation of antigen presenting cells such as dendritic cells. These cytokines include type 1 interferons, tumour necrosis factor-α (TNF-α), interferon gamma (IFN-γ) and interleukin-12 (IL-12) \(^68\). These then activate CD4\(^+\) and CD8\(^+\) T cell responses. CD8\(^+\) T cells can develop into cytotoxic effector T cells that move to areas of tumour growth and mediate anti-tumour immunity following recognition of tumour antigens \(^68\). As viruses induce a natural immune response from the host, this can result in oncolytic virus clearance by neutralising antibodies and through cytotoxic T-cell mediated immune response.

Release of tumour associated antigens in combination with cytokines and DAMPs can be beneficial in the generation of an immune response against cancer cells. This is particularly important in the generation of an anti-cancer immune response to tumours that are distant to the site of virus administration \(^68\). The innate immune response can also be activated by Type 1 interferons and DAMPs as they directly activate natural killer cells. Natural killer cells target cancer cells as these cells have a downregulation of major histocompatibility complex (MHC) class 1 expression. There is a balance to the effect of the activation of NK cells, whilst these are able to target cancer cells as described they can also eliminate virally infected cells, with a subsequent reduction in viral efficacy \(^68\). The factors influencing the balance between immune-mediated clearance of virus and the induction of anti-tumour immunity are incompletely understood.
Figure 1.4 Induction of the immune system by oncolytic virus. Following infection of cancer cells by oncolytic virus, endoplasmic reticulum and genotoxic stress occurs within the infected cells. Reactive oxygen species and antiviral cytokines are produced and then released from the infected cells with subsequent initiation of the immune system. Immune cells such as antigen presenting cells, CD8+ T cells and natural killer cells are stimulated. Oncolytic of the infected cancer cell occurs and this causes a release of viral progeny, pathogen associated molecular patterns (PAMPs), which include: viral capsids, viral proteins and viral DNA/double stranded or single stranded RNA; danger associated molecular patterns (DAMPs) which include heat shock proteins (HSP), high mobility group box 1 protein (HMGB1), calreticulin, adenosine triphosphate (ATP) and uric acid; and tumour associated antigens/neoantigens. PAMPs and DAMPs stimulate the immune system through the activation of Toll-like receptors. Antigen presenting cells take up and tumour associated antigens and neoantigens which also stimulates the immune system whilst the release of viral progeny acts to spread viral infection further. These events stimulate the immune system to act against cancer cells infected with virus and to generate immune responses to tumour associated and neo-antigens which may be displayed on cancer cells that are not infected by oncolytic virus. Figure adapted from Kaufman et al.68.

1.3.2.2 Overcoming cancer-mediated immune evasion

Tumour cells have developed the ability to evade the immune system in several ways. These include the expression of immune inhibitory receptors on cell surfaces that inactivate effector immune cells. These inhibitory receptors secrete factors that promote the recruitment of immune suppressive cells, these factors include IL-10, TGF-β as well as indoleamine-2,3-dioxygenase (IDO). The immune suppressive cells that are recruited as a result include tumour associated macrophages and MDSCs.
In order to overcome inhibitory responses OVVs modify the inhibitory environment through several mechanisms that alter the cytokine milieu as well as the type of immune cells present in the tumour microenvironment. These changes result in increased recognition of tumour cells by the immune system as well as eradication of these cells. They can also increase spread of tumour-associated antigens. In the presence of DAMPs and following engagement of TLRs there is an increased level of type 1 interferons and other inflammatory mediators with a resultant increase in systemic immunity against cancer cells.

The lysis of cancer cells can lead to the expression of previously concealed antigens, termed neo-antigens. The expression of these leads to their detection by antigen presenting cells, triggering an immune response against these new antigens. If T-cell clones are generated then these may be able to kill antigen-expressing cells that were not originally infected by the virus.

1.3.3 Factors affecting oncolytic virus spread

Barriers that reduce the spread of OVVs include elevated interstitial pressures, dense connective tissue, necrotic tissue, calcification, and reduced vasculature. In an attempt to overcome these physical barriers, the majority of clinical trials utilising OVVs have opted to administer them intratumorally. In clinical practice intratumoral administration of OV is limited as it is only applicable to those tumours that are accessible to injection with OV. However, the phase III clinical trial of talimogene laherparepvec (T-VEC) did demonstrate that a systemic anti-tumour response could be generated from the injection of an index tumour with distant sites of non-injected disease responding to intratumoral OV adminisitration, therefore overcoming physical barriers.

The size of tumours as well as the heterogeneity of the tissue can also affect viral biodistribution. Oncolytic viruses have the ability to infect cancer-associated fibroblasts (CAFs), however infection of these does not result in viral replication. The infection of these fibroblasts can therefore reduce delivery of oncolytic virus to cancer cells.
1.3.4 Oncolytic virus development

As viruses are live, the approach to tumour cell targeting as well as the attenuation of viral pathogenesis are important considerations in OV development. It is desirable for OVs to have limited viral immunogenicity whilst maintaining tumour cell killing with subsequent immune activation. Recombinant engineering has permitted the development of effective oncolytic virus strategies.

1.3.4.1 Cancer cell targeting of oncolytic viruses

Many oncolytic viruses have a natural tropism for aberrant cell surface proteins that are expressed on cancer cells. Examples of these include intracellular adhesion molecule 1 (ICAM-1) and decay accelerating factor (DAF) for coxsackie virus and the herpesvirus entry mediator (HVEM) and nectins for the entry of herpes simplex virus into cancer cells.

Oncolytic viruses can also be engineered so that they target specific cell surface receptors that are expressed on cancer cells. For example, a measles virus has been engineered to target certain tumours that express the tumour antigen carcinoembryonic antigen (CEA). The virus has been engineered to express a single-chain antibody that recognises expressed CEA. Another OV adenovirus that is currently being investigated in clinical trials has been engineered to target integrins that are highly expressed on the surface of ovarian cancer cells.

1.3.4.2 Utilising aberrant signal pathways in cancer for oncolytic virotherapy

Several molecular targets promote virus accumulation and replication within cancer cells. There is frequent overexpression of the B cell lymphoma (BCL) cell survival proteins. One of these proteins (BCL-XL) is targeted by Newcastle Disease virus as its expression prevents apoptosis, enabling viral replication and spread.

The RAS signalling pathway is another pathway that has been targeted in OV therapy (Figure 1.5). The RAS signalling pathway is involved in the regulation of several aspects of carcinogenesis including resistance to cell death and cell proliferation. This has been targeted by the OVs vaccinia virus and
reovirus as these have a natural selectivity for cancer cells with an over-active pathway \(^{78,79}\). In healthy, normal cells, there is activation of the PKR pathway following viral entry. Once activated this pathway inhibits protein translation and prevents the production of virus particles halting viral spread \(^{68}\). Cancer cells that have a RAS transformation do not activate the PKR pathway and this results in cell infection by OV and subsequent replication, leading to oncolysis ultimately \(^{68}\). Vaccinia virus depends on epidermal growth factor receptor (EGFR)-induced RAS signalling for viral replication. This virus encodes ligands that trigger EGFR signalling and in cancer cells that over-express EGFR there is increased viral infection through the EGFR-induced RAS signalling pathway \(^{78}\).

Cancer cells can also have defects in their antiviral defences through type 1 interferon signalling. Type 1 interferons are essential in antiviral responses in healthy cells through the promotion of host immune response against the virus and through the reduction of cellular proliferation and activation of the apoptotic protein p53. In many cancers this pathway is inactivated through the reduction of type 1 interferons or by limiting signalling via reduced receptor expression or through alteration of downstream signalling. Oncolytic viruses therefore have an increased specificity for cancer cells and in cells in which the type 1 interferon response is limited (as in normal healthy cells) there is viral clearance through interferon-mediated responses \(^{68}\).
Figure 1.5 Targeting of the Ras signalling pathway by oncolytic vaccinia virus. In cancer cells regulation of cell cycle and cellular proliferation are disrupted. This promotes viral replication and encourages oncolytic virus-induced cell death. Increase in the GTPase Ras blocks protein kinase R (PKR) and can facilitate oncolytic virus replication through the inhibition of apoptosis in Ras-mutant cancer cells. In the cell nucleus there is a resultant decrease in the cell cycle regulators p16 and retinoblastoma which renders cancer cells more susceptible to oncolytic vaccinia virus. Figure adapted from Kaufman et al.

1.3.4.3 Limiting the viral pathogenesis of oncolytic viruses

As oncolytic viruses are live particles they have the potential to be able to cause host toxicity and even latent disease and subsequent chronic infection. The potential pathogenicity is dependent on the virus, natural or engineered attenuation factors and the host immune response. Most oncolytic viruses utilise attenuated vectors or less virulent strains of virus to limit patient toxicity.

Talimogene leherperevec (T-VEC) is an example of an engineered attenuated virus. Herpes simplex virus is known to cause neurovirulence and latent infection. The viral gene product ICP34.5 mediates toxicity as it counteracts the type 1 interferon response and antagonises PKR signalling in non-dividing cells.
Vaccinia virus has also been attenuated to limit its activity to cancer cells. In non-attenuated vaccinia virus infections, the viral protein vaccinia growth factor (VGF) is secreted and acts on the host EGFR activating the RAS signalling pathway. This activation results in increased production of thymidine kinase through increased cell proliferation. Thymidine Kinase helps to promote viral replication. Deletion of VGF results in attenuation of vaccinia virus, permitting cell replication in cells with aberrant EGFR-RAS signalling such as rapidly proliferating cancer cells.

Vaccinia virus can also be attenuated through the deletion of the B18R protein that is responsible for blocking type 1 interferon signalling. This results in the susceptibility of normal healthy cells to the host type 1 interferon response with subsequent limitation of the viral infection. As cancer cells commonly disrupt the type 1 interferon pathway in order to evade the immune system they are susceptible to viral infection and oncolysis. B18R deletion may also increase vaccinia virus activity as type 1 interferons produced as a consequence of viral infection are not blocked by the B18R protein.

1.3.4.4 Stimulation of the immune system by oncolytic viruses

Stimulation of the immune system by OVs to clear tumour is a key aspect of the anti-cancer effects of oncolytic viruses. Several approaches have been utilised to stimulate this anti-tumour response in oncolytic virotherapy including the viral expression of pro-inflammatory cytokines or T-cell co-stimulatory molecules such as with the vaccinia virus JX-594 that expresses granulocyte macrophage colony stimulating factor (GMCSF), promoting robust T cell responses. An oncolytic adenovirus that expressed heat shock protein 70 (HSP70) has been used to increase antigen presentation. This is because HSP70 increases the delivery of proteins to proteases with a resultant increase in protein breakdown and processing. In the use of herpes simplex virus as an oncolytic virus such as with T-VEC there has been a deletion of the ICP47 protein. This protein acts to block the function of the transporter associated with antigen processing. The action of this protein results in a reduced presentation of infected cells to CD8 T cells.
1.3.4.5 Limiting viral clearance by the host immune system

Whilst activation of the immune system is an important aspect of creating a host immune response towards cancer cells, there is a balance at play as the same immune response can result in rapid clearance of virus from the host and a subsequent reduction in its efficacy. Through vaccination or naturally, we become exposed to oncolytic viruses and generate neutralising antibodies, thus generating cellular immunity against those that we have been exposed to.

One mechanism by which this can be overcome includes the utilisation of various serotypes of virus. It is possible to switch serotypes between administration for certain viruses such as adenovirus and vesicular stomatitis virus (VSV) which prevent the development of neutralising antibodies. Other ways that this can be overcome include covalent conjugation with polyethylene glycol of the viral coat and polymer coating to reduce binding of antibodies to viruses and subsequent neutralisation.

Another approach is to alter the host’s immune response towards oncolytic viruses. Pre-clinical studies have shown a reduced oncolytic viral neutralisation through the dampening of the host immune system with cyclophosphamide. Pre-treatment with cyclophosphamide prior to oncolytic virotherapy with measles virus and VSV resulted in a reduction of host circulating antiviral antibodies.

1.3.4.6 Improving oncolytic virus tumour infiltration

As well as trying to limit oncolytic virus neutralisation and elimination following administration strategies have been sought to optimise their distribution and bioavailability. In an attempt to improve viral penetrance proteolytic enzymes have been applied to tumours prior to treatment with oncolytic virus to break-down the extracellular matrix that poses a barrier to oncolytic virus treatment. Oncolytic viruses have also been engineered so that they express proteolytic enzymes such as hyaluronidase with studies showing an increased efficacy of virus expressing proteolytic enzyme compared with parental oncolytic virus alone.
1.3.5 Oncolytic viruses in clinical development

There are currently two oncolytic viruses that have been approved for clinical use. These include the FDA and NICE approved herpes simplex virus T-VEC, and the Chinese approved adenovirus H101. Several oncolytic viruses are discussed below before focusing more closely on the use of vaccinia virus as an oncolytic virotherapy.

1.3.5.1 Adenovirus

Adenovirus is a non-enveloped double-stranded DNA virus with a 35Kb genome. Due to its large genome it is able to tolerate multiple engineering modifications. Most of the population will have had exposure to the virus and will therefore be seropositive for virus exposure.

Adenovirus enter the cell using the coxsackie-adenovirus receptor before being trafficked to the cell nucleus where it expresses its early genes required for viral propagation. These early genes encode the proteins E1A and E1B that target the tumour suppressor gene p53 and retinoblastoma associated protein, promoting cell-cycle entry. In healthy cells targeting of these host cell cycle regulators results in abortive apoptosis and viral clearance. Treatment with adenoviruses have resulted in very few adverse events. Coupled with its safety profile, the relative ease with which it is possible to modify its genome without adversely affecting its infectivity have made them a good virus for development. Adenoviruses have been engineered to target cell surface receptors that are upregulated on cancer cells. A modification to the adenovirus Ad5/3-Δ24 enabled cell entry through binding of cell receptors αvβ3 and -5 as well as through the binding of the coxsackie-adenovirus receptor (CAR). This modification is beneficial in the treatment of ovarian cancer where the expression of the CAR receptor is variable and the expression of the receptors αvβ3 and -5 is high. Further adenovirus modifications include the combining of serotypes termed ‘mosaics’. CD46 is expressed on normal cells and prevents complement recognition and elimination. Cancer cells can also express this in order to prevent recognition and elimination by
complement. One adenovirus serotype, Ad35 gains cell entry through the binding of CD46. A mosaic adenovirus has been developed with the CAR-binding sequence of Ad5 replaced with the CD46 binding of Ad35, this modification enhances the cytocidal effect of the oncolytic adenovirus in cancers with a low expression of CAR.

The adenovirus H101 has a deletion in the portion of E1B that inactivates p53. This adenovirus has been approved for clinical use in the treatment of nasopharyngeal cancers in China. In a phase III clinical trial of patients with advanced squamous cell carcinoma of the head and neck or oesophagus, it was found that combination treatment with H101, cisplatin and 5-FU showed a higher rate of response compared with treatment by cisplatin and 5-FU alone. The trial also demonstrated a good safety profile of the virus with feverish symptoms, injection site reactions and flu-like symptoms being the main adverse reactions reported. No survival data was reported in this study.

Following publication of the results of this clinical trial the virus was approved for clinical use in China.

1.3.5.2 Herpes Simplex Virus

Another oncolytic virus that has been approved for clinical use is the herpes simplex virus, T-VEC. HSV-1 is a member of the alphaherpesvirus family. It is a double stranded DNA virus and has a larger genome than that of adenovirus at 152kb. HSV-1 replicates in cell nuclei however its replication does not result in mutations of the host cell DNA (insertional mutagenesis). Its large genome and lack of insertional mutagenesis make HSV-1 an excellent oncolytic virus candidate, it can however cause skin lesions in patients as well as causing infection of peripheral nerves and latent infection.

T-VEC contains deletions of the neurovirulence genes ICP34.5, which results in better cancer selectivity of the virus and reduced infection of neurons. It also has an ICP47 deletion which usually acts to inhibit antigen presentation. The deletion of ICP47 leads to the presentation of antigens by both normal cells and cancer cells, leading to immune-mediated killing of cancer cells whilst leading to containment of the infection in healthy tissues. The ICP47 deletion also causes early activation of US11 which blocks PKR phosphorylation and prevents cancer cells from undergoing abortive apoptosis.
once infected which increases the therapeutic activity of T-VEC\textsuperscript{98}. As well as these deletions, T-VEC has had GM-CSF inserted in place of the ICP34.5, improving the induction of anti-tumour immunity.

T-VEC has been evaluated in clinical trials for several types of cancer including melanoma, pancreatic cancer as well as head and neck cancers. T-VEC was assessed in phase II clinical trial on patients with unresectable melanoma. Patients were treated with intratumoral virus followed by a further injection 3 weeks later and 2weekly injections subsequent to this until a maximal clinical response or tolerated dose was reached or until disease progressed. This phase II study found an objective response rate with T-VEC treatment of 26\%. As well as this the virus was well tolerated with flu-like symptoms being the predominant side effect reported. A phase III prospective, randomised, clinical trial then followed in which 436 patients with advanced, unresectable melanoma were treated with intratumoral viral injection. The trial found that 16.3\% of patients treated with T-VEC responded to treatment with the virus within 12 months of commencing treatment and maintained a response for 6 months subsequent to that. This was in comparison to the other arm of the study in which patients were treated with GM-CSF alone. In this group of patients 2.1\% of participants achieved a durable response. This was found to be statistically significant \textsuperscript{67}. There was an objective response rate of 26.4\% in patients treated with T-VEC whilst a complete response rate of 10.9\% was also noted and there was also an increased median overall survival when compared to patients treated with GM-CSF alone \textsuperscript{67}.

Following the success of this clinical trial T-VEC was approved for use in the treatment of melanoma by the FDA in the United States and subsequently by NICE who have also approved the use of T-VEC for treating inoperable metastatic melanoma.

\textit{1.3.5.3 Vaccinia virus}

Vaccinia virus belongs to the poxviridae family. Poxviruses are large enveloped viruses with a linear double stranded DNA genome \textsuperscript{99}. Vaccinia has an approximate 190kb genome and is a large and complex virus of which there are many strains. The central genomic region is highly conserved amongst poxviruses; however, the terminal regions encode viral factors that modulate the immune
response or affect host ability to produce an antiviral response\textsuperscript{99}. Unlike many other viruses no specific host-cell receptor for virus binding and entry have been identified for vaccinia virus\textsuperscript{100}. It replicates entirely in the cell cytoplasm of infected cells and therefore the risk of viral integration into host chromosomes does not occur. With regards to the treatment of cancer, oncolytic vaccinia virus is a promising candidate and has been shown to replicate and lyse cancer cells within 72hrs of infection\textsuperscript{101}. It also has a broad tumour tropism and is stable in the bloodstream enabling treatment of distant metastases. It also spreads rapidly within tumours, whilst having a large transgene-encoding capacity\textsuperscript{78,101}. Vaccinia virus also has an established safety profile in humans given its use in the smallpox vaccination process\textsuperscript{102}.

There are four forms of vaccinia virus that are produced during the life-cycle of the virus. These include the intracellular mature virion (IMV), intracellular enveloped virion (IEV), cell-associated enveloped virion (CEV) and the extracellular enveloped virion (EEV)\textsuperscript{81}. Assembly of these virions takes place in cytoplasmic ‘factories’ and these involve the use of non-infectious precursors called crescents\textsuperscript{103}. The IMV consists of single lipid bilayer and is the most abundant form of vaccinia virus. Cells infected by vaccinia virus produce IMVs in the cell cytoplasm and these become wrapped virions following the acquisition of two extra membrane envelopes derived from the trans Golgi network or from the endosome. Microtubules then transport these to the cell surface where they fuse with the cell membrane, acquiring an additional membrane and becoming EEVs\textsuperscript{99}. These can transform into EEV as a result of exiting cytoplasmic crescents via microtubules and undergoing additional modifications including the addition of an extra membrane\textsuperscript{81}. The EEV differs in that it expresses fewer viral antigens on its surface whilst also incorporating host cell proteins, which enables it to evade the host immune response\textsuperscript{104}. This ability to limit its exposure to the host immune response facilitates its arrival at the target tumour\textsuperscript{104}.
1.3.5.3.1 Cell entry and replication of vaccinia virus

The mechanism of cell entry for vaccinia virus is not completely understood. A number of pathways and cell signalling factors have been shown to contribute to its entry into cancer cells. Cell entry of vaccinia virus can vary depending on the strain of vaccinia. Entry can occur through direct fusion with the plasma membrane or through endocytic routes.

1.3.5.3.2 Direct fusion to the plasma membrane

With regards to cell entry via direct fusion to the plasma membrane, 4 proteins are responsible for the attachment of the mature virion (MV). These proteins include H3, A27, and D8 that bind to glycosaminoglycans on the cell surface; and protein A26 that binds with the extracellular matrix protein laminin and mediates viral binding independent of glycosaminoglycans. Extracellular enveloped virion (EEV) binding moieties have not been identified. Once MV and EEV have attached to the plasma membrane an 11 protein entry fusion complex completes the fusion of vaccinia virus to the plasma membrane. As well as this EFC, it has been reported that tumour necrosis factor associated factor 2 (TRAF2) which is a cytoplasmic adapter protein, facilitates entry of vaccinia virus via direct fusion at the plasma membrane.

1.3.5.3.3 Endocytosis

Endocytosis appears to be the predominant mechanism for cell entry by vaccinia virus. The advantage of endocytosis in cell entry is that there is no evidence of virus presence on the host plasma membrane and this avoids an immune mediated reaction. The enclosure of activated viral proteins within endocytic vesicles also protect them from neutralisation by the host immune circulating antibodies. A low pH, commonly found within the tumour microenvironment has also been shown to facilitate endosomal mediated entry of vaccinia viruses. MVs and EEVs utilise micropinocytosis, an endocytic process dependent on actin that is mediated by tyrosine kinases, to gain entry into tumour cells. MVs utilise their ubiquitously expressed surface phosphatidylserine (PS) reported to target the MV
producing vaccinia virus for apoptosis. This results in MVs being mistaken for apoptotic debris and is taken up by tumour cells \(^{110}\). It is thought that PS enables viral entry due to the presence of EGF-like domains present within PS receptors. These domains are thought to interact with EGFRs present on the cell surface with subsequent induction of blebbing (detachment of a patch of cell membrane from the surface of the cell) \(^{110}\). The induction of blebbing leads to cytoskeletal rearrangements that lead to the accumulation of actin and this enables the blebs to retract further leading to the formation of out-pocketings. These go on to form the macropinosome. These move deeper into the cytoplasm and the pH lowers within becoming more acidic. This change in pH as well as other cues prepare the virus for its deposition into the host cytoplasm \(^{110}\).

1.3.5.3.4 Factors promoting vaccinia virus entry into cells

It has been shown that vaccinia utilises a pH dependent route to enter into cells. To confirm this Villa et al. utilised two drugs to raise the pH of the endosome through the inhibition of vacuolar-ATPase. This enzyme is responsible for generating the acidic pH in early endosomes. The study found that inhibition of vacuolar-ATPase resulted in compromised delivery from early to late endosomes \(^{105}\). The study also noted that when vaccinia virus was in an environment with a low pH it was able to partially overcome the inhibitory effects of these drugs, thus indicating that the pH surrounding the endosome plays a part in vaccinia virus entry \(^{105}\).

Hypoxia and low oxygen tension have also been shown to be factors that affect vaccinia virus cell entry. In the presence of vascular endothelial growth factor (VEGF-A), a pro-angiogenic growth factor that is induced by hypoxic conditions, expression in tumours was shown to increase viral internalisation into tumour cells through the Akt pathway \(^{111}\). Inhibition of the Akt pathway was shown to reduce viral cell entry however it did not completely reduce vaccinia virus internalisation, indicating that other pathways and mechanisms are likely to be involved also \(^{111}\).
1.3.5.4 Vaccinia virus oncolytic virotherapy

Vaccinia virus is the most well-studied of the poxvirus family. It exhibits a wide range of tropism in both normal and cancerous mammalian cells. In its use as an oncolytic virus, vaccinia has been attenuated to improve its selectivity for infection of cancer cells. Several versions are currently in use in clinical trials. Genetic modifications of vaccinia virus include thymidine kinase (TK) gene deletion. TK is an essential enzyme for pyrimidine synthesis, and its deletion ultimately results in selectivity of virus replication in cells with high nucleotide pools, such as rapidly dividing cancer cells\(^\text{112}\). In studies in which there has been a VACV double deletion (vvDD), VGF is also deleted, leading to further attenuation and dependence on the cell cycling status of target cancer cells. VGF is expressed early in VACV infection and is secreted as a ligand of EGFR to stimulate adjacent quiescent cells; it therefore impacts upon the spread of infection within normal tissues\(^\text{113}\). Despite this deletion causing a decreased pathogenicity of VACV, its ability to replicate within and destroy cancer cells is not affected\(^\text{114}\).

1.3.5.4.1 JX-594

The first VACV to be tested in phase I clinical trials was pexastimogene devacirepvec (Pexa-Vec, JX-594). Derived from the Wyeth vaccinia strain this oncolytic virus has a TK deletion as well as having the immunomodulatory gene encoding for GM-CSF and β-galactosidase, with the aim of stimulating the immunotherapeutic effects of the virus as well as stopping the intra-tumoral (IT) vascular supply\(^\text{66,115,116}\). In a phase I clinical trial JX-594 was given intratumorally to 14 patients with primary or metastatic liver tumours. This was an open-label, phase I dose-escalation trial with patients receiving one of four intratumoral doses of JX-594 (1x10^8 pfu, 3x10^8 pfu, 1x10^9 pfu and 3x10^9 pfu) every 3 weeks. All patients experienced grade I – III flu-like symptoms, four developed transient dose-related thrombocytopenia whilst two developed Grade III hyperbilirubinaemia at the highest dose. The maximum tolerated dose of JX-594 was therefore determined as 1x10^9 pfu. The trial also showed evidence of dissemination of virus in the blood through infection of distant tumour sites that were not
injected. 10 of the 14 patients underwent radiographic evaluation of their disease following injection with JX-594, and this showed that three had had a partial response to treatment, 6 had stable disease whilst one had progressive disease (according to response evaluation criteria in solid tumours (RECIST)) 66. Mastrangelo et al. also administered JX-594 intratumorally in the treatment of patients with incurable melanoma 117. Patients were given twice-weekly intratumoral injections of escalating dose from 1x10⁴ up to 2x10⁷ pfu/lesion for 6 weeks. Viral treatment was well tolerated with patients experiencing low-grade flu-like symptoms lasting less than 24 hours. Of the seven patients that were involved in the trial, two with the largest tumour burden did not respond to treatment with vaccinia virus. Three patients had a mixed response with treated tumours and some distant tumours regressing whilst other distant ones progressed. One patient had a partial response with regression of injected and uninjected dermal tumours. This patient went on to have disease resected and was subsequently rendered disease free. One patient developed a complete response to treatment with vaccinia virus 117. A phase II dose-ranging study was conducted to evaluate the safety and anti-tumour efficacy of JX-594 at high dose (1x10⁹ pfu) vs low dose (1x10⁹ pfu) in patients with hepatocellular carcinoma 118. Virus was administered intratumorally and was well tolerated at both doses. Patients received intratumoral JX-594 at three time-points: day 1, 15 and 29. 30 patients were enrolled and randomised to either arm of the study. Based on the modified response evaluation criteria in solid tumours (mRECIST) following magnetic resonance imaging four patients responded to treatment with one complete response and 3 partial responses, whilst 10 patients had stable disease. Responses to treatment were also seen in both injected and non-injected tumours. The overall survival was significantly longer in the high-dose arm compared with the low dose arm (14.1 months vs 6.7 months, HR: 0.39, P=0.02), whilst the overall survival was 9 months for the whole cohort 11. Another phase Ib clinical trial in patients with hepatocellular carcinoma who had failed sorafenib treatment was completed and did not reach its primary endpoint of prolonging overall survival in patients treated with JX-594 compared with best supportive care 119. Heo et al. conducted a pilot study exploring the safety and efficacy of JX-594 treatment followed by sorafenib in 3 patients with hepatocellular carcinoma. In all three patients
sequential treatment was well tolerated, resulted in reduced tumour perfusion and was associated with objective tumour responses on MRI scanning according to the Choi criteria. A phase III trial is currently underway comparing the treatment of hepatocellular carcinoma with Pexa-Vec and sorafenib to sorafenib treatment alone.

Breitbach et al conducted a phase I dose-escalation trial of a single intravenous infusion of JX-594 in 23 patients with other advanced solid tumours refractory to treatment. Tumours included lung, colorectal, melanoma, thyroid, gastric and mesothelioma. Patients were treated in one of six dose cohorts ranging from $1 \times 10^5$ to $3 \times 10^7$ pfu/kg. JX-594 delivery, gene expression and replication in solid tumours was assessed. Safety, pharmacokinetics and antitumour activity were evaluated. The treatment was well tolerated, and dose escalation proceeded without dose limiting toxicities. As with other trials assessing JX-594 the most common adverse effects were low grade flu-like symptoms lasting up to 24 hrs. Neutralising antibodies to vaccinia were detectable in 6 patients at baseline and developed in all patients treated at a high concentration of the virus by day 15. Despite this there was no correlation between neutralising antibody titres whether raised at baseline or induced, and JX-594 replication, safety or anti-tumour activity. Biopsies were assessed 8-10 days post infusion of JX-594. 87% of patients treated with a high dose of virus ($>1.5 \times 10^7$ pfu/kg) had biopsies that were positive for JX-594 on immunohistochemical and quantitative polymerase chain reaction (qPCR) analysis compared to those treated at lower doses with no detection of virus on biopsies.

Park et al conducted a phase Ib clinical trial with JX-594 in patients with colorectal cancer. In this clinical trial 15 patients with treatment refractory colorectal cancer were treated with two-weekly intravenous administration of JX-594. The purpose of this study was to establish the maximum tolerated dose following intravenous administration with doses of $1 \times 10^6$, $1 \times 10^7$ and $3 \times 10^7$ pfu/kg administered. In this trial no dose-limiting toxicity was reported, and the maximum tolerated dose was not reached. Common side effects of systemic virus administration were low grade flu-like symptoms which lasted less than 24 hours. Following intravenous administration plaque forming units
were detectable in the patient circulation up to 2 hours following the first cycle and up to 30 minutes following the 4th cycle of virus administration. Of the 15 patients treated in this trial ten had radiologically stable disease. 122.

1.3.5.4.2 Oncolytic vaccinia viruses in clinical trials

Other strains of VACV currently in clinical trials include GLV-1h68 and a double deleted VACV (vvDD). GLV-1h68 has been attenuated by the deletion of viral TK gene F14.5L, J2R and the haemagglutinin gene A56R. 123,124 Despite these deletions the virus remains highly tumour selective and has been shown to exhibit higher oncolytic efficacy than the parental Lister strain of virus and has been shown to cause efficient regression and in some cases, eradication of tumour in nude mice bearing several solid tumours including colorectal tumours. 123. It is believed that activation of innate immunity and infiltration of immune cells at tumour sites play a role in this effect.

The double-deleted VACV (vvDD) has both TK and VGF genes deleted, increasing replication selectivity in cancer cells. In pre-clinical work involving mice with paediatric solid tumours this virus inhibited tumour growth and prolonged survival. 125.

1.3.5.4.3 Oncolytic vaccinia virus in combination treatment

Most cancer therapies rely on the use of multiple agents in conjunction with one another. One such example is the use of the FOLFOX and FOLFIRI regimens that are used in the treatment of patients with colorectal cancer. These combine several chemotherapy agents such as leucovorin, 5-FU, oxaliplatin and irinotecan and have both been shown to improve 5-year survival of patients compared with 5-FU alone. 54.

Similarly, it has been demonstrated that there is a superior effect when oncolytic virotherapy is used in combination with traditional therapies. An example of this includes the treatment of patients with advanced hepatocellular carcinoma. JX-594 oncolytic vaccinia virus was combined with Sorafenib, which is a small-molecule multikinase inhibitor with antiproliferative and antiangiogenic effects. The
combination of these two agents was found to induce tumour necrosis in patients and limit disease progression, whilst use of the virus alone did not achieve such results. Based on these results a phase III trial is currently underway comparing combination treatment with sorafenib monotherapy.

A similar result from combination treatment was seen in patients with renal cancer. In a single patient with a prognosis of less than 6 months, repeated JX-594 administration followed by treatment with sunitinib (small-molecule VEGF inhibitor) resulted in complete tumour response with a prolonged disease free survival.

1.3.5.5 Vaccinia virus encoding a fusion suicide gene (FCU1)

For many years 5-FU has been the principle active agent to treat colorectal cancer and is still included in chemotherapy regimens for the treatment of colorectal liver metastases. 5-FU is able to exert a bystander effect through passive diffusion into and out of cells, resulting in a bystander effect. Its cytotoxicity is determined by its conversion into 5-fluorouridine triphosphate (5-FUTP) and 5-fluorodeoxyuridine monophosphate (5-FdUMP). 5-FUTP inhibits RNA synthesis whilst 5-FdUMP inhibits thymidylate synthase which is responsible for the conversion of deoxyuridine monophosphate into thymidine monophosphate which is a key molecule in DNA synthesis (Figure 1.6). 5-FU can also be directly incorporated into cell RNA, interfering with RNA transcription, and less often it can be incorporated into DNA, inhibiting replication.

Systemic toxicity of 5-FU lead to the development of new approaches aiming to concentrate it within tumour tissue. One such technique is suicide gene therapy that results in the intracellular conversion of non-toxic prodrugs into potent chemotoxins delivered by oncolytic viruses directly to the cancer cells. One example of this type of suicide gene therapy is the incorporation of the fusion suicide gene (FCU1). This suicide gene is derived from a fusion of the Saccharomyces Cerevisiae cytosine deaminase (CDase), an enzyme that is present in fungi and bacteria but absent from mammalian cells, that deaminates cytosine to uracil. It is also derived from the uracil phosphoribosyltransferase (UPRTase)
enzyme derived from Escherichia Coli which converts 5-FU into the toxic metabolite 5-fluorouridine monophosphate (5-FUMP). The fusion suicide gene has been shown to efficiently catalyse 5-FC into its toxic metabolites 5-FU and 5-FUMP. This strategy of suicide gene therapy also generates a bystander effect as the generation of 5-FU from infected cells is able to diffuse into non-infected cells, unlike some other prodrug systems that require gap junctions.

Figure 1.6 5-fluorouracil metabolism and effects
1.3.5.5.1 Pre-clinical use of fusion suicide gene using an adenovirus vector

Erbs et al first assessed the fusion suicide gene using a replication-deficient adenovirus to transduce tumour cells. The study found that in vitro the FCU1 gene maintained equivalent effects of UPRTase compared with the gene originating from Saccharomyces cerevisiae, whilst its CDase effects were higher compared to the original Escherichia Coli gene. Tumour cells that were transduced with adenovirus encoding the FCU1 gene were more sensitive to 5-FC, therefore requiring lower concentrations compared to adenovirus expressing the original CDase gene alone. The study also found an increase in the bystander effect in cells treated with adenovirus expressing FCU1 compared to virus expressing either the UPRTase gene or the CDase gene. In vivo testing also found that tumour growth was reduced following intra-tumoral injection of adenovirus expressing FCU1 followed by intravenous administration of 5-FC. There was a statistically significant suppression of tumour growth in BALB/c nude mice that were implanted with SW480 and LoVo colorectal cancer cell lines. 4 weeks following injection both SW480 and LoVo tumours were between 50 and 65% smaller compared with controls, however relatively large doses of 5-FC were required to achieve these results. The results of this study suggested that the use of an oncolytic virus to deliver FCU1 suicide gene therapy was potentially an effective candidate for cancer gene therapy strategies.

1.3.5.5.2 Delivery of FCU1 using non-replicative vaccinia virus

In an attempt to improve the efficacy of the FCU1/5-FC strategy Erbs et al. assessed a propagation-deficient vaccinia virus. In this study the Modified vaccinia virus Ankara (MVA) was used as a vector for gene therapy. This virus was chosen due its safety profile, it is also non-propagative in animal and mammalian cells but viral infection results in rapid replication of viral DNA and therefore amplification of the FCU1 transgene. This study demonstrated the ability of the MVA vector to transduce a variety of tumour cells including colorectal cell lines. During in vivo experiments, nude mice with colorectal tumours (LoVo) were treated with a single intratumoral injection of MVA-FCU1, given in combination with oral 5-FC administration. The conversion of 5-FC to 5-FU was evaluated. After a single dose of 5-
FC the highest levels of 5-FU in tumours were reached at 3-8 days following infection, whilst 5-FU was still present within tumour tissues up to 14 days afterwards. The level of 5-FU detected within treated tumours was equivalent to the level achieved with 5-FU administered systemically at 20-fold the maximum tolerated dose, furthermore the serum 5-FU level following treatment with MVA-FCU1 was undetectable, resulting in tumour killing without systemic toxicity. In comparison with his initial study Erbs found that the vaccinia virus vector was able to suppress tumour growth at relatively lower doses of 5-FC whereas no reduction in tumour growth was seen using the adenovirus vector 127.

1.3.5.5.3 VV-FCU1

Foloppe et al. first used a replication competent Copenhagen strain vaccinia virus expressing the transgene FCU1 in the pre-clinical setting (Figure 1.7) 130. Due to the efficient replication, cell lysis and spread of vaccinia virus as well as its broad host range and safety record in human use it was deemed to be an excellent candidate to use. The vaccinia virus was of the Copenhagen strain and was attenuated, having had a TK deletion. As a result, the virus was less pathogenic than wild-type vaccinia whilst preserving its ability to infect and replicate in cancer cells due to its dependence on host cell nucleotides. The study found VV-FCU1 in the presence of 5-FC mediated anti-tumour effects in vitro and in vivo in a murine model of a human colorectal cancer (nude mice with subcutaneous LoVo tumours). In vitro experiments found that there were increased levels of UPRTase and CDase levels at a multiplicity of infection (MOI) of 0.0001 in VV-FCU1 treated cells in comparison with those that had previously been treated with the MVA virus expressing FCU1 at an MOI of 0.01. The study found that the combination of VV-FCU1 and 5-FC resulted in greater toxicity to human cancer cell lines compared with virus alone, whilst the addition of 5-FC to cells treated with virus not encoding the FCU1 gene did not increase the toxicity to these cell lines. These results reinforced the theory that the enhanced killing by the combination of 5-FC and VV-FCU1 was due to the expression of FCU1 and its ability to convert 5-FC to 5-FU. In vivo experiments were also conducted in nude mice bearing subcutaneous LoVo colorectal tumours. VV-FCU1 was injected intravenously or intratumorally in these mice and they
received oral 5-FC at 7-days post virus administration for 3 weeks. Both routes of administration showed a significant reduction in tumour growth compared with controls. The same was also found in a model of liver metastasis. There were no adverse events noted following viral treatment *in vivo*, however some did develop cutaneous lesions that healed over 6 weeks\(^{130}\). This study demonstrated the potency of the Copenhagen strain VV-FCU1/ 5-FC system as a cancer gene therapy and provided further evidence to support the use of this in translational models.

**Figure 1.7 Schematic representation of VV-FCU1 by Foloppe et al\(^ {130}\). Thymidine kinase (TK) has been deleted and in its place of the new virus contains the fusion suicide gene (FCU1) under the control of the p11k7.5 promoter.**

### 1.4 Summary

Oncolytic viruses are a developing field of anti-cancer agents that have multi-modal action and recent advances have seen the licensing of two such viruses, an adenovirus and herpes simplex virus for clinical use in patients. Vaccinia virus is an oncolytic virus that lends itself to oncolytic virotherapy due to its broad tissue tropism, large transgene encoding capacity, its ability to spread in the blood as well as its excellent safety profile in human use.

The immune-mediated effects of oncolytic virotherapy are an important aspect of treatment as they prime the host to act against tumour cells. The benefit of this is that this enables the host immune system to recognise tumour associated antigens at other sites of disease and to create an immune response against these, preventing the development of metastases.
Vaccinia virus encoding the fusion suicide gene is a promising candidate for patients with colorectal cancer and liver metastases. The ability of the virus to convert non-toxic fluorocytosine into its active metabolite 5-fluorouracil lends itself to the treatment of these cancers as this is one of the predominant chemotherapy agents used in the treatment of patients with colorectal cancer that has metastasised to the liver.

1.5 Hypothesis

We hypothesise that VV-FCU1 is an effective oncolytic virotherapy that effectively kills colorectal cancer cells through its modes of action.

The objectives of this study were:

- To evaluate the effects of VV-FCU1 on immortalised colorectal cancer cell lines as well as in human tissue through the development of an organotypic culture model in our laboratory
- Assess the immunogenicity of vaccinia virus in the treatment of colorectal cancers through the assessment of mode of cell death and through the identification of markers of immune cell death
- To use a precision cut tumour slice model to investigate methods of enhancing oncolytic virotherapy in solid tumours
<table>
<thead>
<tr>
<th>Poxvirus</th>
<th>Study type</th>
<th>Tumour site</th>
<th>Administration</th>
<th>Phase</th>
<th>Status and outcomes</th>
<th>National clinical trial ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pexa-Vec (JX-594)</td>
<td>Clinical</td>
<td>Colorectal cancer</td>
<td>IV</td>
<td>1</td>
<td>Completed. No dose-limiting toxicities reached, maximum tolerated</td>
<td>NCT01469611</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>Colorectal cancer</td>
<td>IV</td>
<td>1</td>
<td>Completed. Repeat IV injections were well tolerated. No dose limiting toxicities noted</td>
<td>NCT01380600</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>Solid tumours</td>
<td>IV</td>
<td>1</td>
<td>Completed. JX-594 infects, replicates and expresses transgene products in dose-related fashion and is tumour selectiv</td>
<td>NCT00625456</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>Colorectal cancer</td>
<td>IV</td>
<td>1/2ab</td>
<td>Completed. Results not published</td>
<td>NCT01394939</td>
</tr>
<tr>
<td></td>
<td>Clinical</td>
<td>Colorectal cancer</td>
<td>IV</td>
<td>1b</td>
<td>Completed. Maximum tolerated dose not reached, multiple injections well tolerated</td>
<td>NCT01469611</td>
</tr>
<tr>
<td>GLV-1h68/GL-ONC1</td>
<td>Clinical</td>
<td>Solid tumours</td>
<td>IV</td>
<td>1</td>
<td>Completed. Well tolerated with minimal toxicity</td>
<td>NCT00794131</td>
</tr>
<tr>
<td>GLV-1h68</td>
<td>Pre-clinical</td>
<td>Colorectal</td>
<td>IV</td>
<td></td>
<td>Inhibited primary tumor growth and metastatic lesion formation; disrupted tumor vasculature</td>
<td>NA</td>
</tr>
<tr>
<td>VV-FCU1</td>
<td>Pre-clinical</td>
<td>Colorectal cancer</td>
<td>IV/IT</td>
<td></td>
<td>Significant reduction in tumour growth of colon tumours and liver metastases with both IV and IT administration compared with controls</td>
<td>NA</td>
</tr>
</tbody>
</table>

Table 1.3 A summary of clinical and pre-clinical trials using oncolytic vaccinia virus in the treatment of colorectal cancer
Chapter 2

Materials and methods
Chapter 2 – Materials and methods

2.1 Cell lines, treatment and media

2.1.1 Vaccinia Virus

Vaccinia viruses (Copenhagen strain) used in this study were supplied by Transgene SA, France. Details are listed in Table 2.1 below:

Table 2.1 Vaccinia viruses supplied by Transgene SA, France and storage conditions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>VVTK-I4L-/GFP</td>
<td>Transgene SA, France</td>
<td>Stored at -80°C in aliquots at 2.5x10^9 pfu/mL</td>
</tr>
<tr>
<td>VVTK-I4L-/FCU</td>
<td>Transgene SA, France</td>
<td>Stored at -80°C in aliquots at 2.3x10^8 pfu/mL</td>
</tr>
</tbody>
</table>

2.1.2 Cell culture media

All cell culture media used in this study are displayed in Table 2.2 along with their supplier and supplements. Working cell culture medium was prepared in the same way but supplemented with 10% fetal bovine serum (FBS).

Table 2.2 Cell culture media and additional supplements used in experiments

<table>
<thead>
<tr>
<th>Medium</th>
<th>Supplements</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-15 Medium (Liebowitz)</td>
<td>100 U/mL penicillin (Sigma, UK), 100µg/mL streptomycin (Sigma, UK), 2mM L-Glutamine (Sigma, UK) and 10% FBS (Life Technologies, UK)</td>
<td>Sigma (UK)</td>
</tr>
<tr>
<td>McCoys 5A Medium (modified)</td>
<td>100 U/mL penicillin (Sigma, UK), 100µg/mL streptomycin (Sigma, UK), 2mM L-Glutamine (Sigma, UK) and 10% FBS (Life Technologies, UK)</td>
<td>Thermofisher (UK)</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle's Medium (DMEM)</td>
<td>100 U/mL penicillin (Sigma, UK), 100µg/mL streptomycin (Sigma, UK), 2mM L-Glutamine (Sigma, UK) and 10% FBS (Life Technologies, UK)</td>
<td>Sigma (UK)</td>
</tr>
</tbody>
</table>
2.1.4 Cell lines

All cell lines were recently purchased from the American Type Culture Collection (ATCC, USA). No cell line validation was required. The cells were adherent lines that were maintained at 37°C in an atmosphere of 5% CO₂ within an incubator. All cell culture work was carried out under sterile conditions in a laminar flow biosafety cabinet class II (Kendro, UK). Tissue culture flasks and other plasticware was provided by Nunc. Mycoplasma testing was carried out regularly using the MycoAlert™ Mycoplasma Detection Kit (Lonza, UK).

Table 2.3 Cell lines used in tissue culture experiments

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell culture medium</th>
<th>Tissue Type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>McCoys (Modified) 5A Medium</td>
<td>Human colorectal adenocarcinoma</td>
<td>ATCC, USA</td>
</tr>
<tr>
<td>SW620</td>
<td>L-15 Medium</td>
<td>Human colorectal adenocarcinoma</td>
<td>ATCC, USA</td>
</tr>
<tr>
<td>HCT116</td>
<td>McCoys (Modified) 5A Medium</td>
<td>Human colorectal carcinoma</td>
<td>ATCC, USA</td>
</tr>
</tbody>
</table>

2.2 Tissue Culture

2.2.1 Passaging of adherent cells

Tissue culture flasks were removed from incubation conditions (37°C, 5% CO₂). Media was removed from each flask and the cell layer was washed with Hanks’ Balanced Salt Solution (Sigma, UK) before being discarded. Cells were then detached using Trypsin-EDTA (1x) solution (Sigma, UK) for 5-10 minutes in incubation conditions. Once detached cells were transferred into a universal tube and culture media was added to disperse cells. The universal tube was then centrifuged at 1500rpm for 3
minutes after which the supernatant was discarded, leaving a cell pellet. This was re-suspended in culture media and the cells were split. These were then added to a new flask containing fresh culture medium and placed in incubation conditions. To set up cells for an experiment, re-suspended cells were counted to enable seeding at an appropriate cell concentration.

2.2.2 Calculating cell number

A 1:10 dilution of cell suspension (10µL) in trypan blue (90µL) (Sigma, UK) was made and 10µL of the mixture was pipetted onto a haemocytometer. Trypan blue can only penetrate dead cells as it is membrane impermeable, therefore live cells remain unstained. Only live cells were counted in the four corners of the haemocytometer.

The following formula was used to determine the cell number:

\[
\text{Mean number of cells/quadrant} \times \text{dilution factor (10)} \times \text{chamber depth (10^4)}
\]

2.2.3 Cryopreservation of cells

Cells were harvested in culture medium and centrifuged at 1500rpm for 3 minutes. The supernatant was removed, and the cell pellet re-suspended in medium containing 50% cell culture medium, 40% FBS (Life Technologies, UK) and 10% dimethyl sulphoxide (DMSO) (Sigma, UK). The cell suspension was transferred to sterile cryovials and stored down at -80°C in a cryo-freezing container before being transferred to liquid nitrogen for storage.

2.2.4 Revitalisation of cryopreserved cell lines

Cryovials containing cells were removed from liquid nitrogen storage and thawed in a water bath for 30 seconds to 1 minute. Cell suspension was then transferred to a universal tube containing 10mL of warmed culture medium and was centrifuged at 1500rpm for 3 minutes. Supernatant was then discarded leaving behind a cell pellet. This was re-suspended in media and transferred in to a tissue culture flask before being placed in an incubator at 37°C, 5% CO₂.
2.3 Evaluation of cell line treatment with vaccinia virus

2.3.1 Calculating the amount of virus needed for specific multiplicity of infection (MOI)

MOI is the ratio of virus particles to the number of cells in a defined area. The number of virus particles that enter cells at a particular time is a statistical process as some cells will absorb varying amounts of virus particles or not absorb them at all.

To calculate the number of vaccinia virus needed, the following calculations were performed:

\[
\text{Number of cells/well} \times \text{required MOI} = \text{volume of virus required (pfu/well)}
\]

\[
\text{Volume of virus required (pfu/well)} \div \text{virus stock concentration} = \mu\text{L/well virus needed}
\]

2.3.2 Cell Titre 96® Aqueous non-radioactive cell proliferation (MTS) assay

The cell titre 96® aqueous non-radioactive cell proliferation (MTS) assay (Promega, UK) contains an MTS tetrazolium compound and an electron coupling reagent (PES). PES is chemically stable enabling its combination with MTS to form a stable solution. MTS is then bio-reduced by cellular oxidoreductase enzymes into a coloured formazan product, directly proportional to the number of living cells in culture.

100µL cells were seeded in a 96-well plate at the required seeding density and left in culture media for 24 hours at 37°C, 5% CO₂. This ensured that cells were 80% confluent in culture. After 24 hours supernatant was aspirated from each well and replaced with 100µL of diluted vaccinia virus in growth medium and left for a specified period of time in incubation conditions. Cell titre 96® aqueous one solution reagent (Promega, UK), diluted in RPMI media at a ratio of 1:10 was added to all wells in the 96-well plate. Plates were incubated for up to 1 hour before being placed in the Varioskan® Flash plate reader (Thermo Scientific, UK) and the Optical Density (OD) absorbance readings measured (wavelength 492 nm). Data were then analysed. The average OD of background control was subtracted.
from the average OD of samples from each treatment. The percentage of cell survival was then calculated relative to untreated cells:

\[
\% \text{ survival} = \frac{\text{av. OD cells treated with virus at specified MOI}}{\text{av. OD un-treated samples}} \times 100
\]

2.3.3 Immunogenic cell death (ICD) – determinants and markers

All cell lines were plated in 6-well plates at specified seeding densities and were incubated at 37°C 5% CO₂ overnight. Cells were then treated with either vaccinia virus, heat inactivated vaccinia virus or were left untreated. Mitoxantrone was used as a positive control for ICD. Treatment was left on cell lines for 24, 48 and 72 hours respectively. Supernatants were initially taken off from each cell line and reserved, these were then retained for HMGB1 ELISA (Sandwich-enzyme immunoassay, IBL international, Switzerland) (see later). Cells were washed with PBS (Fisher Scientific, UK), once removed PBS wash was retained in a falcon tube. Accutase (Thermofisher Scientific, UK) was used to dissociate adherent cells, removed and the wells were once again washed with PBS. Accutase, adherent cells and PBS wash was taken up and pipetted into the same falcon tube as the initial wash. Suspension was spun, re-suspended in PBS (Fisher Scientific, UK) and 100µL of cells were aliquoted into a 96-well plate with each well corresponding to a specific antibody per treatment for each of the three cell lines. Cells were incubated for one hour with primary antibodies (see Table 2.4 below). Cells were then washed, and secondary antibodies (Table 2.4) were added for a further 30 minutes to non-labelled primary antibodies. Cells were washed once again and then analysed by flow cytometry using a MACSQUANT Analyzer and MACSQuantify software (Miltenyi Biotec, UK).

Table 2.4 Primary and secondary antibodies used in the assessment of immunogenic cell death

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal antibody to calreticulin</td>
<td>Abcam, UK</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit monoclonal antibody to HSP70</td>
<td>Abcam, UK</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit polyclonal antibody to FAS</td>
<td>Abcam, UK</td>
<td>1:20</td>
</tr>
</tbody>
</table>
Table 2.5 Cell viability staining kit used in the assessment of immunogenic cell death

<table>
<thead>
<tr>
<th>Cell viability staining</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live/Dead fixable violet dead cell stain kit</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

2.3.4 High mobility group box 1 protein (HMGB1) – enzyme linked immunosorbent assay (ELISA)

ELISA was used for the quantitative determination of HMGB1 protein from supernatants collected and stored during initial experiments for assessment of ICD in cell lines. Supernatants and standards were added to the relevant wells of a 96-well plate coated with purified anti-HMGB1 antibody. The plate was incubated at 37°C 5% CO₂ overnight. Enzyme conjugate was then added for 2 hours followed by a wash. The plate was then incubated with a colour substrate solution for 30 minutes at room temperature before a stop solution was added. The optical density was then measured at 450nm using a Variaskan® flash plate reader (Thermo Scientific, UK) to determine HMGB1 concentration.

2.3.5 Phycoerythrin (PE) Annexin V apoptosis detection

Apoptosis is a normal physiological process which occurs in maintenance of tissue homeostasis. Apoptosis is characterized by certain morphologic features that include the loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and the nucleus, and internucleosomal cleavage of DNA. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is
translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36kDa calcium dependent phospholipid binding protein that has a high affinity for PS. Annexin V may be conjugated by fluorochromes, retaining its binding affinity to PS and enabling flow cytometric analysis of cells that are undergoing apoptosis. 7-amino-actinomycin D (7-AAD) is a dye which allows identification of early apoptotic cells. Cells with intact membranes are not permeable to the dye whereas dead and damaged cells are permeable to it. This allows the distinction to be made between cells in early apoptosis, late apoptosis and necrosis.

All three colorectal cancer cell lines were plated in 6-well plates and incubated overnight at 37°C 5% CO₂. Cells were treated with VV, heat inactivated VV, cisplatin (positive control) or were left untreated in media and left for 24, 48 and 72 hours respectively. At each time-point cells were dissociated using Accutase (Thermofisher Scientific, UK) and spun down to create a cell pellet. Supernatant was then discarded, and cells were re-suspended in Annexin V binding buffer (BD Biosciences, UK) and 100μL aliquots were pipetted into 96-well plates. 5μL of 7-AAD and PE Annexin V were added to each well before subsequently analysing them by flow cytometry using a MACSQUANT Analyzer and MACSQuantify software (Miltenyi Biotec, UK).

2.3.6 Autophagy detection

When subjected to certain conditions that threaten survival, eukaryotic cells employ a lysosome mediated pathway for digesting their own cellular contents. This process is known as autophagy. Various constituents of the cytoplasm such as organelles and proteins are sequestered into double-membraned autophagosomes. Autophagosomes subsequently fuse with lysosomes and their contents are degraded. Autophagy plays a variety of important roles including tumour suppression, cellular differentiation and regulation of innate and adaptive immunity.

Cyto-ID® (Enzo Life Sciences, UK) is a 488nm-excitable green fluorescent detection reagent that becomes brightly fluorescent in vesicles produced during autophagy enabling detection of autophagy in live cells using flow cytometry.
Rapamycin is a lipophilic macrolide antibiotic that is used as an inhibitor of the mammalian target of Rapamycin (mTOR) and is a well-established inducer of autophagy in a wide range of cells. mTOR is a serine/threonine kinase, important in the control of cell growth, proliferation and survival. mTOR inhibits autophagy by phosphorylating proteins encoded by autophagy-related genes (Atgs). Rapamycin forms a complex with the intracellular Rapamycin receptor, immunophilin FK506-binding protein 12 (FKBP12), which interacts specifically with the TOR proteins and inhibits signalling to downstream targets. Rapamycin induced autophagy is characterized by the accumulation of autophagic vacuoles and the stimulation of autophagic flux.

All HCT116, HT29 colorectal cancer cell lines and B16F10 mouse melanoma cells were plated in a 6-well plate and incubated overnight at 37°C 5% CO₂. Cells were treated with VV, rapamycin positive control or left untreated in media following 24 hours of incubation. After 72 hours' treatment cells were detached using Accutase (Thermofisher Scientific, UK) and centrifuged at 1000rpm for 5 minutes to create a pellet. Cells were then washed and resuspended in 1x Assay Buffer (Enzo Life Sciences, UK) and were collected by centrifugation once more. Cells were then resuspended in 250\(\mu\)L of 1x Assay Buffer (Enzo Life Sciences, UK) and 250\(\mu\)L of Cyto-ID® green (Enzo Life Sciences, UK) was added to each sample and mixed. Samples were incubated for 30 minutes at 37°C in the dark. After 30 minutes cells were collected by centrifugation and re-suspended in 500\(\mu\)L of 1x Assay Buffer (Enzo Life Sciences, UK) before being analysed by flow cytometry using a MACSQUANT Analyzer and MACSQuantify software (Miltenyi Biotec, UK).

2.3.6 Western Blotting for protein detection in cell lysates

2.3.6.1 Lysate preparation and protein separation using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Colorectal cancer cell lines treated with VV or untreated controls were washed with PBS (x2) (Fisher Scientific, UK) and then lysed using 250\(\mu\)L cold radioimmunoprecipitation (RIPA) buffer (Life Technologies, UK) and with Halt™ protease and phosphatase inhibitor cocktail mix 1:100 (Life
Technologies, UK). Following 5 minutes of gentle shaking on ice, cell lysates were sonicated for 15 seconds (x3). Sonicated lysates were then centrifuged at 13,000 rpm for 5 minutes at 4°C before transferring supernatants into fresh eppendorfs and storing down at -80°C.

Total protein in the lysate was determined using Pierce™ bicinochonic acid (BCA) Protein Assay Kit (Life Technologies, UK) by following the manufacturer’s protocol. 25µL samples or albumin containing standards were added in duplicate to a 96-well plate prior to the addition of 200µL working reagent for 30 minutes at 37°C. The reaction product was measured at 562nm using a Variaskan® flash plate reader (Thermo Scientific, UK). The protein concentration was then interpolated from the standard curve produced.

To ensure uniform protein loading, samples were diluted in RIPA buffer (Life Technologies, UK) to the required concentration. 31µL of diluted lysate, 4µL of NuPAGE® sample reducing agent (Life Technologies, UK) and 10µL NuPAGE® LDS loading buffer (Life Technologies, UK) was mixed before being placed on a heating block at 90°C for 5 minutes. The XCell Surelock™ Mini-Cell Electrophoresis apparatus (Life Technologies, UK) was assembled according to the manufacturer’s instructions. 40µL of lysate samples were loaded onto a NuPAGE® 4-12% Bis-Tris protein gel, 1.0mm, 10 well (Life Technologies, UK). 5µL of BLUeye pre-stained protein ladder (10-245kDa, Geneflow, UK) was also loaded onto the gel. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene which is constitutively expressed in cells, was used as a loading control to ensure equivalent protein loading of all samples. Samples were run in NuPAGE® 3-(N-morpholino) propansulfonic acid (MOPS) sodium dodecyl sulfate (SDS) running buffer (Life Technologies, UK) that was diluted 1:20 in water. Electrophoresis was carried out at 180V for 60 minutes using a PowerPac (Bio-Rad, UK).

2.3.6.2 Protein transfer, blocking, antibody probing and band detection

Proteins from the gel were transferred to a nitrocellulose membrane by electroblotting at 20 volts for 7 minutes using the iBlot® gel transfer device (Life Technologies, UK). The membrane blot was incubated for 1 hour at room temperature in blocking buffer (PBS 0.1% Tween 20) (Sigma, UK)
containing 5% milk powder, and left on a roller. Primary antibody (Table 2.6) was then added and left at 4°C overnight on a rotating plate. The membranes were then washed (x3) with PBS 0.1% Tween 20 (Sigma, UK) before the secondary antibodies (Table 2.7) were added for a further hour and left to incubate on a rotating plate at room temperature for 1 hour. Membranes underwent a further three washes with PBS 0.1% Tween 20 (Sigma, UK). The blot was then covered in SuperSignal® West Pico chemiluminescent substrate or SuperSignal® West Femto maximum sensitivity substrate (Life Technologies, UK) for up to 5 minutes. Following exposure to substrates the protein bands were visualised using the ChemiDoc-It² imager (UVP, UK). Direct comparison of the protein bands was confirmed by comparison to the BLUeye prestained protein ladder (Geneflow, UK). The blot was then returned to PBS 0.1% Tween 20.

Table 2.6 Primary antibodies used in western blotting

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Molecular weight (KDa)</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified mouse anti-RIP antibody</td>
<td>74</td>
<td>1 in 1000</td>
<td>BD biosciences, UK</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-RIP3 antibody</td>
<td>57</td>
<td>2.5 in 1000</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>Rabbit polyclonal PARP antibody</td>
<td>116 and 89</td>
<td>1 in 1000</td>
<td>Cell Signalling, UK</td>
</tr>
<tr>
<td>Mouse monoclonal anti-GAPDH antibody</td>
<td>35.9</td>
<td>1 in 2000</td>
<td>Origene, USA</td>
</tr>
<tr>
<td>RIP and PARP controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jurkat apoptosis cell extracts (untreated or treated with etoposide)</td>
<td>116 and 89</td>
<td>N/A</td>
<td>Cell signalling, UK</td>
</tr>
<tr>
<td>NIH 3T3 cell lysate</td>
<td>74</td>
<td>N/A</td>
<td>ATCC, UK</td>
</tr>
</tbody>
</table>

Table 2.7 Secondary antibodies used in western blotting

<table>
<thead>
<tr>
<th>Secondary antibodies</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse IgG</td>
<td>1 in 1000</td>
<td>Origene, USA</td>
</tr>
<tr>
<td>Donkey anti-rabbit IgG</td>
<td>1 in 5000</td>
<td>Fitzgerald, UK</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG</td>
<td>1 in 2000</td>
<td>Cell Signalling, USA</td>
</tr>
</tbody>
</table>
2.3.6.3 Membrane stripping and antibody re-probing

15mL of Restore™ PLUS stripping buffer (Fisher Scientific, UK) was added to the blot and left on a rocker for 10 minutes before washing (x3) in PBS 0.1% Tween 20 for 5 minutes. They were then blocked using non-fat milk 5% with TBS 0.1% tween-20. Membranes were incubated overnight at 4°C with purified mouse GAPDH 2D9 monoclonal antibody (Insight Biotechnology, UK) to detect GAPDH. The membranes were then washed again 3 times for 5 minutes using TBS 0.1% tween and incubated for an hour with goat anti-mouse IgG Horseradish Peroxidase (HRP) (Insight Biotechnology, UK). Membranes were then washed, and bands were detected as previously described.

2.4 Organotypic culture

2.4.1 Tissue collection and preparation

Ethical approval was obtained for the ‘Evaluation of the effects of treatment on cancer tissue’ 12/LO/1661. Written consent was obtained from patients undergoing hepatic resection at the Royal Surrey County Hospital, Guildford.

Tumour samples were collected at the time of surgical resection from the Royal Surrey County Hospital, Guildford. At the time of surgical resection 8mm punch biopsies (Kai Medical Europe, Germany) were taken from tumours and transported in a sterile container on ice to the laboratory. To enable slicing of tumour cores, a 4% agarose (Sigma, UK) solution was made in advance and stored at 4°C. At the time of receipt of tumour this was heated in a microwave until liquid and then placed into a water bath at 42°C. Tumour cores were embedded in agarose within a 6-well plate and allowed to set on ice for 10-20 minutes. Once set, the agar was cut into the shape of a cube containing the tumour core. This was removed from the 6-well plate and fixed onto a metallic support block using an ethyl cyanoacrylate-based glue (Carl Roth, Germany). This was allowed to set on ice for 5-10 minutes.
Whilst the glue was setting the Leica VT1200 s vibrating microtome (Leica Microsystems Ltd, UK) was calibrated and set up to move at a speed of 0.4mm/s, oscillation 3.0mm and slice thickness 300µM. The decision to slice the tissue at 300µm intervals was based on existing literature regarding the use of organotypic cultures as well as our experience in cutting tumours. Whilst thinner slices can be procured we found that the cutting of tissue cores at this thickness was less consistent. The metallic support block and embedded tissue were then inserted into a purpose built media tray within an ice tray with DMEM (supplemented with 10% FBS and 100 U/mL penicillin (Sigma, UK), 100µg/mL streptomycin (Sigma, UK)) within, submerging the embedded tissue. Having already been calibrated consecutive 300µm slices were produced until the tumour was completely sliced or until the agar mould gave way and the tissue was no longer fixed in place (Figure 2.1).

Slices were then transferred onto Millipore® Millicell® cell culture inserts (Sigma, UK) within a 6-well plate. 1mL of either media alone or containing virus at desired MOI was added to the bottom of these wells with an additional 250-500µL being placed within the insert containing the tissue slice. Once treated the tissue was placed inside an incubator at 37°C, 5% CO₂ and placed onto a rotating tissue slice incubation unit (Alabama Research & Development, USA).

After a defined period, tissue slices were removed from culture conditions and fixed in 10% formalin (Sigma, UK). They were then transferred to the University of Surrey, vet school pathology lab and were processed in an automated Sakura Tissue-Tek VIP6 (Sakura Finetek Europe B.V., Netherlands) tissue processing machine and left overnight. Following processing tissue slices were placed in a Sakura Tissue-Tek TEC (Sakura Finetek Europe B.V., Netherlands) paraffin embedding machine and were manually embedded within cassettes.
A plaque-forming unit (pfu) is a measure of the number of virus particles capable of forming plaques per unit volume. It is a functional measurement rather than a measurement of the absolute quantity of particles: viral particles that are defective or which fail to infect their target cell will not produce a plaque and thus will not be counted.

To calculate the number of virus particles required the following calculations were performed:

\[
\text{Desired virus concentration/Stock concentration of virus} \times 1000\mu L = \text{volume of virus particles required per ml}
\]

\[
\text{Vol. of treatment per tissue slice} = 1.5\text{ml/slice}
\]

\[
\text{Vol. virus required/ml} \times 1.5\text{ml/slice} = \text{pfu required/experiment}
\]
Visualisation of virus presence, evidence of apoptosis and cell proliferation was assessed using IHC. Firstly, paraffin embedded tissue slices were deparaffinised in three 5-minute washes in 100% xylene (Sigma, UK) for 5 minutes. They were then washed in 100% ethanol (Fisher Scientific, UK) twice. Following washes in ethanol the tissue slices were placed in 0.3% methanol/hydrogen peroxide (Sigma, UK) for 20 minutes, blocking endogenous peroxidases. Tissue slices were then rehydrated in 70% and 50% ethanol (Fisher Scientific, UK) before being rinsed in distilled water for 5 minutes. Antigen retrieval was performed to break methylene bridges formed during tissue fixation, exposing antigenic sites for antibody binding. This was done by placing tissue into boiling 0.01M citrate buffer pH6.0 for 12 minutes. Slices were subsequently left to cool down for 1 hour. Slides were washed in distilled water for 3 minutes followed by two 3-minute washes in phosphate buffered saline (PBS) (Fisher Scientific, UK).

Tissue slices were blocked by the addition of 2.4% horse serum in 1% PBS/BSA (Vector Laboratories, UK) in a moist chamber for 15 minutes. This was tapped off and Avidin D (Vector Laboratories, UK) was added to tissue slices and left for 15 minutes before the slices were washed three times in PBS (Fisher Scientific, UK). Biotin (Vector Laboratories, UK) was then added to the tissue slices and left for 15 minutes before being tapped off. The desired primary antibody was then applied to each of the tissue slices (100µL/slice) and the slices were left overnight at room temperature in a moist chamber. The dilutions at which antibodies were used was determined through previous optimisations in the laboratory. Optimisation was performed for the following newly acquired antibodies: anti-GFP, anti-VV as well as anti-HMGB1 antibodies. Three further washes with PBS (Fisher Scientific, UK) were performed before the addition of Universal secondary antibody (Vector Laboratories, UK) for 30 minutes to rabbit monoclonal or polyclonal antibodies. These slices were then washed with PBS (Fisher Scientific, UK) before adding Vectastain R.T.U ABC reagent (Vector Laboratories, UK). This was left for 30 minutes before slices were once again washed with PBS (Fisher Scientific, UK). For slices to
which a goat polyclonal primary antibody was added these were washed three times in PBS before adding ImPRESS™anti-goat HRP reagent (Vector laboratories, UK) and leaving for 30 minutes before washing with PBS.

DAB peroxidase substrate solution (Vector Laboratories, UK) was added to each of the sections for 3-10 minutes. Sections were then rinsed in distilled water before being counterstained by Haematoxylin QS (Vector Laboratories, UK) for up to 45 seconds. They were then immediately rinsed and placed in running water for 5 minutes.

Tissue was then dehydrated in a series of 50%, 70%, 100% ethanol (Fisher Scientific, UK) and in three changes of 100% xylene (Sigma, UK). Slices were left to dry completely before being mounted with Vector mounting media (Vector Laboratories, UK) and a glass coverslip (VWR International). Slides were left to set until dry before being assessed under a light microscope.

Table 2.8 Primary antibodies used for immunohistochemical staining

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Description</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Ki67 (ab16667)</td>
<td>Rabbit monoclonal</td>
<td>1:100</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>Cleaved Caspase 3 (Asp175)</td>
<td>Rabbit monoclonal</td>
<td>1:50</td>
<td>Cell signalling Technology®, USA</td>
</tr>
<tr>
<td>Anti-green fluorescent protein (ab6673)</td>
<td>Goat polyclonal</td>
<td>1:2000</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>Anti-Vaccinia virus (ab35219)</td>
<td>Rabbit polyclonal</td>
<td>1:6000</td>
<td>Abcam, UK</td>
</tr>
<tr>
<td>Anti-HMGB1 antibody (ab79823)</td>
<td>Rabbit monoclonal</td>
<td>1:400</td>
<td>Abcam, UK</td>
</tr>
</tbody>
</table>

2.4.4 Quantification of 3,3'-Diaminobenzidine (DAB) immunohistochemical staining

Evaluation of immunohistochemical staining was performed using the open resource digital image analysis software, ImageJ. The IHC profiler application developed by Varghese et al 131 enables automated digital IHC analysis for unbiased, quantitative assessment of antibody staining intensity in tissue sections. To create this programme the authors adopted the spectral deconvolution method of DAB/haematoxylin colour spectra by using optimised optical density vectors of the colour.
deconvolution plugin for separation of the DAB colour spectra. The plugin has been assessed on thousands of scored DAB stained IHC images. A comparison study between use of the plugin and manual analysis resulted in an 88.6% match with a statistical significance of P<0.0001.

IHC staining was performed as outlined in materials and methods. Using the Zeiss Primo Star microscope digital images were taken at three random areas of each DAB stained tissue slice and analysed using the IHC profiler application for Image J. The percentage of highly positive DAB staining for each digital image was calculated using the software and then an average reading was taken per tissue slice.

2.5 Evaluation of VACV in organotypic culture

2.5.1 Detection of virus presence in 300µm tissue slices by confocal microscopy

Following treatment of tissue with virus containing GFP, slices were fixed in 4% formalin solution (Sigma, UK) and left overnight. The following day tissue slices were washed with two washes in PBS (Fisher Scientific, UK) for 5 minutes each. Tissue was then blocked with Tris-buffered saline (Sigma, UK) with 0.2% Tween-20 (Sigma, UK) and 1% FBS (Life Technologies, UK) for 15 minutes. TOPRO®-3 nuclear stain (Life Technologies, UK), diluted 1:400 in 1% PBS/BSA, was added and tissue was left for 30 minutes. Tissue slices were then mounted onto Dako slides (Dako, UK) and Vectashield hard set mounting medium (Vector laboratories, UK) was added and a cover slip placed on top. Tissue was then taken to the confocal microscope (Nikon A1M confocal laser scanning microscope for assessment.

2.5.2 Detection of virus presence in paraffin embedded 4µm tissue slices by confocal microscopy

Paraffin embedded tissue slices, treated with virus containing GFP, were deparaffinised in three 5-minute washes in 100% xylene (Sigma, UK) for 5 minutes. They were then washed in 100% ethanol (Fisher Scientific, UK) twice. Following washes in ethanol the tissue slices were placed in 0.3% methanol/hydrogen peroxide (Sigma, UK) for 20 minutes, blocking endogenous peroxidases. Tissue
slices were then rehydrated in 70% and 50% ethanol (Fisher Scientific, UK) before being rinsed in distilled water for 5 minutes.

Slides were then air dried before the addition of TOPRO®-3 nuclear stain (Life Technologies, UK), diluted 1:400 in 1% PBS/BSA. This was left for 30 minutes. Following this, Vectashield hard set mounting medium (Vector laboratories, UK) was added and a cover slip placed on top. Tissue was then taken to the confocal microscope (Nikon A1M) for assessment.

2.5.3 Enzymatic assays

FCU1 activity was assessed in treated organotypic cultures through the measurement of 5-FC and 5-FU as substrates. Organotypic cultures were left untreated, treated with media 100µM 5-FC (Abcam, UK) treated with virus alone or treated with virus and 100µM 5-FC. 5-FC was added at 48 hours following infection with VV-FCU1. At 24-hour time intervals 50µL supernatant was taken and frozen up to 96 hours following treatment. The samples were then transported on ice to Transgene laboratories in France where high performance liquid chromatography (HPLC) was performed. After thawing the frozen supernatant samples 5-FC and 5-FU were separated isocratically using HPLC (Hewlett Packard HP 1100 liquid chromatograph with UV detection at 260nm and 280nm). A Supelco supelcosil LC-18-S (5µm packing; 4.6 x 250mm) column and a guard cartridge (10 x 3 mm; Varian, Les Ulis, France) with a flow rate of 1ml min⁻¹. The mobile phase was 20mM KH₂PO₄, 5mM tetrabutylammoniumsulfate, 5% methanol adjusted to pH5 with potassium hydroxide.

2.5.4 Focused ultrasound delivery and cavitation detection

Ultrasonic exposures were carried out using the System for Acoustic Transfection (SAT) chamber. This system was based on prior design but modified to allow a decrease in the exposure area for the prepared tumour slices. Tumour slices and treatment agents were contained in a sonolid assembly that consisted of a 35mm cell culture dish (µ-dish) (Ibidi, Germany) fitted to a polydimethylsiloxane (PDMS) lid (Dow Corning, USA). Details of the construction and assembly are the same as in Carugo et
al. except that for this experiment a 12mm Millipore® Millicell® cell culture insert (Sigma, UK) was inserted between the cell culture dish and the sonolid. The sonolid was sealed over the cell culture dish with Millipore® Millicell® cell culture insert (Sigma, UK) within it ensuring that no air pockets were within the assembly (Figure 2.2).

The sonolid was held in the SAT by a circular bracket in the pre-focal region of 40mm radius, 120mm radius of curvature, 1.1MHz center frequency ultrasound transducer (Sonic Concepts, USA), such that the incident pressure field was focussed on the Millipore® Millicell® cell culture insert. The transducer drive signal path consisted of a waveform generator (Agilent Technologies, UK), low-pass filter (Mini-Circuits, UK) and power amplifier (E&I Ltd, USA).

When the cell culture plate inserts, culture dishes and sonolids were in the ultrasound chamber they were exposed to 1.1MHz, 150mV, 40 cycles at 10ms pulse duration for 20 seconds in total. These conditions were kept constant throughout the experiment.

Figure 2.2 Schematic representation of instrument set up and organotypic culture position for exposure to cavitational ultrasound. Set up based on previous published work by Carugo et al. 132. Following preparation of organotypic cultures these were then placed inside a 12mm Millipore® Millicell® cell culture insert fixed within a cell culture dish. The cell culture insert was then submerged in the desired treatment with or without 50µL SonoVue microbubbles. This was sealed with a Sonolid, ensuring that there were no bubbles within the set up. This was then placed into supporting apparatus and submerged in the SAT chamber above the ultrasound transducer and exposed to ultrasound frequency as described.
2.5.5 Experimental protocol

Prepared tumour slices were placed within 12mm Millipore® Millicell® cell culture inserts (Sigma, UK) within a cell culture disc. The cell culture insert containing the tissue slice was filled with 850µL of the desired treatment:

1. DMEM alone
2. DMEM plus 50µL SonoVue microbubbles (Bracco Imaging, France)
3. Vaccinia Virus (Transgene, France) in media plus 50µL SonoVue microbubbles (Bracco Imaging, France)

Tissue slices were completely submerged within the cell culture insert. The sonolid was attached as described and the dish was then taken for ultrasound application in the SAT.

Following treatment exposure, tissue slices were removed from the SAT apparatus and transferred onto Millipore® Millicell® cell culture inserts (Sigma, UK) within a 6-well plate. 1mL 10% FBS DMEM media was added to the bottom of these wells with an additional 250-500µL being placed within the insert containing the tissue slice. The tissue was then placed inside an incubator at 37°C, 5% CO₂ and placed onto a rotating tissue slice incubation unit (Alabama Research & Development, USA).

2.6 Statistical Analysis

All statistical calculations were performed using GraphPad Prism Version 7.04 software. Two-way analysis of variance (ANOVA) with Bonferroni correction was performed for multiple comparisons. In the assessment of immunogenic cell death two-way ANOVA was also performed for the comparison of multiple means with the Tukey post-hoc test or the Bonferroni correction. Results are shown in graphs with error bars shown. For all tests, a 5% significance level was used. Therefore, the following symbols were used in figures to show the level of significance: *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001.
Chapter 3

Evaluation of the effects of oncolytic vaccinia virus in colorectal cancer cell lines
Chapter 3 – Evaluation of the effects of oncolytic vaccinia virus in colorectal cancer cell lines

3.1 Introduction

Colorectal cancer (CRC) is the fourth most common cancer in the UK. At the time of diagnosis approximately 20% of patients will present with distant metastatic disease \(^{48}\). Only 10-20% of patients with metastatic colorectal liver metastases are candidates for surgical resection, with the vast majority presenting with liver metastases that are not amenable to resection \(^{14}\). This group of patients will receive systemic chemotherapy, either given with palliative intent or as a neo-adjuvant treatment with the aim of downsizing tumour burden and converting patients to operable disease. Fluorouracil is the mainstay of chemotherapy treatment and has been shown to reduce recurrence rates by 17% and to improve overall survival by 13-15% \(^{51}\). A novel approach to the treatment of metastatic colorectal cancer is with oncolytic virotherapy. This utilises viruses that have the ability to replicate within tumour cells naturally or are modified to selectively replicate within tumour cells. It has long been recognised that viruses have the ability to kill cancer cells and recently clinical trials have shown evidence of a therapeutic benefit in patients with multiple tumour types \(^{65-67}\). Following oncolysis, tumour cells release tumour associated antigens. These are then able to promote an adaptive immune response that mediates an anti-tumour effect at distant tumour sites. Following oncolytic cell death tumour cells also release viral PAMPs as well as danger associated molecular patterns (DAMPs) such as heat shock proteins (HSPs), high mobility group box 1 proteins (HMGB1), calreticulin, adenosine triphosphate (ATP), and uric acid. They also release cytokines that promote the maturation of antigen presenting cells such as dendritic cells. Release of tumour associated antigens in combination with cytokines and DAMPs can be beneficial in the generation of an immune response against cancer cells. This is particularly important in the generation of an anti-cancer immune response to tumours that are distant to the site of virus administration \(^{68}\).
Vaccinia virus is one such form of oncolytic virus. It belongs to the Poxviridae family and is the most well studied virus in this family having been utilised as a vaccine during the eradication of smallpox. With regards to the treatment of cancer, oncolytic vaccinia virus is a promising candidate and has been shown to replicate and lyse cancer cells within 72hrs of infection. It exhibits broad tumour tropism and is stable in the bloodstream enabling treatment of distant metastases. It also spreads rapidly within tumours, whilst having a large transgene-encoding capacity. Vaccinia virus has an excellent safety profile in humans given its use in the smallpox vaccination process. In its use as an oncolytic virus, vaccinia has been attenuated to improve its selectivity for infection of cancer cells. Several versions are currently in use in clinical trials, the most prominent of which is JX-594. Most cancer therapies rely on the use of multiple agents in conjunction with one another. Similarly, it has been demonstrated that there is a superior effect when oncolytic virotherapy is used in combination with traditional therapies. For many years 5-FU has been the principle active agent to treat colorectal cancer and is still included in chemotherapy regimens for the treatment of colorectal liver metastases. Due to the systemic toxicity of 5-FU experienced by patients new approaches have been made aiming to concentrate it within tumour tissue, therefore limiting these side effects. One such technique is suicide gene therapy. This technique converts a non-toxic pro-drug into potent chemotoxins delivered by vaccinia viruses directly to the cancer cells. One example of this type of suicide gene therapy is the incorporation of the fusion suicide gene (FCU1). Vaccinia virus encoding the fusion suicide gene has been shown to be a promising candidate for patients with colorectal cancer and liver metastases in in vitro and in vivo studies.
3.1.1 Aims

The aim of this chapter was to determine if colorectal cancer cell lines were susceptible to the oncolytic vaccinia viruses VVTK-I4L-/GFP and VVTK-I4L-/FCU. Furthermore, we wanted to assess the mode of cell death if susceptibility to treatment was demonstrated to assess if cell death was immunogenic.

In order to assess this the following experiments were performed:

1. Infection of 3 representative colorectal cancer cell lines with vaccinia virus and assessment of oncolysis by Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay (MTS assay) to validate previous findings

2. To determine the primary mode of cell death induced by oncolytic vaccinia virus by phycoerythrin (PE) Annexin V apoptosis detection and western blotting

3. To determine if there was evidence of immunogenic cell death through the detection of determinants and markers of immunogenic cell death using flow cytometry and by enzyme linked immunosorbent assay (ELISA)
3.2 Results

3.2.1 Susceptibility of colorectal cancer cell lines to Vaccinia Virus

The first step in this experiment was to confirm the susceptibility of colorectal cancer cell lines to vaccinia virus. Three standard colorectal cancer cell lines were selected to assess this, these were HT29, HCT116 and SW620 colorectal cell lines. To assess the susceptibility of each of these, cells were plated on a 96-well plate and were then treated with serial dilutions of vaccinia virus at multiplicity of infection (MOI) ranging from 1x10^{-6} up to an MOI of 1 for up to 72 hours (Figure 3.1). Cells lines were treated with media alone as a comparative negative control. Cell survival was then analysed using an MTS assay as described in methods.

The response to vaccinia virus infection varied in this panel of colorectal cell lines. MTS assay (see methods) was used to assess for a reduction in cell proliferation. Both the HCT116 and SW620 cell lines demonstrated a reduction in cell proliferation using this assay following treatment with vaccinia virus. At the highest MOI in these experiments, less than 25% of cells remained viable after 72 hours of incubation with virus. Using the MTS cell proliferation assay, the HT29 cell line did not demonstrate any reduction in cell proliferation following 72 hours of treatment. At the maximum MOI of 1, more than 90% of these cells treated with vaccinia virus appeared to remain viable according to the results of the assay (Figure 3.1).
3.2.2 Assessment for presence of vaccinia virus through expression of green fluorescent protein in cell lines using fluorescent microscopy

As one form of the vaccinia virus used in our experiments encoded green fluorescent protein (GFP) this enabled us to assess for viral presence through its expression using simple fluorescent microscopy in the first instance.
We plated each of the 3 cell lines in 6-well plates and treated them with serial dilutions of vaccinia virus and these were incubated for up to 72 hours. At each 24-hour interval the cell lines were assessed using a fluorescent microscope to assess for expression of GFP as a marker of vaccinia virus presence on the plate. Over 72 hours each cell line was found to have increasing expression of GFP on fluorescent microscopy suggesting proliferation of vaccinia virus (figure 3.2).
HCT116 treated with vaccinia virus expressing GFP at 72 hours

MOI 0  MOI 0.000001  MOI 0.0001  MOI 0.001

MOI 0.001  MOI 0.01  MOI 0.1  MOI 1

HT29 treated with vaccinia virus expressing GFP at 24 hours

MOI 0  MOI 0.000001  MOI 0.00001  MOI 0.001

MOI 0.001  MOI 0.01  MOI 0.1  MOI 0.1
Having confirmed virus presence in tissue culture experiments through the expression of GFP using immunofluorescence, it was unclear as to why these MTS results were seen in the HT29 colorectal cancer cell line. The immunofluorescent images obtained also demonstrated an apparent increased expression of GFP over time as with the other cell lines, suggestive of viral replication in this cell line (Figure 3.2). As there was evidence of increasing GFP expression in the HT29 colorectal cancer cell line under direct visualisation, but there was no evidence of reduced cell proliferation with MTS assay we wanted to further assess this cell line for evidence of any vaccinia virus effects in the treated cell line prior to progressing with further experiments. To do this we utilised PE annexin V to assess for evidence of cell death. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36kDa calcium dependent phospholipid binding protein that has a high affinity for PS. Annexin V may be conjugated by fluorochromes, retaining its
binding affinity to PS and enabling flow cytometric analysis of cells that are undergoing apoptosis. 7-amino-atinomycin (7-AAD) is a dye which allows identification of early apoptotic cells. Cells with intact membranes are not permeable to the dye whereas dead and damaged cells are permeable to it. This allows the distinction to be made between cells in early apoptosis, late apoptosis and necrosis and would give us the information required to assess for an effect of vaccinia virus on the HT29 cell line. HT29 cells were treated in the same way as with previous experiments with the same range of MOI (1x10^6 to 1) of vaccinia virus. The results of this experiment demonstrated an increase in cell death of this colorectal cancer cell line when treated with oncolytic vaccinia virus (Figure 3.3). At the maximum MOI of 1, less than 20% of cells were alive after 48 hours. This finding therefore confirmed that all three colorectal cancer cell lines intended for use in further experiments were susceptible to treatment with oncolytic vaccinia virus.

**Figure 3.3 Evidence of cell death in HT29 cells following treatment with vaccinia virus.** PE annexin V assay assessing cell survival in HT29 colorectal cancer cells treated with oncolytic vaccinia virus at 72 hours with varying MOIs.
3.2.3 Control experiments

To confirm that these were accurate results secondary to vaccinia virus infection control experiments were performed on all three cell lines. Vaccinia virus expressing GFP was first heat inactivated at 70°C for 10 minutes based on preliminary experiments (Figure 3.4)

![Image of SW620 cell lines treated with vaccinia virus expressing GFP at an MOI of 1, that had been heat inactivated at varying temperatures for varying times. The lowest temperature and duration of heating were chosen for further experiments. We therefore heat inactivated the virus at 70°C for 10 minutes. Immunofluorescent image also taken following heat inactivation of virus at 70°C for 10 minutes with no GFP expression following this mode of inactivation. Two experimental repeats were performed.](image)

Figure 3.4 Effects of vaccinia virus following heat inactivation. Heat inactivation of virus and percentage cell survival based on MTS assay. SW620 cell lines were treated with vaccinia virus expressing GFP at an MOI of 1, that had been heat inactivated at varying temperatures for varying times. The lowest temperature and duration of heating were chosen for further experiments. We therefore heat inactivated the virus at 70°C for 10 minutes. Immunofluorescent image also taken following heat inactivation of virus at 70°C for 10 minutes with no GFP expression following this mode of inactivation. Two experimental repeats were performed.

Untreated cells in media and heat inactivated virus were used as negative controls, whilst cisplatin was used as a positive control, having been previously shown to have a cytotoxic effect in colorectal cancer cell lines. The results from our control experiments demonstrated that there was no reduction in cell survival in colorectal cancer cell lines left in media and also treated with heat inactivated vaccinia virus over 72 hours. The cisplatin positive control did cause a reduction in cell survival and this was less marked than for cells treated with virus (Figure 3.5).
3.2.4 Comparison of effects of VV-GFP and VV-FCU1

As we were provided with two types of vaccinia virus, one encoding GFP, and the other encoding the fusion suicide gene FCU1 required for pro-drug activation, we wanted to assess that the effects of both types of virus to ensure that they were similar and to exclude any interference of results by GFP or FCU1. To do this we therefore performed comparative MTS assays using both forms of virus and our colorectal cancer cell lines.

Figure 3.6 shows the MTS assays used to assess cell viability in two of three of our colorectal cancer cell lines SW620 and HT29. Both cell lines were treated with both forms of vaccinia virus as described in methods and left to incubate for 72 hours. The results of our experiments demonstrated that both types of vaccinia exerted similar effects on our colorectal cell lines. This confirmed that the effects that were demonstrated in our tissue culture experiments was due to vaccinia virus rather than GFP or FCU1.
3.2.5 Determination of mode of cell death

The mechanism of tumour cell death is poorly understood following treatment with vaccinia virus. Several modes of cell death have been implicated to varying degrees. These include apoptosis, necrosis, necroptosis and autophagy. Vaccinia virus strain also is a factor in the type of cancer cell death that occurs.

We first wanted to assess for evidence of apoptotic cell death. Characteristics of cells undergoing apoptosis includes chromatin condensation, DNA fragmentation and membrane blebbing. This form of cell death occurs in all normal tissues, causing a silent death where phosphatidylserine (PS) is...
expressed on the cell surface as a signal attracting antigen presenting cells (APCs). There are two main pathways of apoptosis: the extrinsic and intrinsic pathways. The extrinsic pathway is ligand dependent. Tumour necrosis factor alpha (TNF α) and Fas ligand (Fas L) bind to their respective receptors, leading to the recruitment and activation of caspase 8 by dimerization. Activation of caspase 8 results in cleavage of executioner caspses 3 and 7 and this results in target cell degradation. Mitochondrial stress and damage results in the release of cytochrome C from mitochondrial pores. This results in the formation of an apoptosome with caspase 9 as well as adaptor protein apoptotic protease-activating factor-1 (APAF1). This leads to cleavage and recruitment of executioner caspases and to apoptotic cell death.

Poly (ADP-ribose) polymerase (PARP) detects and repairs breaks in DNA. This is a target of the executioner caspases and undergoes proteolysis prior to the onset of apoptosis. Cleavage of PARP at the execution stage of apoptosis is one of the hallmarks of apoptosis.

Firstly, we looked for evidence of apoptotic cell death using the PE Annexin V assay. This assay was carried out as outlined in methods. All three colorectal cancer cell lines were treated for a 72-hour time course (Figure 3.7). In order to validate results of treatment with vaccinia virus, cells were also treated with comparative controls. These controls included: no treatment, heat inactivated vaccinia virus and cisplatin treatment. Over 72 hours there was an increase in Annexin V and 7-amino-actinomycin D (7-AAD) following treatment of all three colorectal cell lines with vaccinia virus (Figure 3.7). These results are consistent with cell death being predominantly apoptotic as the binding of annexin V to externalised phosphatidylserine indicates the presence of apoptosis, whilst 7-AAD staining is indicative of cell death, with early apoptotic cells excluding this dye and late apoptotic cells staining positively when the dye passes into the nucleus and bind to DNA.

Our results showed an increase in apoptosis in the three colorectal cancer cell lines treated with virus over the 72-hour time course, with the percentage of apoptosis increasing in a time-dependent manner. In the untreated and heat inactivated virus negative control groups there was very little cell
death over 72 hours compared with virus and cisplatin treatment (Figure 3.7). The cisplatin positive control group did show an increase in cell death over 72 hours, however this was less than virus treatment.
These findings suggest that treatment with this strain of vaccinia virus resulted in apoptotic cell death in these three colorectal cancer cell lines. To further assess for apoptotic death western blotting was performed as described in methods. Poly (ADP-ribose) polymerase (PARP) is a 116kDa polymerase that appears to be involved in DNA repair in response to stress. PARP can be cleaved and is one of the main targets of caspase-3 which is one of the key mediators of apoptotic cell death in vivo. Given the initial results of the PE annexin V assay which indicated apoptotic cell death in treated colorectal cancer cell lines we anticipated that our western blot would result in reduced presence of the 116kDa PARP band. HCT116, HT29 and SW620 colorectal cancer cell lines were treated with...
vaccinia virus, with control cells left untreated. Jurkat cells were treated with 25\(\mu\)M etoposide as recommended by the manufacturer as a positive control.

In the HCT116 colorectal cancer cell line treated with vaccinia there was a reduction in the 116kDa band on western blotting compared with untreated controls over 72 hours suggesting cleavage of PARP and indicating apoptotic cell death (Figure 3.8). In the SW620 cell line at 72 hours post treatment there was a reduction in the 116kDa band in comparison with untreated control which also indicated apoptotic cell death. There did not appear to be any obvious reduction in this band in the HT29 cell line over 72 hours when compared with untreated controls (Figure 3.8).

For two of the three cell lines used in this experiment the western blotting results supported the findings from the initial experiment with PE annexin V suggesting that the primary mode of cancer cell death following treatment with vaccinia was apoptosis.
Figure 3.8 Assessment of apoptotic cell death in colorectal cancer cell lines treated with vaccinia virus using western blotting. Western blotting assessing for PARP cleavage in colorectal cancer cell lines treated with oncolytic vaccinia virus. All three cell lines were treated for 72 hours as outlined in methods. Western blotting performed as described in methods. In HCT116 colorectal cell line there was an apparent reduction in PARP bands at 24, 48 and 72 hours in virus treated cells (VV) compared with untreated controls (UT). There was reduction in PARP at 72 hours in the SW620 colorectal cell line treated with VV compared to control however this was not evident at earlier time points. There was no reduction in PARP bands at any time points in the HT29 cell line. Positive controls are also displayed and performed as per manufacturers advice. GAPDH bands were measured to assess the amount of protein in each well and to validate findings.

As other forms of cancer cell death have been implicated with oncolytic vaccinia virus, further experiments were undertaken to see if other modes of cell death were also occurring. Necrotic cell death is induced by external factors such as toxins, infection and trauma. This type of cell death is morphologically characterised by cellular swelling, rupture of plasma membranes and loss of cytoplasmic contents. Whilst necrosis has long been viewed as non-programmed cell death its execution has been shown to be controlled by specific signal-transduction pathways and catabolic mechanisms. This form of programmed necrotic cell death is termed necroptosis. Necroptosis is...
induced by TNF signalling involving the receptor-interacting protein (RIP) family. Following inhibition of apoptosis by the caspase inhibitor activation of RIP1 and RIP3 kinase leads to mitochondrial instability and death. When RIP1 and RIP3 kinase become phosphorylated this leads to the generation of the necrosome, a molecular complex that initiates necroptosis. This leads to phosphorylation of mixed lineage kinase domain-like (MLKL) pseudokinase with a resultant influx of calcium into the cell. This results in cellular swelling and plasma membrane rupture, releasing intracellular components. This mode of cell death has been identified as the predominant mode of ovarian cancer cell death following treatment with vaccinia virus by Whilding et al. To assess for programmed necrosis in colorectal cancer cell lines we performed western blotting specifically looking for an increase in RIP1 and RIP3 kinase bands that would be an indication of necroptotic cell death in our colorectal cancer cell lines.

All three colorectal cancer cell lines were treated with vaccinia virus over a 72-hour time-course as in previous experiments. Western blotting was undertaken as outlined in methods. The results from our experiments showed reducing RIP1 and RIP3 bands compared to untreated negative controls, rather than an increase in these bands as would be expected in necroptosis (figure 3.9). These results indicate that there is no evidence of necroptotic cell death in colorectal cancer cells treated with vaccinia virus. For positive controls 3T3 cells were treated with cytochrome C as per manufacturers recommendations. These positive controls showed an increase in RIP1 and RIP3 bands compared with untreated controls. The results of our experiment indicated that cell death in our three colorectal cancer cell lines was not necroptosis.
Figure 3.9 Assessment of necroptotic cell death in colorectal cancer cell lines treated with vaccinia virus. Western blotting assessing for increase in RIP1 and RIP3 bands in colorectal cancer cell lines treated with oncolytic vaccinia virus. All three cell lines were treated for 72 hours as outlined in methods. Western blotting performed as described in methods. There was no obvious increase in RIP1 and RIP3 bands for all three colorectal cancer cell lines treated with oncolytic vaccinia virus throughout the time course compared with untreated controls. Positive controls are also displayed and performed as per manufacturers advice. GAPDH bands were measured to assess the amount of protein in each well and to validate findings.

The results of these experiments suggested that the predominant mode of cell death in colorectal cancer cells treated with oncolytic vaccinia virus was apoptosis. Apoptosis is classified as a type 1 programmed cell death \(^{138}\). Although this has been historically considered to be non-immunogenic
several studies have shown that oncological treatments such as irradiation and other agents such as doxorubicin can trigger immunogenic apoptosis \(^{138,139}\).

3.2.6 Assessment of immune mediated cell death

We then wanted to assess if cell death in colorectal cancer cell lines treated with oncolytic vaccinia virus was immune mediated. We decided to assess a panel of DAMPs that are markers of immune mediated cell death. These DAMPs included: high-mobility group box 1 protein (HMGB1), calreticulin (CRT), and heat shock protein 70 (HSP70). We also decided to look for other proteins that are determinants of immune mediated cell death. These included CD80, human leukocyte antigen (HLA), programmed death ligand 1 (PD-L1) and first apoptosis signal receptor (FAS). CD80 is a protein found on dendritic cells, activated B cells and monocytes that provides a costimulatory signal required for T cell activation. HLA is a gene that encodes the major histocompatibility complex proteins in humans and is responsible for the regulation of the immune system. PD-L1 is a transmembrane protein that plays a role in immunosuppression after it binds to its receptor PD-1 found on activated T cells, B cells and myeloid cells. Expression of PD-L1 may result in immune evasion by cancers and inhibit response to treatment with oncolytic viruses. FAS is a death receptor on the cell surface that leads to apoptotic cell death.

Flow cytometry and ELISA were performed as described in methods to assess for immunogenic cell death in the three colorectal cancer cell lines. Mitoxantrone an anthracycline, was used as a positive control as this has been proven to induce immunogenic cell death \(^{140}\). Negative controls included heat inactivated virus, as used in previous experiments as well as those left untreated. Cells were left for 72 hours before being analysed.

In the HCT116 colorectal cancer cell line there was an increase in calreticulin over 72 hours following treatment with vaccinia virus compared with controls. This was statistically significant at the 72-hour time-point \((p=0.0002)\) (Figure 3.10). There was also an increase in HMGB1. This also increased over the 72-hour time point and was more marked in cells treated with virus compared with controls. At
72 hours the difference in HMGB-1 level was statistically significant in the cells treated with virus compared with all other controls (VV vs UT p=0.0002; VV vs HI p=<0.0001; VV vs MTX p=<0.0001) (Figure 3.10). There was no significant increase in HSP70, FAS, PD-L1 or MHC following treatment with vaccinia.

As there was an increase in both HMGB1 and calreticulin the results of these experiments suggested that cell death in HCT116 colorectal cell lines treated with oncolytic vaccinia virus may be immunogenic.
Figure 3.10 Markers of immunogenic cell death in HCT116 colorectal cancer cell lines treated with vaccinia virus. Flow cytometry and ELISA performed as described in methods. Cells were treated for up to 72 hours and were assessed for markers and determinants of immunogenic cell death at 24-hour intervals. Mitoxantrone (MTX) was used as a positive control. Negative controls were heat inactivated virus (HI) and untreated cells (UT). VV = vaccinia virus. Graphs represent pooled data from two independent experimental repeats. Statistical analysis using two-way ANOVA. Statistical level of significance: *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001

In the SW620 colorectal cancer cell line there was an increase in HSP70 over 72 hours following treatment with vaccinia virus compared with controls, although this peaked at 48 hours. The increase in this marker was found to be significant compared to heat inactivated virus and to positive controls treated with mitoxantrone (p=0.03) (Figure 3.11). There was an increase in FAS over 72 hours also and once again this was maximal at the 48-hour time point. There was a statistically significant increase in FAS in cells treated with vaccinia virus compared with heat inactivated virus (p=0.04), but no significant increase compared with other controls (Figure 3.11). There was also an increase in HMGB1 over 72 hours. This increased over the 72 hours and was more marked in cells treated with virus
compared with controls. At 72 hours the difference in HMGB-1 level was statistically significant in the cells treated with virus compared with all other controls (VV vs UT p=0.001; VV vs HI p=0.0004; VV vs MTX p=0.04) (Figure 3.11). There was no significant increase in CRT, PD-L1 or MHC following treatment with vaccinia.

There was an increase in 3 of the 7 markers that were assessed. This was most marked however in the case of HMGB1 with a gradual increase over the 72 hours. It was also significant compared with all other controls. The results from this cell line suggest that cell death following treatment with oncolytic vaccinia virus may be immunogenic.
In the HT29 colorectal cancer cell line there was an increase in CRT, HSP70 and MHC over 72 hours following treatment with vaccinia virus compared with controls. The increase in these markers were not statistically significant when compared to controls (Figure 3.12). At 48 hours there was an increase in CD80 in cells treated with vaccinia virus and this was significantly raised compared with all other controls (p=0.01), this did not continue to 72 hours (Figure 3.12). As with the previous cell lines there was an increase in HMGB1 over 72 hours. This increased over the 72 hours and was more marked in cells treated with virus compared with controls. At 72 hours the difference in HMGB-1 level was statistically significant in the cells treated with virus compared with negative controls but not
compared with positive controls (VV vs UT \( p=0.01 \); VV vs HI \( p=0.02 \)) (Figure 3.12). There was no increase in FAS, PD-L1 or MHC following treatment with vaccinia virus.

There was a significant increase in 2 of the 7 markers of immunogenic cell death that were assessed. Once again this was most marked in the case of HMGB1 with a gradual increase over the 72 hours. The results from this cell line suggest that cell death following treatment with oncolytic vaccinia virus may be immunogenic.
Figure 3.12 Markers of immunogenic cell death in HT29 colorectal cancer cell lines treated with vaccinia virus. Flow cytometry and ELISA performed as described in methods. Cells were treated for up to 72 hours and were assessed for markers and determinants of immunogenic cell death at 24-hour intervals. Mitoxantrone (MTX) was used as a positive control. Negative controls were heat-inactivated virus (HI) and untreated cells (UT). VV = vaccinia virus. Graphs represent pooled data from two independent experimental repeats. Statistical analysis using two-way ANOVA. Statistical level of significance: *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001
3.3 Discussion

We initially set out to see if three colorectal cancer cell lines that had been shown to be susceptible to treatment by other forms of oncolytic vaccinia virus such as JX-594 were susceptible to the vaccinia virus used in our experiments. As with JX-594 our oncolytic vaccinia virus was genetically modified to improve its tolerability and effectiveness compared with naturally occurring vaccinia virus. Deletion of thymidine kinase ensures that virus replicates specifically in tumour cells rather than in healthy cells. We initially confirmed viral presence and replication through direct immunofluorescence in tissue culture over 72 hours. This preliminary information indicated that the virus was replicating over the course of the experiment as GFP appeared to be increasing over time. To quantify the effects of VV-GFP we then performed MTS proliferation assays. The result from our three colorectal cell lines was mixed as two of the three lines (HCT116, SW620) treated with vaccinia showed an obvious reduction in cell proliferation following treatment for 72 hours at relatively low MOIs whilst a third cell line (HT29) did not. This finding contrasted with the increased visualisation of GFP under direct immunofluorescence in our preliminary experiment. The results of the MTS assay may be attributable to overseeding of the well plate with a resultant high readout due to the reduction of MTS by mitochondrial reductase in the HT29 cell line. The result could also be a product of the cell line being contaminated or contamination of any of the constituents of tissue culture during cell passage. As is known with immortalised cell lines, after serial passaging the resultant cell lines over time develop changes so that they differ from their original cell precursor. If this has happened with the HT29 cell lines in this case then there could be a change in the behaviour of these cells and a resultant resistance to infection by vaccinia virus compared with the other two colorectal cancer cell lines which were assessed in the same way. To further assess the HT29 cell line we then performed a cell death assay using PE annexin V. The results from this experiment found that treatment of HT29 with VV-GFP did result in cell death. These findings reassured us that the virus was having an effect in this cell line as suggested by the increased GFP expression under direct immunofluorescence and in contrast to MTS results. It is not clear why the MTS assay result did not show a reduction in cell proliferation in the
HT29 cell line. This could potentially have been due to over-seeding of the cells in tissue culture compared with other studies however, the cells were always assessed pre-treatment and 80% confluence was visually confirmed, as with the other cell lines. Once treated the cells also appeared to be decimated by the vaccinia virus at 72 hours. Figure 3.13 demonstrates the change in appearance of HT29 cell lines using grey scale microscopy following treatment with VV-GFP at an MOI of 1 at 24 and 72 hours for comparison. The images are in clear contrast with one another as after 72 hours the treated HT29 cells are condensed and aggregated compared with the appearance at 24 hours. They were also no longer attached to the base of the well-plate. As the results of PE annexin V testing showed evidence of cell death in keeping with other published studies utilising the HT29 cell line treated with other types of oncolytic vaccinia virus, we decided to include the cell line in further experiments. This cell line could be further investigated through lower well-plate seeding, a longer incubation period with virus, or even a shorter exposure to MTS. If these results still did not show infectivity of virus then further interrogation into reasons for resistance could be assessed.

![Figure 3.13 Appearances of HT29 cell line following treatment with VV-GFP at MOI 1 at 24 and 72 hours.](image)

Once we had confirmed susceptibility of these cell lines to vaccinia virus we then went on to assess for the mode of cell death. This was assessed using PE annexin V to assess for apoptosis and necrosis as well as western blotting, looking again at apoptosis but also necroptosis. Our experiments found
that the predominant mode of cell death in these treated colorectal cancer lines was apoptosis. The type of cell death is thought to be important with regards to immunogenicity and the influence on immune cells in the tumour microenvironment. Apoptosis has historically been considered to have been non-immunogenic, however some anti-cancer agents have been shown to trigger immunogenic apoptosis. Immunogenicity of oncolytic viruses is beneficial as it can result in the release of tumour associated antigens which are then able to promote an adaptive immune response that mediates an anti-cancer effect at distant sites of disease. This has clinical implications for the delivery of oncolytic viruses and could result in the systemic administration of OVs to treat tumours rather than depending on intratumoral administration which can limit their application in certain circumstances. Mode of cell death was also investigated using flow cytometry for autophagy. Results from our experiments did not show that there was any evidence of autophagy in our three-tested colorectal cancer cell lines (data not shown).

To assess if apoptotic death was immunogenic in our treated cell lines we performed flow cytometry and ELISA to assess for a panel of markers and determinants of immunogenic cell death as described earlier in this chapter. The results of all three colorectal cancer lines treated with oncolytic vaccinia virus were variable however each cell line was noted to have an increase in certain markers and determinants of immunogenic cell death. In the HCT116 cell line there was a statistically significant increase in calreticulin and HMGB1 compared with controls over 72 hours. In the SW620 cell line there was an increase in HSP70 and FAS over 72 hours that peaked at 48 hours but was statistically significant compared with controls. As with the HCT116 cell lines there was a statistically significant increase in HMGB1 over 72 hours compared with controls. In the treated HT29 cell line there was a significant increase in CD80 compared with controls at 48 hours and as with the other two cell lines there was a significant increase in HMGB1 expression over 72 hours. Treatment of all three cell lines with vaccinia virus consistently resulted in significant increases in HMGB1 expression. These results indicate that treatment with vaccinia virus results in apoptotic cell death that is immunogenic in these colorectal cancer cell lines. In its use with other cancer cell lines such as melanoma vaccinia virus was
seen to induce a higher expression of calreticulin as well as HMGB1\(^\text{142}\). HMGB1 can cause additional immunogenic effects so that virally induced cell death can influence immune infiltration in tumours to attract immune cells and stimulate them\(^\text{142}\). Oncolytic viruses appear to be type II inducers of immunogenic cell death, selectively targeting the endoplasmic reticulum. They are able to induce immunogenic apoptosis by altering homeostasis of the endoplasmic reticulum and triggering stress\(^\text{143}\). In our experiments vaccinia virus was not able to induce a full picture of immunogenic cell death, however a mixed type of cell death was seen and this has been shown to be more immunogenic than apoptosis alone by other groups\(^\text{142}\).

3.4 Conclusion

Our work has demonstrated that oncolytic vaccinia virus successfully infects and kills colorectal cancer cells through a mixed pattern of cell death that is predominantly apoptotic but that is also necrotic. This mixed pattern of apoptotic and necrotic cell death has been indicated to be more immunogenic than apoptosis alone by other groups\(^\text{142}\). We have also found that when infected with vaccinia virus colorectal cancer cell lines there was an increase in the expression of some of the markers of immunogenic cell death.

Given the excellent ties with our surgical colleagues at the Royal Surrey County Hospital NHS Trust we wanted to assess the effects of VV-GFP and VV-FCU1 in human tissue. We planned to do this using excess tissue taken at time of surgery in patients undergoing curative liver resections for metastatic colorectal cancer. Moreover, we aimed to assess the pro-drug activation model in human tissue as this had previously been described by Foloppe et al. in a colorectal xenograft model in nude mice\(^\text{130}\). We hoped to develop an organotypic culture method in our laboratory that would enable us to do this.
Development of an organotypic culture model and evaluation of the use of oncolytic vaccinia virus in colorectal cancer
4.1 Introduction

Tumours have been appropriately described as pathological organs consisting of cancerous cells and stromal tissue supporting these. The stroma of tumours consists of an extracellular matrix made up of proteoglycans, hyaluronidase, fibrous proteins and stromal cells. These stromal cells include fibroblasts and adipocytes (mesenchymal supporting cells) as well as immune cells and cells of the vascular system. Peptide factors such as growth factors, cytokines, chemokines and antibodies are also located here. As tumours develop, changes also take place to the stroma and in later stages of tumour development the stroma is more supportive of tumour progression than in the early stages of cancer \(^{144}\). The ability to predict tumour response to treatment is difficult. Whilst molecular and genetic profiling drives the evolution of personalised anti-cancer therapy, the presence of a molecular biomarker does not always translate into a successful clinical outcome \(^{145}\). An example of this in colorectal cancer is the epidermal growth factor (EGFR) inhibitors cetuximab and panitumumab that are approved for use in patients with wild-type KRAS but result in clinical benefit in up to 20% of patients \(^{145}\). The ability to identify drug sensitivity to treatment and give an indication of clinical benefit would be a valuable tool in the treatment of cancers. The ability to conserve the heterogeneity of tumours in vitro would therefore aid the ability to effectively predict tumour response to treatment.

4.1.2 2D monolayers of tumour cells

Following dissociation this model requires tumours to be adherent to the culture dish. Two types of monolayer cultures exist, these include primary cell cultures and immortalised cancer cell lines. Primary cultures taken from tumours are more heterogeneous and are therefore more likely to be representative of the primary tumour but are limited in their proliferative capacity when compared to cancer cell lines. Immortalised cancer cell lines such as those used in tissue culture experiments are
clonal outgrowths from a primary tumour. The benefits of 2D models are that they are easy to handle, have a homogeneous character and result in limitless growth which allow a high throughput for experiments. The immortalisation of cancer cell lines is often inefficient owing to the inability of cells to adhere to tissue culture plates as well as their proliferative ability. This results in clonal outgrowth and as such these cell lines do not represent the heterogeneity of the primary tumour. These cell lines have been shown to have genetic, epigenetic and gene expression differences to the in vivo tumours from which they were derived\textsuperscript{146,147}.

4.1.3 3D culture models

3D culture systems are a more faithful representation of in vivo tumours as cell to cell interactions can occur in these systems. 3D culture systems have been developed so that they are able to grow for many passages. This has been established through the embedding of stem cells into a 3D matrix and exposure to tissue-specific exogenous growth factors as well as growth factors endogenously produced by the stem cell microenvironment and surrounding mesenchyme\textsuperscript{146}. They then organise into epithelia of their organ of origin. Similarly, tumour cells have been cultured into spheroid structures termed ‘organoids’. This technique has demonstrated long-term expansion ex-vivo of tumour cells that maintain the heterogeneity of the original tumour and organoids have been developed for several tumour types including colorectal and pancreatic cancers\textsuperscript{148}.

Limitations of the 3D culture system include the necessity for a collagen gel for culturing as can complicate potential drug screening and also make culturing more labour intensive than the 2D model of culture. Organoids that are developed from stem cells also lack the heterogeneity and microenvironment of tissue in vivo. This can be addressed through the addition of patient co-cultures taken from normal tissue adjacent to the tumour containing stroma. The same method can be adapted for cell cultures through the addition of fibroblasts, endothelial cells and immune cells enabling interaction between tumour cells and the stromal cells of the tissue microenvironment\textsuperscript{146}. The generation of organoids takes several weeks which reduces their utility in directing personalised
therapy for patients as this would be required in a much shorter time frame. Despite this 3D models are valuable tools in the development of anti-cancer therapeutics however they lack the complexity of the tumour microenvironment and are lacking in any vasculature.

4.1.4 Patient derived xenografts

Patient derived xenografts are developed from patient tumours that are implanted on immune deficient mice. These tumours retain the heterogeneity of the original tumour as the tissue is implanted without the necessity for dissociation, they also retain the original tumours characteristics such as histological features and metastatic behaviour. This model has been used for drug screening, the discovery of biomarkers as well as pre-clinical evaluation as in the case of the vaccinia virus used in our experiments. Systematic analysis of xenografts allow biobanking of genomically well-defined tumours and these are useful for developing new biomarkers and individual treatment strategies for patients.

As with the other methods described there are also limitations to this technique. These include variable engraftment success rates, with highly aggressive tumours having the highest engraftment rate thereby potentially skewing the variation of tumours that are seen compared to the population. These models also take a long time to generate and this results in a relatively low throughput as well as being expensive to run. Another limitation is due to the necessity for these xenografts to be implanted into immunocompromised host animals which makes the evaluation of tumour immunology as well as immunotherapeutic agents difficult. As a result, other models are required to assess immunotherapies.

4.1.5 Organotypic cultures

Precision-cut tumour slices or organotypic cultures are a potentially more complete 3D representation of the tumour microenvironment in vitro as they do not necessitate excessive manipulation of tissues. These slices capture the architecture, heterogeneity and native cellular elements of the tumour of
interest rather than surrounding tumour with artificial matrices. Organotypic culture was initially reported in chick embryo cardiac tissue and rat brain tissue in 1957 and 1962 respectively. Tissue is taken from tumour tissue and is quickly sliced using a vibratome before being placed in optimal culture conditions. It is important that this process occur in a short period of time after tissue collection to minimize tissue distortion and to preserve native morphology and architecture for further analysis.

Several studies have assessed the use of organotypic cultures. Van der Kuip et al utilised organotypic cultures to assess anti-cancer drugs in a breast cancer model. Using this method fresh primary breast tumour tissue was taken at the time of surgical resection, in organ transportation medium on ice. 5mm tissue cores were prepared under a sterile hood using a hand-held coring tool. 200µm tissue slices were prepared in cold PBS using a Krumdieck precision cutting tissue slicer. These slices were then submerged in 1ml supplemented mammary epithelial growth medium and incubated in 24-well plates at 37°C 5%CO2 on a shaking platform at 150 rpm. Medium was changed after 24 hours and was performed for an additional 72 hours. This study found that 88% (22/25) of breast tumour samples were viable with three cases being unsuccessful due to a lack of transportation medium in one instance, contamination of the tissue in a second instance and finally due to the tumour being mucinous and difficult to slice. Cell viability was assessed using three-coloured immunofluorescence with living cells detected using tetramethylrhodamine methyl ester (TMRM), picogreen and SYTO®63. Through immunofluorescent assessment of live and dead cells as well as IHC analysis found them to be viable for 4 days when comparing morphology to the paraffin embedded original tumour tissue. In this study there was at least a 3 hour gap between the tissue being resected and preparation of the organotypic cultures. The same method also proved effective when assessing treatment of these breast cancer derived organotypic cultures with Taxol.

Zimmerman et al assessed human precision-cut liver tumour slices as a patient-individual predictive test system for oncolytic measles virus. The aim of this study was to evaluate the use of the
precision cut liver slice model as a predictive test system for the effectiveness of different measles vaccine viruses in primary or secondary liver tumours. Samples were obtained from 20 patients and were transported in ice-cold William’s E medium and transported directly to the laboratory where the slicing procedure started within 1 hour. 8mm cores were produced using a special coring device. The tissue was sliced using the Krumdieck tissue slicer as in the study by Van der Kuip, and these were between 200 and 300µm thickness. After slicing, samples were washed with supplemented and oxygenated William’s E medium and placed in 12-well plates. Slices were cultivated in 2ml WEGG medium in 80% oxygen, 5% CO2 at 37C with gentle shaking. In this study slices were infected one day following slicing with virus and left for 1 hour before virus was removed and replaced with fresh culture medium. Precision cut tumour slices were cultured for up to 5 days in 12-well plates. The study found that the slices created demonstrated heterogeneity in terms of the tissue composition as well as the viability of the slices, although it did not address the variability of tissue slice viability any further than this. The study did confirm that the oncolytic measles virus used did successfully infect organotypic cultures. Using confocal microscopy, the study was also able to demonstrate evidence of measles virus infection in a colorectal liver metastasis model, although the depth of penetrance was not described, and the amount of infection was not quantified. This study utilised the confocal microscope to determine the success of infection and to help determine whether further analysis should be undertaken on the treated tumour slices.

Vaira et al. adapted their methods and incorporated the use of the vibratome VT1200 which was able to cut tumour cores at a defined thickness at a micrometre level, whilst also being able to cut tumour slices in 20-40 seconds. The machine also minimises the vertical deflection of the blade, therefore preserving the integrity of delicate specimens. Vaira et al assessed tissue viability in organotypic cultures from tumours that had just been resected, they excluded any patients that had received neoadjuvant chemotherapy or radiotherapy. Organotypic cultures were cultured on Millipore inserts for up to 120 hours using 1ml of Ham-F12 media, with media being changed every 2 days. Tissue slices were harvested from baseline, every 24 hours up to 120 hours. This was done in
duplicate with one slice being snap-frozen for qPCR and the other being formalin fixed for IHC evaluation. IHC evaluation was assessed using an automated image analysis system. The study found that the general architecture including epithelial structures and their spatial relationships to stroma was confirmed up to 120 hours. Ki67 staining was used to assess proliferative activity and they did not see a significant decrease up to the 120 hour timepoint although there was a gradual decline in this. The study showed that tissue slices were viable for up to 120 hours, however there was a steep decrease in the viability from 72 hours onwards compared with the earlier timepoints. This study assessed the effects of small-molecule targeted therapy with the PI3K inhibitor LY294002 and found a partial dose-response relationship following treatment. The study concluded that organotypic culture was a reproducible method for testing therapeutic agents.\textsuperscript{154}

Preserving tissue slices for a prolonged period of time is a key factor and requires optimisation of culture conditions. Tissue slices have been cultured floating freely in media or on membrane supports. Membrane supports are porous and keep the tissue slice still during culture. This prevents it from moving around the culture dish which could potentially damage the tissue slice and affect the tissue architecture in comparison with the original tumour. When culture plates are placed on rotational plates this increases oxygen diffusion to the tissue which can be beneficial for tissue viability.\textsuperscript{146} Davies et al assessed a variety of incubation methods for organotypic cultures whilst also assessing the impact that transportation and preparation of tissue slices had through the expression of stress proteins.\textsuperscript{155} This study found that the tissue morphology of tissue slices was most similar to that in vivo when they were cultured on porous cell culture inserts. In comparison tissue slices cultivated in media alone and left to float had condensed apoptotic nuclei and vacuolated structures compared to original tissue.\textsuperscript{155} Oxygen levels were also assessed with cultures exposed to low oxygen levels (<5%) and atmospheric oxygen levels (20%). The study determined through immunohistochemical staining that atmospheric oxygen levels were important to retain tumour slice viability.\textsuperscript{155} Incubation of tissue slices on a rotating incubation unit also permitted intermittent immersion of tissue slices in media whilst preventing the establishment of an air-filter interface. Hypoxia is a hallmark of most solid tumours
with oxygen levels <2% considered to be mild hypoxia and oxygen levels <0.1% considered severe hypoxia, whilst tissue normoxia is also much lower than atmospheric levels. As tissue slices do not have an intact circulation oxygen supply is dependent on gas diffusion through tissue and the atmospheric conditions may be sufficient to ensure that deeper layers of the tissue slice have an adequate oxygen supply to maintain viability. The effects of tumour transport and cutting were also assessed as it was felt that this was likely to induce stress in the tissues, however the results of the study found that transportation and cutting did not significantly increase the expression of stress biomarkers in tissue slices. Tumour slice thickness was also not found to be a major determinant of tissue slice viability.

Tissue slices have been used in several studies in the context of precision medicine to assess sensitivities to a variety of anti-cancer drugs, although there has been no standardisation in the method of cultivation. Despite this all studies have confirmed the viability of tissue slices in culture and their suitability in the use of anti-cancer treatments with viability in studies ranging from 3 – 5 days.

Given the comprehensive assessment of the tissue slice culture system and the workflow set out by Davies et al. we decided to try and recreate this system and potentially optimise this for the evaluation of virotherapy. The workflow set out by Davies et al. appeared to be the most completely interrogated method of tissue slice preparation for viability. The results from this study and the conditions used were also extremely promising in terms of the maintenance of tissue slice architecture and viability. This would be an important aspect of our work with the oncolytic vaccinia virus, pro-drug activation system and our tissue slice model.
4.2 Results

4.2.1 Development of an organotypic tissue culture model in our laboratory

The aim of this section of work was to develop a human tumour slice model in our laboratory and to assess the feasibility of developing this model for use in testing novel anti-cancer drugs. As mentioned previously tumour slice viability following harvesting, slicing and during incubation would be a key aspect of this.

4.2.2 Tissue slice viability

Tissue slices were prepared from patient tumours at the time of surgical resection as outlined in methods, and incubated at 37°C, 5% CO₂. Slices were placed in a well plate, floating in culture medium on a vibrating plate at <100 rpm. For viability assessment purposes they were then removed from culture conditions every 24 hours and fixed in formalin solution. This was done up to 10 days following retrieval. After the last tissue slice in the experiment was fixed they were then all processed, paraffin embedded and sliced in preparation for IHC staining (see methods). H&E staining, immunostaining as well as staining for e-cadherin, collagen IV and Ki67 was performed by the laboratory at the Royal Surrey County Hospital, Guildford. Further staining for cleaved caspase 3 and Ki67 was performed in the laboratory at the Leggett Building, University of Surrey.

4.2.3 Preliminary viability results of organotypic cultures

Our initial experiments set out to firstly see if it was possible to develop an organotypic culture system in our laboratory and if so what the optimum conditions required so that the organotypic cultures would remain viable. The decision to slice the tissue at 300µm intervals was based on existing literature regarding the use of organotypic cultures as well as our experience in cutting tumours. Whilst thinner slices can be produced we found that the cutting of tissue cores at this thickness was less consistent. Our choice of media in these experiments was DMEM supplemented with 10% FBS and 100 U/mL penicillin and, 100µg/mL streptomycin. We found that this media which was readily
available in our laboratory, did not impact adversely on the organotypic cultures, furthermore we saw no benefit from using different media which were potentially more costly and also less readily available.

In our initial experiments we assessed the 300µm tumour slices generated by placing them into individual wells of a 24-well plate and submerging them in media before being placed in an incubator with atmospheric oxygen levels on a shaking plate at <100rpm (Figure 4.1). Staining of the slices with Ki67 (cellular marker for proliferation) and cleaved caspase 3 (active version of protein caspase 3, which is the converging point of both the extrinsic and intrinsic pathways, and an indicator of programmed cell death) to assess for evidence of cell proliferation and apoptosis respectively in these culture conditions was also performed.

![Figure 4.1 Organotypic cultures obtained during the preliminary experiment set up. Tumour cores taken from freshly resected patient specimens were prepared as described in methods before being sliced to 300µm thick tissue cultures. These were then placed in 24-well plates and were submerged in media before being placed in an incubator on a rocking plate.](image)

After processing tumour slices using this method it was noted that the architecture of the tissue very quickly became distorted compared with controls fixed at timepoint 0 hours. In these slices there was histological evidence of vacuolisation and disassociation of the tumour slices when compared to control slices and non-sliced tissue fixed in formalin solution at the time of slice preparation (Figure
4.2). This finding was also noted by Davies et al when comparing slices left floating in media compared to those on filter supports. Using this method of culture, the organotypic culture model would not have been suitable for the assessment of our oncolytic vaccinia virus and for assessment of the pro-drug activation system. We therefore adapted our initial method.

Figure 4.2 IHC images of tissue prepared using our initial culture conditions. Organotypic cultures were produced from tumour specimens placed in 24-well plate, submerged in media and left on a rocking plate in an incubator for the duration of the experiment. Organotypic cultures were fixed at 24-hour intervals. Staining was performed for H&E, Ki67 and cleaved caspase 3. Results from our preliminary experiments showed that at 72 hours following incubation there was a marked distortion in tissue architecture with loss of cellular content as well as vacuolisation of the tissues which is highlighted in the IHC images at 72 hours with black arrows. Images captured at x10 magnification to give an overview of the tissue appearances throughout the organotypic cultures.

4.2.4 Adaptation of culture conditions to improve organotypic culture viability

Based on our preliminary findings in viability experiments as well as the results published by Davies et al. the method of culture was updated so that tissue slices were placed on filter supports and incubated on a rotating plate to aid oxygen diffusion to tissue slices. These adaptations were recommended in the work published by Davies et al. Following these adaptations to our organotypic culture methods there appeared to be a reduction in vacuolisation of the tissue slices whilst the tissue structure and architecture was maintained and comparable to that seen in the
original tissue fixed at the time of tissue preparation (T0 hours) when H&E images were reviewed (Figure 4.3).

![Figure 4.3 IHC images of prepared organotypic cultures using adjusted culture conditions up to 264 hours. Tissue slices were incubated in 6-well plates on filter supports with media added so that the slices were not completely submerged. Within the incubator the 6-well plate was placed on a rotating plate to assist with diffusion of oxygen through the tissues. In this viability experiment the tissue slices were left for up to 11 days. Organotypic cultures were fixed in formalin at 24-hour intervals. The results of H&E staining in this experiment show maintenance of tissue architecture compared with 0-hour timepoint. There is also little evidence of any vacuolisation of tissues. These results compare favourably with the results from our initial methods shown in Figure 4.2.]

As well as performing H&E staining to assess overall architecture and appearance of the tissue slices, we also stained the tissue for the immunomarkers cytokeratin 7 (CK7), Cytokeratin 20 (CK20) and CDX2. CK7 is a type II keratin of non-keratinizing squamous epithelium specifically expressed in simple epithelia lining the cavities of internal organs. Staining is usually negative in colorectal adenocarcinoma. CK20 is a major cellular protein found specifically in gastric and intestinal mucosa. Staining is usually positive in colorectal adenocarcinoma. CDX2 is a nuclear homeobox transcription factor that is crucial for axial patterning of the GI tract during embryonic development and involved in the process of intestinal proliferation, differentiation, adhesion and apoptosis. Staining for CDX2 is positive in primary and metastatic colorectal adenocarcinoma. These are the immunomarkers that are routinely assessed and stained for by the histopathology services at the Royal Surrey County Hospital.
when reviewing colorectal tumours. For further detail regarding tissue architecture and viability we assessed staining for E-cadherin, a calcium dependent cell-cell adhesion molecule in colorectal carcinoma that plays an important role in the maintenance of tissue architecture. We stained for collagen IV, of which basement membranes are primarily composed of. Collagen IV provides an intermediate adhesion area between parenchymal cells and interstitial matrix and gives detail regarding tissue architecture. Finally, we also stained for the proliferation marker Ki67. We were then able to compare the staining from our prepared organotypic cultures to the original histopathology blocks that were prepared by the histopathology department at the Royal Surrey County Hospital, Guildford at the time of surgery. The images were also reviewed by Dr Izar Bagwan, Consultant histopathologist at the hospital (Figure 4.4).
Figure 4.4 Comparison of organotypic culture architecture compared with histopathology blocks prepared from the original tumour specimen. Comparison of IHC staining for H&E to assess overall appearances as well as Ki67 to assess for viability through the assessment of cellular proliferation. Staining for E-cadherin and collagen IV performed to further interrogate architecture of the tissue. IHC from original tissue blocks found H&E appearances to be consistent with that of colorectal liver metastasis. The proliferation index was calculated at 80%. Staining for E-cadherin and collagen IV were both positive indicative of preserved basement membrane and therefore architecture within the tissue. In comparison organotypic cultures produced as outlined in methods and left in culture conditions for 48 hours also showed H&E appearances consistent with the original tissue blocks confirming colorectal liver metastasis. The proliferation index was calculated at 70%, slightly less than those of the original tissue at this time point. Staining for E-cadherin and collagen IV were both positive and once again indicative of preserved basement membrane and overall tissue architecture.

To assess our methods, we compared staining for these immunomarkers and markers or architecture and proliferation in the organotypic cultures prepared in our laboratory with the staining of the original tumour specimen (from which our core biopsy was taken, and tissue slices produced). Staining for these markers was performed by the histopathology laboratory at the Royal Surrey County Hospital, Guildford. The results of the staining confirmed that there was no difference between the
original specimen and the organotypic cultures created when assessing these markers. As with the
original specimen our organotypic cultures stained negatively for CK7 and positively for CK20 an CDX2
(Figure 4.5).

![Organotypic cultures](image)

**Figure 4.5 Staining for immunomarkers in prepared organotypic cultures for comparison with original histopathology blocks.** Organotypic cultures stained negatively for CK7 whilst staining positively for both CK20, and CDX2 which is consistent with a diagnosis or colorectal liver metastases. The same staining was also found in the original histopathology blocks prepared by the histopathology laboratory at the Royal Surrey County Hospital, Guildford.

The results of the staining for E-cadherin and collagen IV in combination with H&E staining to assess for tissue viability found that our adapted culture system resulted in a tissue viability of a maximum of 5 days with a minimum viability of 3 days. In all the tumours that were assessed for viability purposes we found that the minimum viability of 72 hours was consistent (Table 4.1).
Table 4.1 Summary of viability assessment for organotypic cultures prepared from resected tumour specimens in four patients

<table>
<thead>
<tr>
<th>Patient no:</th>
<th>CK7</th>
<th>CK20</th>
<th>CDX2</th>
<th>Diagnosis</th>
<th>Proliferation index (Ki67)</th>
<th>Duration of intact tissue architecture (E-cadherin and collagen IV)</th>
<th>Overall viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Colorectal liver metastasis</td>
<td>50% up to 120 hours, followed by a reduction to 5% at 144 hours</td>
<td>Blurring of basement membrane at 120 hours</td>
<td>120 hours</td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Colorectal liver metastasis</td>
<td>25% at 48 hours followed by a reduction to 10% at 96 hours</td>
<td>Staining for E-cadherin lost at 96 hours</td>
<td>48-72 hours</td>
</tr>
<tr>
<td>3</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Colorectal liver metastasis</td>
<td>50% at 72 hours with a reduction to 10% at 144 hours</td>
<td>Cells separating from basement membrane at 72 hours</td>
<td>72 hours</td>
</tr>
<tr>
<td>4</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Colorectal liver metastasis</td>
<td>70% up to 120 hours</td>
<td>Loss of architecture in tissue slice and minimal staining for E-cadherin at 144 hours</td>
<td>120 hours</td>
</tr>
</tbody>
</table>

The results of our adapted methods showed that we had developed an organotypic culture system that was suitable for the assessment of the oncolytic vaccinia virus and for assessment of the pro-drug activation model.
4.2.5 Treatment of organotypic colorectal liver metastases with Vaccinia Virus

Having established that our methods enabled us to assess organotypic cultures for a minimum of 72 hours up to 120 hours, we wanted to use this model to assess the effects of oncolytic vaccinia virus directly applied to tumour tissue slices.

4.2.5.1 Evidence of viral presence and effect on tissue slices using immunohistochemical analysis

Tissue slices were prepared as outlined in the methods. In these initial experiments vaccinia virus encoding green fluorescent protein was then applied to slices at several concentrations: 10⁴ pfu/ml, 10⁵ pfu/ml, 10⁶ pfu/ml and 10⁷ pfu/ml. The treated tissue slices were left for up to 120 hours (5 days). Plaque forming units are a measure of the number of virus particles capable of forming plaques per unit volume. It is a functional measurement rather than a measurement of the absolute quantity of particles: viral particles that are defective or which fail to infect their target cell will not produce a plaque and thus will not be counted. At 24-hour intervals treated slices and comparative untreated controls were fixed in formalin before undergoing processing as outlined in methods. Slices were then generated from the paraffin embedded tissue to enable us to perform immunohistochemical analysis. H&E staining was performed on these slices to assess for the overall appearance of the tumour tissue as well as staining for anti-GFP or anti-vaccinia virus antibody as a marker of viral presence within tissue slices. The reason for using two antibodies for viral detection was due to the two forms of vaccinia virus that we were using in our experiments. As one expressed GFP we decided to use an antibody against this protein, the second form did not have GFP, in its place it expressed the fusion suicide gene (FCU1) responsible for the conversion of pro-drug into active 5-fluorouracil. We therefore used an anti-vaccinia virus antibody to stain directly against vaccinia virus. Cleaved caspase 3 antibody was used to assess for any differences in apoptosis between the treated and untreated tissue slices.

After confirmation of tumour presence in tissue slices on H&E staining, staining with cleaved caspase 3 and anti-vaccinia virus antibody, or cleaved caspase 3 and anti-GFP antibody was performed (Figure 4.6).
Results from our experiments showed that the vaccinia virus treated organotypic cultures stained positively for anti-GFP antibody or the anti-vaccinia antibody compared with the untreated controls. Staining for these two antibodies confirmed viral presence within the treated organotypic cultures. There also appeared to be increased staining for cleaved caspase 3 in the treated tissue slices when compared to untreated controls.
**Figure 4.6 Evidence of viral presence in treated organotypic cultures compared with untreated controls.** Cultures treated with vaccinia virus at increasing pfu/ml. Treatment at the lowest (VV10⁶ pfu/ml) and maximum (VV10⁶ pfu/ml) doses shown. No evidence of virus expression in untreated controls whilst there was evidence of increased anti-GFP staining at higher dose of virus compared with lower dose. Evidence of cleaved caspase 3 expression in organotypic cultures treated with vaccinia virus compared with untreated controls. Increased expression of cleaved caspase 3 in organotypic cultures compared with those treated with lower concentrations of virus. Evidence of an increase in staining of both cleaved caspase 3 and anti-GFP antibody with increasing viral dose suggesting a dose-response relationship.
Staining for cleaved caspase 3 is suggestive of apoptosis within the organotypic culture. The increased staining in the treated organotypic cultures compared with the untreated controls was suggestive of an effect following oncolytic virus application. This was further reinforced in our experiment as there appeared to be co-localisation of both anti-vaccinia virus and anti-GFP stains in areas of increased cleaved caspase 3 staining. Furthermore, we found that there was an increased expression of cleaved caspase 3 with increasing viral concentration suggesting a dose-response relationship. We also treated organotypic cultures with heat inactivated virus to further assess that the changes we were seeing in treated tissue were a consequence of treatment with oncolytic vaccinia virus. In these treated tissue slices there was no positive staining for anti-GFP antibody as compared with the untreated controls (figure 4.7).

Figure 4.7 Control experiment confirming no effect on tissue slices treated with heat inactivated vaccinia virus at $10^7$ pfu/ml. Staining for anti-GFP antibody and cleaved caspase 3 similar in untreated slices and those treated with heat inactivated virus. Positive controls also shown, positive staining for anti-GFP in tumour slice treated with vaccinia virus expressing GFP and tonsil tissue used as a control for cleaved caspase 3 staining.
4.2.5.2 Quantification of 3,3′-Diaminobenzidine immunohistochemical staining

We wanted to quantify the staining for cleaved caspase 3 and GFP in the organotypic cultures that were treated with increasing pfu/ml of vaccinia virus to get an objective measure of the positivity of staining in our slices. Evaluation of immunohistochemical staining was performed using the open resource digital image analysis software, ImageJ. The IHC profiler application developed by Varghese et al.\textsuperscript{131} enables automated digital IHC analysis for unbiased, quantitative assessment of antibody staining intensity in tissue sections. To create this programme the authors adopted the spectral deconvolution method of DAB/haematoxylin colour spectra by using optimised optical density vectors of the colour deconvolution plugin for separation of the DAB colour spectra. The plugin has been assessed on thousands of scored DAB stained IHC images. A comparison study between use of the plugin and manual analysis resulted in an 88.6% match with a statistical significance of $P<0.0001$\textsuperscript{131}.

After performing this analysis, we found that there was a dose response relationship between pfu/ml vaccinia virus and percentage of positive staining for GFP as well as cleaved caspase 3. The percentage of positive staining within each slice was variable in all four cases but tended to be maximal at VV 10\textsuperscript{7} pfu/ml. In three of the four cases there was a significant increase in positive staining for GFP at higher concentrations compared with untreated controls. In one case there was a statistically significant increase in GFP staining between slices treated at VV10\textsuperscript{7} pfu/ml and those treated at VV10\textsuperscript{4} and VV10\textsuperscript{5} pfu/ml (figure 4.8).

When assessing for percentage positive staining for cleaved caspase 3 we saw similar results as to those of staining for GFP. In two out of four patients we saw a significant increase in cleaved caspase 3 positive staining in treated slices compared with untreated controls. In one patient there was a statistically significant increase in staining for cleaved caspase 3 at the highest pfu/ml of vaccinia virus compared with lower concentrations.
The results of this quantification found that staining for cleaved caspase 3 and GFP were similar in all four patients. This is a reassuring finding as we would expect that staining for cleaved caspase 3 and GFP would correlate. If there was a large disparity between staining, then it would be difficult to attribute a relationship between viral infection and effect in tissue as measured by staining of cleaved caspase 3. Our results therefore indicate a relationship between viral infection and apoptosis in the treated organotypic cultures.

Figure 4.8 Quantification of anti-GFP and cleaved caspase 3 antibody staining using IHC in four patients treated with increasing pfu/ml of vaccinia virus expressing GFP compared with untreated controls. Amount of virus used escalated from 1x10^4, 1x10^5, 1x10^6 and 1x10^7 pfu/ml. Untreated control used as a comparison to assess the effects of culture conditions on the organotypic culture. Treatment was for 5 days after which the slices were fixed in formalin in preparation for IHC analysis. These figures show a dose response relationship with an increasing expression of GFP with increasing concentration of virus treatment. CC3 – cleaved caspase 3; GFP – green fluorescent protein. Statistical analysis using two-way ANOVA. Statistical level of significance: *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001
4.2.5.3 Evidence of virus presence in organotypic cultures using immunofluorescence

We wanted to further assess for viral presence in our organotypic cultures following the results of these initial experiments. As the VACV encoded GFP it was possible to assess for the presence of virus using direct immunofluorescence on both the processed paraffin embedded 4µm slices for IHC as well as the 300µm slices fixed in formalin before they were processed.

The 4µm slices were prepared in the same way as those for IHC analysis. These were then de-paraffinised (as outlined in methods) and then counter-stained with the nuclear stain TOPRO®-3. The confocal microscope was used to assess for GFP expression within these treated slices compared with untreated controls.

Using this method, we saw expression of GFP in the cytoplasm of infected cells that were counterstained with the TOPRO®-3 nuclear stain. In comparison the untreated controls only stained positively for the nuclear stain, there was no GFP expression in these slices. These results confirmed viral presence through GFP expression in the treated organotypic cultures (figure 4.9).

Figure 4.9 Confirmation of viral presence through the expression of GFP in treated 4µm tissue slice compared to untreated control. Left to right: untreated control tissue stained with nuclear stain TOPRO®-3; tumour slice treated with virus with

![Figure 4.9](image)
evidence of expression of GFP in cell cytoplasm with nuclear counterstain confirming viral presence in organotypic cultures after treatment with vaccinia virus.

Whilst we saw GFP expression in these fine slices we wanted to assess whether the viral treatment was able to penetrate through the organotypic cultures or whether the findings were confined to superficial aspects of the organotypic cultures. To do this we decided to perform Z-stack imaging through the treated 300µm tissue slices as this would be able to assess the depth of penetrance of the oncolytic virus.

Each tumour core retrieved at the time of surgical resection was prepared as outlined in methods before undergoing slicing using a vibratome to 300µm thick slices. Organotypic cultures were treated with virus and cultured in the conditions stated in methods. Comparative untreated controls were also processed and assessed at the same time. After treatment was completed tissue slices were fixed in formalin before being prepared for confocal microscopy as outlined in methods. Using the confocal microscope, we were able to perform Z-stack imaging at multiple points through the 300µm slices to assess for GFP expression throughout the thickness of the culture. The results from the Z-stack images obtained through our tissue slices showed that there was some evidence of penetrance through the organotypic cultures with GFP expression seen at 60 - 65µm depth (figure 4.10).
4.2.5.4 Evidence of viral replication in tissue slice model

As well as direct visualisation of virus through GFP expression we wanted to confirm viral replication in our model further. To do this we first collected the supernatants of treated tumour slices. These were then frozen and sent to Transgene, France where viral titres were calculated. After calculating the titres at the end of treatment we compared them to the initial concentration of virus used to treat the organotypic cultures initially. This would allow us to assess for any obvious changes, with an
increase in viral titre compared with the original viral concentration being indicative of viral replication in our organotypic culture system.

Results from our experiments using three patients with colorectal tumours found an increase in viral titres when compared with the original viral concentration applied to slices (Figure 4.11). This was assessed from 0 hours and at 24 hour increments thereafter up until 96 hours of treatment. This was also within the maximum period of time of 120 hours that the organotypic cultures could be viable for using our methods. The results from these investigations showed that there was an increase in viral titres in all three treated tumours with all concentrations of virus used. We found that the maximum peak in viral titre compared to the initial treatment tended to be at 24 hours in most cases however this could also be at 48 or 72 hours before viral titres would start to decline (Figure 4.11).

These results confirmed that there was evidence of viral replication in organotypic cultures following the administration of vaccinia virus.
Figure 4.11 Viral titres in supernatants of tissue slices from 3 patients with colorectal liver metastases treated with increasing concentrations of virus and assessed at 24-hour intervals up to 96hrs. Increased concentration of virus compared with concentration added at 0hrs suggestive of viral replication. Graphs represent data from three independent experimental repeats with two samples for each concentration of virus.

Having confirmed the increase in viral replication through the assessment of viral titres in supernatant we wanted to assess this visually also. To do this we aimed to assess GFP expression in our organotypic cultures at 24-hour intervals up to 5 days which was the maximum period of time that we found that
our organotypic cultures could be viable. Organotypic cultures were prepared and treated with virus as outlined in methods. In this experiment we used the highest concentration of virus (1x10^7 pfu/ml) expressing GFP to treat our cultures. At 24-hour intervals slices were fixed in formalin before being processed in the same ways as previously described for confocal microscopy (see methods). Confocal microscopy and z-stack imaging was then performed for each slice to assess for the presence of GFP (and therefore virus) in organotypic cultures and also if this expression changed over time. Our time-course analysis showed that there was an increased expression of GFP throughout the tissue slices up to 72 hours before this began to reduce (Figure 4.12). The findings from this experiment corroborated our findings from the assessment of viral titres in supernatant as there was evidence of viral replication through increased expression of GFP following treatment with oncolytic vaccinia virus.
Figure 4.12 Z-stack imaging using confocal microscopy confirming the spread of virus through tissue slices over a 120 hour time period. Evidence of viral replication through increased expression of GFP throughout the tissue slices.
4.2.6 Assessment of FCU1 pro-drug activation in organotypic model

Having established evidence of viral infection in our organotypic cultures using vaccinia virus expressing GFP we then wanted to assess the pro-drug activation model of our vaccinia virus expressing FCU1. Organotypic cultures were prepared as outlined in methods. They were then treated for 48 hours with virus before the addition of 100µM of the pro-drug 5-FC and left for up to 120 hours before being fixed in formalin. At the same time 50µL of supernatant was taken after the onset of the addition of 5-FC and frozen. These specimens were then sent on ice to Transgene, France where HPLC was performed (see methods) to assess the activity of the FCU1 gene in converting 5-FC to 5-FU (figure 4.13).

Results from three experiments found variable rates at which 5-FC was converted to 5-FU. In all three experiments there was an increase from 72 to 96 hours following the addition of pro-drug to organotypic cultures treated with virus expressing FCU1. In two of the three cases was a high percentage conversion at 96 hours, this was 48 hours following the addition of 100µM of 5-FC to the
organotypic cultures treated with virus. In patient 2 there was a very high conversion of 5-FC to 5-FU just 24 hours after the addition of pro-drug.

The variability in the conversion rates is likely to represent heterogeneity of the tumours of each of these patients. The results were encouraging with regards to the pro-drug activation model as there was evidence of 5-FU in the supernatant of treated organotypic cultures of all three patients. The high percentage of conversion from 5-FC to 5-FU in two of three cases was also promising.

To further assess the pro-drug activation model, we also assessed treated organotypic cultures through immunohistochemical staining with anti-vaccinia virus antibody and anti-cleaved caspase 3 antibody (Figure 4.14).

Figure 4.14 IHC staining for cleaved caspase 3 in organotypic cultures left untreated in media; treated with media and 5-FC; treated with virus alone and treated with virus and 5-FC. No obvious difference in staining for cleaved caspase 3 in organotypic culture treated with media and 5-FC when compared with untreated control. Similar appearance of staining for organotypic cultures treated with virus alone or those treated with pro-drug and virus in combination.
Results from our IHC staining for cleaved caspase 3 did not show an obvious difference in staining between untreated controls and those slices treated with pro-drug and media. These results were encouraging and indicate that 5-FC alone does not induce apoptosis in organotypic culture. There appeared to be similar staining for cleaved caspase 3 in those organotypic cultures treated with vaccinia virus at $10^7$ pfu/ml and those treated with the same concentration of virus and 100µM of 5-FC.

As with our previous IHC experiments we used the IHC profiler application for image J to quantify our staining. This was performed as described in methods.
to assess if this had any impact on the effects of the vaccinia virus on treating organotypic cultures as we had seen in earlier experiments. In these two experiments as with previous experiments the percentage of positive staining for virus and cleaved caspase 3 was similar suggestive of a relationship between virus presence and cleaved caspase 3 expression. There was no significant difference in cleaved caspase 3 expression in organotypic cultures treated with media alone or treated with media and 100µM 5-FC. As anticipated there was an increase in staining for cleaved caspase 3 and vaccinia virus in cultures treated with virus compare to controls. There was a statistically significant increase in cleaved caspase 3 staining in cultures treated with virus or virus and pro-drug when compared to controls. In one of the two experiments that was a statistically significant increase in cleaved caspase 3 staining in organotypic cultures treated with virus and pro-drug compared to virus alone. This result was encouraging with regards to the pro-drug activation model but given that it is an isolated result it is not possible to conclude a strong difference between treatment with virus alone and virus in combination with 5-FC.

4.2.6.1 Assessment of immunogenicity through HMGB1 expression in tissue slice model

Results from our work with colorectal cancer cell lines suggested that vaccinia virus induced predominantly apoptotic cell death, and that this was immunogenic given the increase in various markers and determinants for this. Given the upregulation of the marker HMGB1 in all three colorectal cell lines, this was felt to be an appropriate marker to assess in the organotypic culture model. HMGB1 is a DAMP and is released extracellularly in immunogenic cell death. We postulated that if our experiments were successful in demonstrating immunogenicity of vaccinia in tissue slices, this would be manifest through the extracellular presence of HMGB1 on staining.
Figure 4.16: HMGB1 staining in original histopathology blocks in four patients and staining of treated and untreated control in prepared organotypic cultures. Evidence of variable HMGB1 expression, with cytoplasmic expression in patients 1 and 2 whilst staining was nuclear for patients 3 and 4. In untreated organotypic cultures and those treated with vaccinia virus at maximum pfu/ml there were no obvious differences in expression with HMGB1 in both being cytoplasmic.
Results from our experiments found that whilst we were able to stain for HMGB1 in IHC, the pattern of staining did not correspond to whether patients had been treated with vaccinia virus or not. Figure 4.16 shows the cytoplasmic expression of HMGB1 in both untreated control and at maximal pfu/ml of virus. Similarly, its expression in the original patient histopathology blocks was variable with two of four patients having cytoplasmic HMGB1 and the others having nuclear HMGB1 expression.

As there was no clear relationship between HMGB1 and treatment in the organotypic culture model we felt that this was not a marker that would help to establish immune mediated cell death in this instance.
4.3 Discussion

Work in our experiments lead to the successful development of an organotypic culture system in our laboratory. Through our own preliminary work and based on the recommended workflow for organotypic cultures published by Davies et al\textsuperscript{155} we were able to develop an organotypic culture system that would enable the interrogation and treatment of tumours from a minimum of 3 days up to a maximum 5 days. As per Davies et al\textsuperscript{155} we found a marked improvement in organotypic culture tissue architecture when placing our organotypic cultures on filter supports with tissue morphology being most similar to tissue in vivo when incubated on these. The filter supports hold the organotypic cultures in place during incubation and this therefore mechanically prevents tissue distortion, as the filter support is porous it also allows nutrients from media to bathe the underside of the culture. Leaving organotypic cultures to be submerged in media and on a rocking plate led to mechanical damage of the organotypic cultures as they washed around unsupported within their wells during incubation. This naturally led to the tissue becoming distorted compared with the original and manifest in separation of the tissue slices and in severe cases loss of part of the organotypic culture or disintegration of the organotypic culture. In our modified version the organotypic cultures were placed within wells on filter supports whilst being placed on a rotating plate inside the incubator. This enabled nutrients from the prepared media to reach the organotypic cultures as it was continually washed over the whole tissue slice repeatedly during rotations, rather than the tissue being permanently submerged in media whilst rocking. This improved our incubation methods as it had the dual effect of preventing tissue from becoming ‘water-logged’ when constantly submerged in media whilst also permitting oxygen to reach the organotypic culture. In the workflow produced by Davies et al it was noted that organotypic cultures floating in low oxygen levels led to a significant alteration in a number of stress pathways which was accompanied by a loss of tissue integrity. Placing organotypic cultures on filter supports in atmospheric oxygen levels ameliorated this. As solid tumours are usually hypoxic our atmospheric oxygen conditions produced a hyperoxic environment which assisted in the maintenance of the viability of the organotypic cultures\textsuperscript{156}. 
As with all in vitro models for cancer research there are limitations with this one also. In order to develop a high throughput process there needs to be excellent communication between the team in the lab and the surgical team to enable prompt processing of these samples at the time of resection. There also needs to be a sufficient availability of patient tumour to enable experiments to be carried out. This requires excellent ties with the oncologists and surgeons at the Royal Surrey Hospital, Guildford in this instance which we were fortunate to have. Tissue availability is dependent on the number of oncological resections being performed as well as the suitability of tumours to be included in the study. As well as surgical and institutional factors there are patient or tumour factors that also need to be taken into consideration. For instance, certain small tumours cannot be biopsied as there is a risk of interfering with the resection margins of clearance and interfering with the histopathological processing performed in the pathology department which can have an impact on the perceived success of surgery, the need for further treatment and the patients’ prognosis. The consistency of tumours is also variable, with larger tumours often possessing necrotic cores that can either not be biopsied or that are extremely fragile resulting in the inability of slices to be cut from them. In patients undergoing liver resection, many will receive neoadjuvant chemotherapy of variable efficacy and this can also unpredictably affect slice viability. Tumour size factors as well as tissue composition are often not certain until the time of resection. During these experiments I was present at all surgical procedures at the time of tumour resection which enabled me to assess the tumours first-hand and assess the suitability for biopsy. This improved over time as my experience working with this tissue improved. My presence at these resections also enabled the prompt retrieval of the tissue and processing of these tumour cores. Another limitation of the system is the uncertainty of the numbers of slices that we would be able to procure from each biopsy taken. This often limited what we were able to do experimentally and often would mean that planned experiments would need to be adapted in terms of number of duplicates and comparison of differing treatments. Each organotypic culture would also be slightly different from the previous one owing to the heterogeneity of tumour tissue within each biopsy. Tumour cores that are obtained and sliced at 300µm intervals
will still demonstrate variability from the top of the core to the bottom despite it coming from the same area of tumour. Although anticipated, this variability can have an impact when trying to compare to treatments, especially if the organotypic culture has varying amounts of cellular and stromal material within it. As well as patient or tumour factors, processing factors can also influence the culture method. The duration of the process, from being present at the time of surgical resection to the production of 300µm slices is extremely time-consuming. It was essential that I would be able to process these tumour cores immediately following retrieval to prevent tissue degradation and to optimise outcomes. The whole process from theatre to the production of these cultures would often take upwards of 3 hours and so it involved a lot of man-hours, often running late into the evening. On numerous occasions following the successful processing of organotypic cultures to the point where they are ready for histological analysis the results can show that the experiment has been unsuccessful in terms of the tissue obtained at the time of biopsy. This can result in the time-consuming process of analysis on tissue that ultimately cannot be assessed for these purposes. In total we processed 30 patient tumours using this method with varying success. We found that of the 30 patient tumours that were processed, 21 were of sufficient quality to assess in terms of IHC or immunofluorescence. This equals a success rate of 70% for tumour cores that completed processing. When considering the number of hours required to process 9 unsuccessful tumour samples it is easy to see how this method has not come to be used regularly in the laboratory to assess novel anticancer treatments. The number of unsuccessful tumour cores being processed is likely to be higher given that we also assessed further tumour cores that were not of sufficient quality to produce 300µm slices. As mentioned earlier, with greater experience it was easier to determine whether or not an experiment would be successful in terms of the tissue obtained and to decide whether or not to proceed with processing or not.

Despite these limitations organotypic cultures represent a high content experimental platform for the interrogation of tumours in vitro. The organotypic culture model is a powerful technique that is an accurate representation of the tumour in vivo including its stroma and heterogeneous microenvironment, permitting the interrogation of tumours in the laboratory. We have demonstrated
that with close surgical and oncological ties with our local hospital we were able to develop a high throughput experimental model for the purposes of our study.

Satisfied with our culture methods and results from preliminary experiments we wanted to assess if it would be possible for us to examine the effects of oncolytic vaccinia virus on organotypic cultures. We treated cultures with increasing pfu/ml of virus up to a maximum of $10^7$ pfu/ml in the first instance to see if there was any effect of treatment and if there was an effect to see if dose of virus impacted on this. We found through IHC staining and immunofluorescence that our organotypic cultures did become infected by vaccinia virus during incubation. We found there to be a dose response relationship between treatment with virus and expression of GFP and cleaved caspase 3 using IHC staining. In order to quantify the staining for cleaved caspase 3 and GFP in the organotypic cultures that were treated with increasing pfu/ml of vaccinia virus to get an objective measure of the positivity of staining in our slices we used the IHC profiler application developed for imageJ by Varghese et al \(^{131}\). These results confirmed the perceived visual dose-response relationship that we had seen in our IHC staining. The increase in positivity of staining was statistically increased in 3 of the four patients assessed for cleaved caspase 3 staining and GFP at the highest doses of vaccinia virus ($10^6$ pfu/ml and $10^7$ pfu/ml) compared with untreated organotypic cultures or those treated with lower doses of vaccinia virus. We also confirmed viral replication and therefore infection using direct visualisation of virus through confocal microscopy and through the assessment of viral titres in supernatants. This confirming the findings from previous studies of viral replication within 72 hours of infection \(^{101}\). The ability to utilise organotypic cultures to assess treatment this way is further evidence as to the utility of the culture method and the additional information that this in vitro method can provide during in vitro work with novel oncological therapies.

Our experiments have highlighted oncolytic vaccinia virus to be an excellent oncolytic virus choice in the treatment of colorectal liver metastases. The attenuated vaccinia virus has demonstrated good tumour selectivity in our organotypic cultures with no evidence of effects in normal healthy liver in
our experiments. As vaccinia virus has an excellent safety profile in human use as well as its ability to evade the host immune response and given the tumour specificity that we have seen it lends itself to systemic administration which has been a limitation in the development of oncolytic virotherapy thus far. Work using the z-stacking feature of the confocal microscope has also demonstrated that vaccinia virus is also able to penetrate our tumour cultures rather than simply having an effect on the slice surface alone. Our results found that staining for vaccinia virus extended down to 60 \( \mu \text{m} \) depth. Tumour penetrance is an important consideration in the treatment of solid tumours with oncolytic viruses. Barriers that reduce the spread of oncolytic viruses include elevated interstitial pressures, dense connective tissue, necrotic tissue, calcification, and reduced vasculature. Certain software packages available for the confocal microscope enable quantification of staining. We were unable to perform this in our laboratory during our experiments as we did not have this software available. Quantification of confocal microscopy would have been a useful tool in our experiments and ideally would have been performed.

For many years 5-FU was the principle agent used in the treatment colorectal cancer and is included in chemotherapy regimens for the treatment of colorectal liver metastases today. Foloppe et al. first used a replication competent Copenhagen strain vaccina virus expressing the transgene FCU1 in the pre-clinical setting. The study found that the combination of VV-FCU1 and 5-FC resulted in greater toxicity to human cancer cell lines compared with virus alone. The same was also found in the treatment of nude mice bearing LoVo colorectal tumours subcutaneously that were treated with systemic or intratumoral virus administration. Our experiments assessed the use of this pro-drug activation model in organotypic cultures. Following treatment with virus, frozen supernatants stored at 24-hour intervals were sent to Transgene, France. At their laboratory HPLC was performed to assess for the conversion of 5-FC into 5-FU. Results from these experiments confirmed a high conversion of 5-FC to 5-FU suggesting that the pro-drug activation model worked and that it was possible to assess this using organotypic cultures. To further assess the effects of the pro-drug activation model we assessed the IHC staining for these treated slices and compared them to those treated with virus alone.
and untreated controls. Both experiments found that there was a significant increase in cleaved caspase 3 expression in organotypic cultures treated with VV-FCU1 compared with untreated controls. In one of the two experiments, the effect of treatment using the pro-drug activation model was significantly greater than treatment with virus alone. Whilst this result is encouraging, it is in isolation and further experiments would be required before it would be possible to reach a conclusion as to whether this is a better treatment than virus alone. In a study conducted by Heinrich et al they found that the same vaccinia virus and pro-drug activation system did not result in a significant additional effect on melanoma cells\(^\text{142}\). This may be a result of the inhibition of viral replication by 5-FU that has been shown with other viruses such as HSV-1\(^\text{159}\).

We had hoped to evaluate the organotypic model for evidence of immunogenicity of treatment with oncolytic virus. Given our findings of consistently raised levels of HMGB1 in our tissue culture experiments we had hoped that we would be able to stain for this using IHC. Unfortunately, we found HMGB1 staining to be an unreliable marker in this model. Staining for this was inconsistent as demonstrated in Figure 4.14. Original tissue blocks for different patients stained differently for HMGB1 with regards to staining being nuclear or extracellular. This appeared to be variable in all four patients rather than us seeing it predominantly being stained for in the nuclei. This made the assessment of ICD in our organotypic cultures treated with vaccinia virus difficult. Of the prepared organotypic cultures that were treated we found no obvious effect of treatment with oncolytic vaccinia virus. Staining was extracellular in both the untreated and maximally treated slices. This finding could indicate that in making the organotypic cultures we alter the expression of HMGB1. Another important consideration is that as cancers evolve they are detected by the host immune system thus triggering anti-tumour responses. Immune damage of cancers is a natural occurrence which can affect ICD assessment as this natural response to tumour will increase markers of ICD within the tumour tissue, and we may not see a difference in this following virotherapy compared to pre-treatment. Other markers of ICD can be stained for in IHC including calreticulin and CD8 and these could also be used to assess for ICD in the organotypic culture model\(^\text{160}\).
4.4 Conclusion

We have developed a high-throughput, high content organotypic model in our laboratory with which to assess patient tumours. In optimal culture conditions these organotypic cultures are viable for a minimum of 72 hours and a maximum of 120 hours which enables the assessment of oncological treatments such as oncolytic viruses, as we have done. Oncolytic vaccinia virus was able to replicate in and exert anti-tumour effects in this model as evidenced by direct immunofluorescence, assessment of viral titres in supernatants and through IHC staining. The effect on organotypic cultures of virus alone was similar to that of virus expressing FCU1 and the addition of pro-drug although one experiment did demonstrate a significant increase in expression of cleaved caspase 3 in the virus and pro-drug model compared with virus alone. Having demonstrated in a tissue culture model that cell death following treatment with vaccinia was immunogenic we were unsuccessful in our attempts to assess this using staining for HMGB1 in IHC. Further work is required to assess this in an organotypic culture model.
Chapter 5

Treatment of organotypic cultures derived from colorectal liver metastases with oncolytic vaccinia virus and exposure to cavitational ultrasound and sulphur hexafluoride microbubbles
Chapter 5 - Treatment of organotypic cultures derived from colorectal liver metastases with oncolytic vaccinia virus and exposure to cavitational ultrasound and sulphur hexafluoride microbubbles

5.1 Introduction

Oncolytic virotherapy is a powerful emerging tool in the treatment of cancer. Clinical trials have confirmed the therapeutic effects of oncolytic virotherapy in the treatment of multiple solid tumours. A limitation of this novel treatment is the restricted ability of oncolytic viruses to reach and penetrate target tumours following intravenous administration. This hurdle has limited their clinical utility with many oncolytic virotherapies reliant on intratumoral administration. Whilst this approach provides a localised anti-tumour effect and has the ability to stimulate the generation of systemic anti-tumour immunity it does not utilise the unique ability of viruses to infect and destroy metastatic tumour deposits. This route of administration can also pose a problem in certain patients such as those with colorectal liver metastases in whom some tumours may not be amenable to intratumoral injection. This can be due to their anatomical location within the liver as well as their proximity to major vessels in the abdominal cavity such as the vena cava.

Oncolytic viruses are unique as a cancer therapeutic in their ability to replicate and self-amplify within the target tumour bed and subsequently spread within and between tumours. For localised tumours intratumoral administration of virus is easily implemented. For patients with metastatic disease such as those with colorectal liver metastases, intravenous administration of oncolytic virus is seen as the preferred method of administration as it provides access to all vascularised tumour sites within the body. The goal of systemic therapy is to exceed the viremic threshold above which the virus infect a critical number of tumour sites and replicate with a resultant destruction of tumour deposits. Current research in oncolytic virus delivery has focused on limiting sequestration of virus in the liver.
and spleen, evading viral neutralisation in patient serum, targeting viruses to vascular endothelia cells lining tumour blood vessels and selectively enhancing their permeability.

5.2 Strategies to improve intravenous delivery of oncolytic virus therapy

5.2.1 Limiting viral sequestration in the liver and spleen

Intravenous viruses are rapidly cleared from the circulation following sequestration by the mononuclear phagocytic system of the spleen and liver. Prior to clearance viruses are opsonised with antibodies, complement, coagulation factors and other proteins that result in their recognition by the macrophages of the spleen and the Kupffer cells of the liver. Their recognition results in phagocytosis and clearance from the circulation.

Strategies used to overcome sequestration have included chemical modification of viral coats by conjugation of biocompatible polymers such as polyethylene glycol (PEG). The result of polymer coating in clinical practice has been shown to increase the circulating half-life of treatments. The result of polymer coating can be a reduction in viral infectivity, however this can be restored by re-engineering receptor binding ligands onto the surface of shielded particles. A different approach to limit the sequestration of viruses readily bound by IgM and complement proteins is to decrease the amount of these factors in patient serum by pre-treatment agents such as cyclophosphamide. This has been shown to facilitate the initial infection of tumours by oncolytic viruses.

Tao et al confirmed that it is possible for the mononuclear phagocytic system to become saturated, leading to increased circulating volumes of virus. Sequestration of the mononuclear phagocytic system can be achieved by pre-conditioning to saturate scavenger receptors or by poisoning the endothelial cells. Saturation of scavenger receptors has been demonstrated in mouse studies in which pre-dosing with polyinosinic acid which binds to scavenger receptors on endothelial cells and macrophages in the liver and spleen reduced sequestration of treatment with oncolytic adenovirus.
Depletion of Kupffer cells of the liver and splenic macrophages has been achieved in mouse models by treatment with clodronate-loaded liposomes. This technique has been combined with oncolytic virotherapy to improve treatment of oncolytic adenoviral treatment. Other methods of sequestration include pre-treatment with gadolinium chloride, gamma globulins or pre-dosing with high doses of viral particles prior to administering a second dose after a 4 hour period.

5.2.2 Evasion of viral neutralisation by serum factors

Attempts have been made to overcome factors in serum such as neutralising antibodies, complement or scavenging by Kupffer cells by hiding onvolytic viruses inside carrier cells. In pre-clinical models techniques such as infusing ex vivo infected cell lines or using normal primary cells such as dendritic and T-cells that can home to tumour beds. Other cell types used to carry oncolytic viruses to tumours include mesenchymal stem cells. These cells preferentially engraft in solid tumours and have been shown to deliver virus in the presence of neutralising antibodies.

5.2.3 Increasing the permeability of tumour blood vessels

The presence of fenestrae and intracellular gaps between the tumour endothelial cells far larger than those of normal blood vessels causing the tumour vasculature to be more susceptible to the passage of macromolecules, viruses and nanoparticles. Poor lymphatic drainage and the increased interstitial pressure arising from the presence of dense stromal tissue impedes virus extravasation and diffusion. To overcome this issue the increased vascular permeability of tumours can be increased by the administration of factors such as interleukin-2 and tumour necrosis factor alpha prior to treatment with the desired anti-cancer agent. Another method of reducing the interstitial pressure within tumours and thereby overcoming the reduced extravasation in tumours is through treatment with chemotherapeutic agents. The tumour vascular permeability is maintained following chemotherapy. Another agent that has been shown to increase the vascular permeability of tumour blood vessels and improve oncolytic virotherapy is an inhibitor vascular endothelial growth factor.
(VEGF). In a mouse model, the administration of VEGF inhibitors along with oncolytic virus treatment resulted in an improved oncolytic virus infection.

5.3 Systemic administration of vaccinia virus

Vaccinia virus has biological characteristics that make it particularly encouraging for use as an oncolytic agent. These characteristics include its stability intravenously and its ability to reach target tumours in the blood, its rapid spread within tumour tissues, the ability for the virus to be engineered to encode large transgenes as well as its safe historical use in humans as a vaccine against smallpox. Moreover genetically modified vaccinia virus is able to selectively target tumour cells whilst sparing non-malignant cells, thereby reducing damage to healthy tissues. As well as its selective targeting of tumour cells its safety is further enhanced due to its site of replication in the cytoplasm of cells, which prevents the integration of viral DNA into host chromosomes and the passage of viral progeny into daughter cells.

The infectious forms of vaccinia virus include the intracellular mature virion, the intracellular enveloped virion, the cell associated enveloped virion and the extracellular enveloped virion. Of these the most commonly produced during assembly are the intracellular mature virion and the extracellular enveloped virion, with assembly taking place in viral factories within the cytoplasm.

The intracellular mature virion is the simplest form of vaccinia virus, and consists of a single phospholipid bilayer. Sometimes this will change into an extracellular enveloped virion. This occurs as a result of intracellular mature virions exiting the cytoplasm via microtubules and undergoing extra modifications including the assembly of an additional membrane that is formed by viral transport through endosomal or trans-golgi cisternae. The extracellular enveloped virion is able to spread more rapidly from cells compared with the intracellular mature virion due to its early release from cells following replication. The extracellular enveloped virions also have fewer viral antigens exposed on their outer surface as well as incorporating host cell proteins which enable it to go
undetected by the hosts immune system and limits viral destruction in circulation prior to arrival at the target tumour site\textsuperscript{104}.

5.3.1 Evasion of the immune response by Vaccinia Virus

Vaccinia virus also has several other countermeasures that enable them to replicate despite the innate immune response of the host. The host complement system is a an important host defence that destroys virus or virus infected cells and promotes phagocytosis of virions that have been opsonised by antibody via recognition of the Fc region bound to immunoglobulin\textsuperscript{186}. To counteract the complement system vaccinia virus expresses an abundant protein called vaccinia virus complement protein (VCP)\textsuperscript{186}. This protein is secreted from infected cells and binds to the complement components C3b and C4b, working as a co-factor with factor I in promoting cleavage of these two components and subsequently inhibiting activation of the complement cascade\textsuperscript{187}. It has also been found on the surface of infected cells as well as extracellular enveloped virions helping to defend these infectious virions against the complement system to\textsuperscript{186}. A second protein, B5 is a type 1 integral membrane protein that is found on the outer membrane of extracellular enveloped virions\textsuperscript{188}. Rather than protecting against complement this protein is required for the formation of extracellular enveloped virions and promotes dissemination of virus\textsuperscript{188}. Another defence against the host complement system is the acquisition of host protein CD55 in the extracellular enveloped virion envelope\textsuperscript{189}. This protein helps the extracellular enveloped virion to evade destruction by the complement system and is implicated in the greater resistance of these particles compared to intracellular mature vaccinia virions, which are extremely sensitive to the complement system\textsuperscript{186}.

Interferons (IFN) are another part of the host-immune response against viral infection. These are grouped into three classes: Type 1 interferons include IFN-\(\alpha\) and IFN-\(\beta\), and these exert their effects via the ubiquitously expressed type I IFN receptor\textsuperscript{186}. Type II IFN is secreted by activated immune cells such as natural killer cells and T-cells and activates macrophages and promotes cell mediated adaptive immune response\textsuperscript{186}. Type III IFN are also secreted following viral infection however these have a
more limited distribution within tissues. Each of these three interferons have a role in the host defence against vaccinia infection 186.

Interferon response is initiated after sensing viral associated pathogen-associated molecular patterns (PAMPs) through host pattern recognition receptors (PRRs) 186. Vaccinia viruses inhibit the host interferon response at multiple levels. The virus restricts PAMP production or minimises PAMP recognition by expressing proteins that block the pattern recognition receptor induced signalling pathway that activates transcription factors that lead to interferon induction, and by inhibiting host protein synthesis 186. Vaccinia virus also secrete proteins from the infected cell that capture interferons in solution or on the cell surface, preventing interferon from reaching receptors 186. They also block signal transduction of interferons by binding to their receptors 186. Finally they produce additional proteins within the infected cells that inhibit the action of interferon induced anti-viral proteins 186.

Tumour necrosis factor, interleukin-1 (IL-1) and IL-18 are pro-inflammatory cytokines that amplify the innate immune response to virus infection and influence the subsequent adaptive immune response. The cytokine tumour necrosis factor also has direct antiviral activity inducing apoptosis of infected cells 186.

Similar to the inhibition of interferons, vaccinia virus has developed strategies to block these cytokines at various levels. This includes the inhibition of proteolytic maturation of cytokines, the expression of soluble decoy receptors and inhibiting cytokine induced signal transduction.

Against IL-1 vaccinia virus acts by inhibiting the production of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is required for the transcription and synthesis of pro-IL-1β (an inactive intracellular form of IL-1) 186. A second signal that is required to activate caspase-1, which cleaves pro-IL-1β into active IL-1β, is provided by the activation of large protein complexes containing pattern recognition receptors called inflammasomes 190. Vaccinia virus has developed mechanisms to inhibit this process. Firstly, it expresses the F1 protein that is able to bind directly to the nucleotide-binding domain, leucine-rich repeat and pyrin domain containing protein 1 (NLRP1)
inflammasome and reduce the amount of active caspase-1 and IL-1β secretion. Certain strains of vaccinia virus also have a functional B13R gene that produce the protein B13 that binds caspase-1 and inhibits pro-IL-1β cleavage within infected cells, though this is not present in the Copenhagen strain of the virus. Secondly it combats the production of IL-1β from uninfected cells by expressing soluble IL-1R that is released from infected cells, capturing IL-1β in solution. It also expresses the protein B15 that is similar to the extracellular ligand binding domain of cellular IL-1R, therefore binding to IL-1β with high affinity and preventing it from binding to its natural receptor.

IL-18 is another pro-inflammatory cytokine. Similarly, with IL-1β it is synthesised from as an inactive precursor that requires cleavage by caspase-1. IL-18 helps clearance of infected cells by inducing IFN-γ synthesis and activating the natural killer and CD8+ T cells. Vaccinia virus has been shown to inhibit IL-18 through the production of the B13 protein that inhibits caspase-1, the secretion of a soluble IL-18 binding protein (C12) that prevents IL-18 reaching its receptor on cells and diminishes the production of IFN-γ and therefore diminishes NK cell and CD8+ T cell responses.

TNF-α and -β are pro-inflammatory cytokines that can induce cell death. TNF-α is thought to be produced predominantly from macrophages and activated T-cells. Its activity is attributed to the promotion of an anti-viral state in neighbouring, uninfected cells, selective cytolysis of infected cells as well as apoptosis and the recruitment of lymphocytes to sites of infection. Vaccinia virus acts to secrete proteins that mimic TNF receptors (vTNFRs) and prevent cellular binding as well as proteins that interfere with cellular signalling. TNF is also a target gene of NF-κB and therefore intracellular inhibitors of this also affect the production of TNF.

Chemokines are small chemoattractant cytokines that act to recruit leukocytes to sites of infection and inflammation. They are produced in response to infection and inflammation and bind to glycosaminoglycans on the surface of adjacent endothelial cell walls, establishing a concentration gradient. In the circulation, leukocytes express chemokine receptors on their surface and bind to
chemokines via their receptor-binding site. Chemokine production from the first infected cells by virus is important in the induction of the host immune response.

Vaccinia virus inhibits the production or function of chemokines in several ways. Following infection, engagement of pattern recognition receptors leads to interferon related factor 3 (IRF3) and NF-κB activation and the transcription of chemokine genes driven by these transcription factors. Varying strains of vaccinia virus such as Western Reserve, Wyeth and the Lister strain of the virus express immunomodulatory proteins that block intracellular signalling leading to chemokine production.

Vaccinia virus also expresses chemokine-binding proteins such as vaccinia virus CC chemokine inhibitor (vCCI), which binds to CC chemokines and is secreted from infected cells early during infection and is able to prevent the binding of chemokines to their target receptors. Another protein A41, like vCCI, is also secreted from cells early during infection, binding with chemokines with a lower affinity than vCCI with the resultant effect of preventing the establishment of the chemokine concentration gradient on endothelial cell walls which reduces the release of chemokines at sites of infection.

To prevent the initiation of the caspase cascade and the onset of apoptotic cell death vaccinia virus directly subverts the intrinsic pathway of the caspase-cascade by expressing F1 and N1 proteins. F1 protein adopts a Bcl-2-like (anti-apoptotic protein Bcl-2) fold and binds the apoptotic effector protein Bak at the mitochondrion preventing apoptosis. The F1 protein also binds to NLRP-1 which is an upstream activator of caspase-1 and reduces the inflammatory response. The N1 protein also has a Bcl-2-like fold and has a groove in its surface enabling it to bind with the pro-apoptotic Bcl2 proteins Bid and Bad and therefore inhibiting apoptosis.

As well as these anti-apoptotic, Bcl-2-like proteins some strains of vaccinia virus also express the proteins B13 and viral Golgi anti-apoptotic protein (vGAAP). The B13 protein has been shown to inhibit the activity of capsase-1 as well as the extrinsic pathway of apoptosis triggered by TNF-α or Fas ligand. The vGAAP protein is similar to the human version of the protein. These proteins are part of the
transmembrane Bax inhibitor-containing motif (TMBIM) family, including anti-apoptotic and calcium-modulating proteins like Bax inhibitor I. They act by modulating intracellular calcium stores. These proteins can protect cells from apoptosis via both the intrinsic and extrinsic pathways. These proteins however are less commonly expressed by various strains of vaccinia virus than the proteins F1 and N1.

Natural killer cells are specialised cells that act against virus-infected and tumour cells. These granular lymphocytes are highly cytolytic and are a host first-line defence against viral infection prior to the development of specific antibody against the virus and a cytotoxic T lymphocyte response. Despite being a first-line defence, natural killer cells still require activation prior to action. This is mediated by several cytokines (IFN-α and -β, as well as IL-12 and -18) but is mainly regulated by several activating and inhibitory receptors that integrate signals that are received from the surface of target cells. Vaccinia virus is able to modulate natural killer cell activation through the effects that it exerts on cytokines and interferons as highlighted earlier. Several receptors are involved in the activation of natural killer cells including the natural cytotoxicity receptors including NKp30. The vaccinia virus protein A56 (haemagglutinin), which is a known virulence factor of the virus, has been identified as a novel viral ligand for the natural killer cell activating receptors. The A56 protein, present on the surface of infected cells has been shown to block the activation of natural killer cells through the NKp30 receptor. This interaction between A56 and the NKp30 receptor has been found to lead to a decrease in the lysis of infected cells by natural killer cells when present on the surface of vaccinia virus infected cells. The A56 protein of vaccinia virus is the only known modulator of natural killer cell activity.
5.4 Challenges faced in the systemic administration of oncolytic virus to treat solid tumours

5.4.1 Tumour vasculature

The broad ability of vaccinia virus to evade the host immune response, enabling infection and viral replication highlight its potential as a candidate for intravenous oncolytic therapy. Solid tumours such as colorectal liver metastases pose a treatment difficulty for anti-cancer treatments. This is because of several factors that result in a non-uniform distribution of treatment as well as insufficient levels of treatment reaching the tumour \(^{199}\). These occur due to abnormalities in the tumour architecture and vasculature which lead to deficiencies in vascular and interstitial transport of these agents to tumours \(^{199}\).

In comparison with healthy tissue, tumour vasculature is leaky with endothelial cells containing large gaps between endothelial cells. The vasculature is also distributed in a chaotic fashion with varying vessel length and diameter. These vessels can also be tortuous and saccular as well as having haphazard interconnections, all of which render the tumour vasculature functionally abnormal \(^{199}\). The proliferation of tumour cells can also exert extra-luminal pressure on these vessels with a resultant impairment in tumour blood flow \(^{199}\). As the tumour vasculature is leaky and there is a lack of functional lymphatic drainage of the tumour there is a resultant increase in the interstitial pressure of the tumour compared to normal tissues. There is also a reduction in the plasma to interstitial gradient of oncotic pressure. Both the increased interstitial pressure and the reduction in the plasma to interstitial gradient of oncotic pressure contribute to the sub-optimal delivery of anti-cancer agents to solid tumours \(^{199}\).

Another factor that results in insufficient delivery of anti-cancer therapy to tumours is the increased distance between tumour cells and their blood vessels. High rates of tumour proliferation can also result in tumour cells forcing vessels apart, this can result in tumour being more than 100μm from their surrounding vasculature which can already be haphazard as described earlier \(^{199}\). This can also
result in the tumour becoming hypoxic – limiting the efficacy of radiotherapy treatment, as well as the lowering of tumour pH through the accumulation of metabolic products such as lactic acid which can affect the cellular uptake of some anti-cancer drugs.\textsuperscript{199}

5.4.2 Tumour extracellular matrix

The extracellular matrix can also prove to be a barrier in the successful delivery of anti-cancer treatment to solid tumours. The extracellular matrix consists of proteoglycans, collagens and other additional molecules produced and assembled by stromal and tumour cells.\textsuperscript{199} Higher collagen content in the extracellular matrix has been shown to reduce the diffusion of large macromolecules such as viruses through the tumour, thus limiting their ability to reach tumour cells.\textsuperscript{199} It can also physically obstruct the delivery of virus if the virus particles are larger than the gaps between these fibres in the extracellular matrix.\textsuperscript{199} Treatment with collagenase has been shown to increase interstitial transport of oncolytic viruses through the extracellular matrix.\textsuperscript{200} Collagen can also bind and stabilise glycosaminoglycans and hyaluronic acid which can result in increased interstitial pressure and reduce interstitial transport.\textsuperscript{201}

The tumour microenvironment is a significant obstacle when aiming to achieve uniform and sufficient delivery of anti-cancer therapy to solid tumours. Once overcome this could substantially improve the treatment of these types of tumour. Several strategies have been developed in an attempt to overcome this issue. These include co-injection of anti-cancer treatment with collagenase as well as the use of external sources of energy in combination with specific carriers that respond to the external energy source for targeted treatment release amongst others.\textsuperscript{199} One such technique that has been developed to help achieve this is ultrasound. Ultrasound can be used in several ways to help overcome the problems posed in the treatment of solid tumours by the extracellular matrix.
5.5 Therapeutic Ultrasound

Ultrasound has been traditionally used in healthcare for diagnostic purposes as an imaging modality. Ultrasound is usually produced from a piezoceramic crystal in short pulses. Diagnostic ultrasound is usually characterised by the center frequency of these pulses (between 2-12 MHz) which is usually a frequency related to the thickness of the crystal. Increasing the pressure amplitude, the frequency or the propagation length can distort the waveform and can result in tissue heating as well as enhancing non-thermal mechanisms. A decrease in the frequency increases the chance of cavitation and gas body activation. Power increase or increase in intensity tends to increase the likelihood and the size of all the bio-effect mechanisms of ultrasound.

5.5.1 Ultrasound induced heating

This is the result of the absorption of ultrasound energy in tissue. In comparison with diagnostic ultrasound, temperature elevations and the potential for bio-effects are kept relatively low by carefully described indications for use, applying the ALARA (as low as reasonably achievable) principle, limiting temporal average intensities and short exposure durations. In its use in therapy, ultrasonic heating utilises longer duration of heating with unfocused ultrasound beams or utilise higher intensity focused ultrasound compared to diagnostic ultrasound. Unfocused heating using ultrasound can be used in physical therapy to promote healing in highly absorbing tissues such as bones and tendons. Alternatively, focused beams can concentrate heat in tissue and this results in tissue coagulation of the purposes of tissue ablation. The result of ultrasound heating can vary from mild heating to coagulative necrosis to tissue vaporisation depending on temperature gradients.

5.5.2 Cavitation and gas body activation

Ultrasonic cavitation and gas body activation are similar mechanisms that rely on the rarefractional pressure amplitude of ultrasound waves. The transmission of ultrasound into tissues can exert rarefractional pressure amplitudes of several megaPascals. High rarefractional pressure can result in
cavitation activity in tissue when suitable cavitation nuclei are present, or directly induce pulsation of pre-existing gas bodies in tissues such as lungs and intestines, or with contract agents. Both cavitation and gas body activation primarily cause local tissue injury including cell death and haemorrhage of blood vessels 202.

5.5.3 Cavitation Nuclei

Gas bubbles with a stabilising shell are used in ultrasound imaging and have been used in pre-clinical studies of enhanced drug delivery. The shell of the microbubble can be coupled to therapeutics such as liposomes or can even be coated with drug. The gas core can also be utilised to transport oxygen or other useful cargo 203. Ultrasound wave reflections from tissue increase in proportion to variations in density and compressibility of the medium and gas bubbles that are highly compressible produce strong ultrasound echoes. In response to ultrasound waves these bubbles expand and contract. When they are exposed to a frequency that is near to the resonance frequency of the bubble they can expand in size. As they subsequently collapse the microbubble wall can reach hundreds of meters per second 203. The collapse of microbubbles can also lead to the formation of small jets that can impact nearby cell membranes with a resultant increase in transport of materials to the cell 203. In addition to the formation of jets additional mechanisms in microbubble-enhanced delivery include radiation forces and microstreaming of fluid. Radiation forces occur where micro-bubbles are displaced following exposure to ultrasound in the direction that the waves are displacing these. Local motion of fluid surrounding the oscillating bubble is known as microstreaming and has been shown to increase the uptake of therapies at a cellular level 203.

5.5.4 Thermal ultrasound applications

It is important that there is tight control of gene therapy. The aim of strategies trying to control gene therapy is to improve targeting and efficiency of gene delivery. Methods have been developed to control temporal transgene expression using gene promoters that that respond to external chemical
factors such as small molecules or antibiotics. Systemic administration of agents such as these however result in delivery of transgene to non-target tissues.

Heat shock protein transcription is initiated in tissues, within moments of exposure to temperature elevations higher than those required for maximum growth. This has been identified as a selective way of regulating gene transcription in tissues. Heat shock protein 70 (HSP70) is one such protein and has been identified as having the best overall response as well as being the first of the heat shock proteins to be repressed in the absence of a stimulus.

High intensity frequency ultrasound (HIFU) is able to generate increases in temperatures within tissues and subsequently turn on genes through HSP70 promoters. HIFU can be guided with imaging modalities such as magnetic resonance imaging (MRI), thus enabling accurate placement of the ultrasound beam and tight control of gene expression within target tissues.

The use of heat sensitive liposomes to encapsulate drugs is a method of improving drug delivery to solid tumours. Enhancement of drug accumulation in tumour can be achieved by incorporating polyethylene glycol (PEG) into the membrane. This results in a prolonged clearance time due to the protective effects of the PEG barrier against interactions with plasma proteins and the reticuloendothelial system. Liposomes can be designed to undergo change when heated and result in release of drug into tissues. By combining these liposomes with the heat induced by ultrasound waves there is a resultant improvement in delivery of drug to target tissues.

HIFU can also be used to cause thermal ablation of tissues. Pre-clinical studies have highlighted the potential of combining HIFU with chemotherapy for the treatment of cancers. It is believed that the combined treatment of tumours with chemotherapeutic agents and HIFU either sensitises the tissues to the usually sub-lethal effects of HIFU, or that the HIFU exposure increases the uptake of chemotherapeutic agents to these tissues.
5.5.5 Non-thermal ultrasound applications

Acoustic cavitation is defined as the growth, oscillation, and collapse of small stabilised gas bubbles under the pressure field of an ultrasound wave in a fluid medium. Two types of cavitation exist, these are called inertial and stable cavitation. Stable cavitation occurs where a gas bubbles oscillates around its equilibrium radius over several acoustic cycles. Inertial cavitation refers to bubbles growing several times their initial radius before collapsing under the inertia of the media that they are in.

Factors affecting acoustic cavitation include the number of gas bubbles available, also termed cavitation nuclei. There is an increase in cavitation with increasing numbers of gas bubbles. These are scarce in human tissues. Other factors include the availability of physical space for bubbles to form and grow, and it can be difficult for cavitation to be induced inside intact cells and in the extra-cellular matrix.

In open medium, following the collapse of cavitating bubbles, it has been noted that there are large increases in temperature locally and that these changes lead to the formation of hydroxyl molecules from dissociated water molecules. This can result in DNA damage, inactivation of proteins and enzymes and lipid peroxidation as a result of free radical formation. More pronounced effects of inertial cavitation occur within an area enclosed by a rigid boundary. As a result of being enclosed within an area there will be constraints in fluid flow. There is a resultant asymmetrical collapse of the bubble and the far side impacts and penetrates the bubble surface close to the boundary creating a jet. These have been shown to cause cracks in tissue surfaces, leaving them damaged.

Acoustic cavitation has been used as a therapeutic tool to disrupt the blood-brain barrier. Whilst continuous HIFU has been utilised to cause targeted ablation of plaques in the basal ganglia of patients with Parkinsons disease it can also be used in a pulsed manner. This subsequently lowers the rate of energy deposition within tissues and is not destructive. When this is utilised in a pulsed manner it leads to the reversible disruption of the blood-brain barrier. This is thought to occur due to the mechanical stress induced by the stably oscillating bubbles causing mechanical stress in adjacent
blood vessel walls without causing damage to brain tissue. Disruption at the blood brain barrier allows the extravasation of therapeutic agents. This has been utilised with some anti-cancer treatments that are usually unable to cross the blood brain barrier in the attempt to treat brain malignancies.

Inertial cavitation occurs where an ultrasound frequency causes the violent collapse of bubbles. This can be used to improve the permeability of individual cells and increase delivery of drugs and genes at a cellular level. This process is known as sonoporation and results following the creation of pores in cellular membranes by ultrasound waves. Sonoporation is a reversible process and as opposed to other mechanisms that attempt to improve gene delivery it is non-invasive and has the ability to be used in all tissues.

5.5.6 Use of ultrasound with oncolytic virotherapy

Cavitational ultrasound has been predominantly used on drug or gene therapy agents that do not have the capacity to replicate. It is potentially a strategy that could improve systemic oncolytic virotherapy. Oncolytic viruses are tumour selective, able to self-amplify and express therapeutic proteins from within solid tumours. The use of these anti-cancer agents is likely to continue to develop over the forthcoming years as clinical testing ensues in combination with conventional anti-cancer therapies as well as immuno-oncological approaches. Combining these two technologies could result in enhanced systemic delivery of oncolytic virus and more successful therapeutic outcomes in patients with solid tumours. In combination with systemic oncolytic adenovirus treatment, cavitational ultrasound and sulphur hexafluoride microbubbles were found to significantly increase tumour transgene expression without causing tissue damage in a mouse model. As well as sulphur hexafluoride microbubbles, inertial polymeric nano-cups have also been assessed as the nuclei to establish inertial cavitation following focused ultrasound exposure in tumours of mice treated with systemic oncolytic virus.

Bazan-Peregrino et al described the use of cavitational ultrasound to enhance treatment of tumours with oncolytic adenovirus. In this study an adenovirus expressing luciferase or green fluorescent
protein was used in the treatment of breast cancer in a murine model. Tumours were treated with intravenous or intratumoral adenovirus with sulphur hexafluoride microbubbles. In tumours that were treated intratumorally, these were sonicated using cavitation ultrasound for 20 seconds 2 minutes post injection. Fluorescence was measured 24 hours following treatment. Mice treated intravenously were pre-treated 24 hours prior to injection with clodronate liposomes. Adenovirus was injected at the start and 120 seconds later whilst exposure to cavitation ultrasound went on for 240 seconds from the initial administration of virus. The study saw an overall enhanced delivery of adenovirus to tumours exposed to sulphur hexafluoride microbubbles and ultrasound at the same time. There was no tissue damage seen following the addition of ultrasound and microbubbles with adenovirus through optimisation of these parameters. The study found that stable cavitation did not increase the effects of oncolytic adenovirus in tumour however inertial cavitation did result in an increased luciferase expression in tumours in vivo. This was variable from mouse to mouse and is due to tumour heterogeneity. The maximum increase in expression was up to 50-fold. The study concluded that oncolytic adenovirus delivery was enhanced with the addition of cavitation ultrasound and that this improved intratumoral spread of virus and increase its bioavailability 

Carlisle et al also assessed cavitation ultrasound and microbubbles with oncolytic adenovirus but also assessed ‘stealthing’ of oncolytic adenovirus using polymers that degraded upon exposure to the tumour milieu. A murine breast cancer model was again used. Mice were pre-treated with clodronate liposomes 24 hours to IV administration of virus. Virus was injected intravenously at 0 and 240 seconds with sulphur hexafluoride microbubbles whilst being exposed to cavitation ultrasound. The study found that stealthing of the oncolytic adenovirus reduced its clearance by complement. The low tumour pH resulted in uncoating of virus. There was an increased half-life of adenovirus that was coated compared with uncoated adenovirus. The study saw a two-fold increase in virus in tumour tissue following exposure to cavitation ultrasound and sulphur hexafluoride microbubbles. After 72 hours a 30-fold increase in virus expression was seen in tumours compared to unstealthed controls. Overall Carlisle et al concluded that stealthing oncolytic adenovirus in combination with ultrasound
and microbubbles led to a longer circulating time of virus as well as a greater reduction in tumour growth\textsuperscript{206}.

Myers et al assessed the use of specialised polymeric nanocups that were more efficient in instigating acoustic cavitation in tumours than the sulphur hexafluoride microbubbles \textsuperscript{205}. This study utilised oncolytic vaccinia virus in conjunction with cavitational ultrasound and polymeric nanocups in the treatment of liver tumours. The same cavitational ultrasound parameters and set up were used as in the Bazan-Peregrino and Carlilse studies \textsuperscript{204,206}. Virus was administered intravenously and cavitational ultrasound exposure lasted for up to 10 minutes with exposure at different points on the tumour. This study found that there was an increased expression of luciferase in tumours treated with virus and polymeric cups compared with microbubbles or virus treatment alone. It also saw a 10,000-fold increase in viral gene expression in tumours treated with ultrasound, nanocups and vaccinia virus compared with those that were treated with ultrasound, microbubbles and vaccinia virus after 5-days \textsuperscript{205}.

Based on these studies we felt that the organotypic culture model would be an effective way to assess the effect that cavitational ultrasound and microbubbles had on colorectal liver metastases that were treated with oncolytic vaccinia virus.
5.6 Aims

In this chapter we hoped to evaluate the use of cavitation ultrasound and sulphur hexafluoride microbubbles with vaccinia virus in our established organotypic model. We had previously established that oncolytic vaccinia virus infects and replicates in these organotypic cultures as well as exerting its anti-cancer effects through the increased expression of cleaved caspase 3 in treated slices when compared to untreated controls on immunohistochemical staining as reported in chapter 4. The aims of the chapter were to:

1. Assess viral infection through immunofluorescence in organotypic cultures treated in combination with virus, sulphur hexafluoride microbubbles and cavitation ultrasound compared with controls.

2. Assess the effects of cavitation ultrasound and sulphur hexafluoride microbubbles on organotypic cultures through the expression of cleaved caspase 3.

3. Assess the effect of virus on organotypic cultures through immunohistochemical staining for cleaved caspase 3 in organotypic cultures treated in combination with virus, sulphur hexafluoride microbubbles and cavitation ultrasound compared with controls.
5.7 Results

5.7.1 Assessment of the combination of oncolytic vaccinia virus with ultrasound and sulphur hexafluoride microbubbles in the treatment of organotypic cultures derived from colorectal liver metastases

As described in methods organotypic cultures were prepared and placed in culture conditions. To assess the effects of combination treatment organotypic cultures were placed inside a Millipore® Millicell® cell culture insert within a tissue culture plate. The specified treatment was then added within the cell culture insert containing the tissue slice and sealed with the sonolid. The apparatus was then placed within the system for acoustic transfection (SAT) chamber and exposed to the cavitational ultrasound frequency. Ultrasonic exposures were carried out using the System for Acoustic Transfection (SAT) chamber. This system was based on prior design by Carugo et al.[132] but modified to allow a decrease in the exposure area for the prepared tumour slices. We were able to utilise an existing system that was brought to our laboratory allowing us to utilise this technology in conjunction with oncolytic virotherapy.

In this first experiment using combination therapy the concentration of virus that was used was $10^7$ pfu/ml. This was the highest concentration of virus that had been used in previous experiments with organotypic cultures. Using $10^7$ pfu/ml vaccinia virus to treat organotypic cultures we had previously demonstrated viral presence in tissues through the expression of green fluorescent protein compared to untreated controls. It was not possible to quantify the amount of positive staining using this method due to the software available with the software available. It was therefore necessary to determine differences in staining subjectively.
Figure 5.1 Confocal microscope image of organotypic cultures from the same patient treated with vaccinia virus expressing green fluorescent protein compared with untreated control. Evidence of expression of green fluorescent protein in tissue slice treated with vaccinia virus compared to untreated control. Images taken at x40 magnification. Liver sections were prepared from human tissue obtained at the time of surgical resection. Organotypic cultures created from 8mm punch biopsies were left untreated or treated with vaccinia virus expressing green fluorescent protein at a concentration of $1 \times 10^7$ pfu/ml in culture for 72 hours. Cultures were fixed in formalin at 72 hours. Fixed tissue was then counterstained with nuclear stain TOPRO®-3 and left for 30 minutes before being mounted on a slide with a coverslip. Images were obtained by confocal microscopy. Blue staining corresponds to TOPRO®-3.

In this initial experiment, we assessed the following treatments:

1. Untreated control
2. Exposure to cavitation ultrasound and sulphur hexafluoride microbubbles without virus
3. Exposure to cavitation ultrasound without microbubbles and virus
4. Exposure to vaccinia virus alone
5. Exposure to vaccinia virus, cavitation ultrasound and sulphur hexafluoride microbubbles
These variations in the treatment of organotypic cultures were performed to ensure that both exposure to cavitation ultrasound alone and exposure to cavitation ultrasound and microbubbles did not exert any effects on the tissue slices by themselves. To assess the effects of these exposures on the organotypic cultures z-stack imaging was performed as outlined in methods to assess for the presence of green fluorescent protein as an indication of viral presence as well as immunohistochemical staining to assess for expression of the apoptotic marker cleaved caspase 3 as an indication of apoptotic cell death.

Using the confocal microscope to create z-stack images through the 300µm thick organotypic cultures we compared the untreated control with virus treatment alone and virus treatment plus cavitation ultrasound and the sulphur hexafluoride microbubbles. On z-stack images there was evidence of expression of green fluorescent protein in both organotypic cultures treated either with vaccinia virus alone or with vaccinia virus in combination with cavitation ultrasound and sulphur hexafluoride microbubbles when compared with untreated controls (Figure 5.2). There was no obvious difference in the amount of green fluorescent protein expressed with vaccinia virus treatment alone or in combination therapy in this instance.
Immunohistochemical staining was performed to assess for cleaved caspase 3, a marker of apoptotic cell death as described in materials and methods. After staining was performed images were taken using the Zeiss Primo Star microscope at x40 magnification at three random areas throughout each stained organotypic culture. Review of these images indicated an increased expression of the apoptotic marker, cleaved caspase 3 in the slices treated with vaccinia virus alone and vaccinia virus plus cavitation ultrasound and sulphur hexafluoride microbubbles, compared to the control (Figure 5.3).
To quantify immunohistochemical staining the digital IHC profiler application for ImageJ was utilised as outlined in methods. This software assessed positivity of diaminobenzide (DAB) substrate staining in treated organotypic cultures. The average score was taken from three separate images taken at x40 magnification from each slice, giving an overall impression of the staining throughout each slice.

In untreated controls, organotypic cultures treated with media and ultrasound, and organotypic cultures treated with media, ultrasound and sulphur hexafluoride microbubbles the percentage

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**Figure 5.3** Immunohistochemical staining for cleave caspase 3 in organotypic cultures from the same patient either untreated or treated with cavitational ultrasound alone, cavitational ultrasound and sulphur hexafluoride microbubbles, vaccinia virus alone or vaccinia virus plus cavitational ultrasound and sulphur hexafluoride microbubbles. Images were taken at x40 magnification. These are representative images taken for each treatment. Liver sections were prepared from human tissue obtained at the time of surgical resection and following a specific period of time fixed in formalin and then paraffin embedded. Slides were then prepared using 4µm slices before undergoing immunohistochemical staining for cleaved caspase 3. Staining was performed using diaminobenzide substrate (brown) for peroxidase. These images demonstrate an increased staining for cleaved caspase 3 in tissue slices that had either been treated with vaccinia virus alone or as well as ultrasound and microbubbles. Combination treatment with virus, cavitational ultrasound and microbubbles appeared to show the most staining for cleaved caspase 3 indicating increased apoptosis in these treated tumour slices.
positive staining for cleaved caspase 3 was 2.83%, 5.43% and 3% respectively. In the organotypic cultures treated with vaccinia virus at $10^7$ PFU/ml the percentage positive staining was 13.73%. The highest percentage of positive staining for cleaved caspase 3 was seen in the organotypic cultures that were treated with a combination of vaccinia virus at $10^7$ PFU/ml, cavitation ultrasound and sulphur hexafluoride microbubbles. In these cultures, the average percentage staining was 25.67% (Figure 5.4); this represents a near 2-fold increase compared to treatment with vaccinia virus alone. There was no significant difference between the cleaved caspase 3 staining between all three controls (untreated; media and ultrasound; media, ultrasound and sulphur hexafluoride microbubbles) suggesting that none of these exerted any apoptotic effects on the organotypic cultures. There was a statistically significant difference in cleaved caspase 3 staining between untreated controls and vaccinia virus alone ($p=0.0199$) as well as cultures treated with ultrasound and microbubbles compared to virus alone ($p=0.0220$). Combination therapy with vaccinia virus, ultrasound and microbubbles showed a statistically significant increase in positive staining for cleaved caspase 3 compared to all other treatments (Figure 41) (vs untreated control ($p<0.0001$); vs media and ultrasound ($p=0.0002$); vs vaccinia virus at $10^7$ pfu/ml ($p=0.0107$)). Combination treatment showed a significant increase in cleaved caspase 3 staining indicative of an increased effect of this treatment through apoptosis in these treated tumour slices compared with other treatments.
5.7.2 Treatment of organotypic cultures with vaccinia virus in combination with cavitational ultrasound and sulphur hexafluoride microbubbles at a lower concentration of virus

The combination of cavitational ultrasound and sulphur hexafluoride microbubbles has previously been shown to cause a fifty-fold increase in tumour transgene expression in combination with oncolytic adenovirus \(^{204}\). Following on from our successful initial experiments with combination therapy, we went on to assess whether it was possible to use a lower concentrations of vaccinia virus to achieve evidence of infection of organotypic cultures derived from colorectal liver metastases.

In preliminary experiments, we found that organotypic cultures showed evidence of infection by vaccinia virus through the expression of green fluorescent protein on confocal microscopy and z-stack imaging as well as through staining for the apoptotic marker, cleaved caspase 3 during immunohistochemical analysis.
From a translational medicine viewpoint, the ability to utilise a lower concentration of virus is desirable as it reduces the risk of the side effects associated with the systemic administration of oncolytic virotherapy. In previous clinical trials that have utilised the systemic administration of oncolytic vaccinia virus to treat cancer, doses as high as $3 \times 10^7$ PFU/kg have been utilised. Whilst oncolytic vaccinia virus is a fairly well tolerated oncolytic virotherapy, all patients will experience episodes of pyrexia or chills that last less than 24 hours following intravenous administration, moreover they commonly experience transient episodes of hypotension often requiring pre-hydration or intravenous fluid administered post-infusion. The majority of side effects experienced are grade 1 (mild) or 2 (moderate) as per the MedDRA grading system. There have not been any severe (grade 3), life threatening (grade 4) or deaths (grade 5) related to the systemic administration of oncolytic vaccinia virus. The ability to use a lower concentration of virus whilst maintaining clinical efficacy is an ideal situation.

In earlier experiments, we had found that there was a dose-response relationship in the treatment of organotypic cultures derived from colorectal liver metastases by vaccinia virus. Immunohistochemical staining for cleaved caspase 3 and for viral presence showed improved results of treatment with the highest concentration of virus ($1 \times 10^7$ pfu/ml) compared to lower concentrations ($1 \times 10^4$, $1 \times 10^5$ and $1 \times 10^6$ pfu/ml) as detailed in results chapter 1.

The highest concentration of vaccinia virus was utilised in the first experiments performed in the assessment of combination therapy. As described earlier there was evidence of infection of these tissues with vaccinia virus through the expression of green fluorescent protein on confocal microscopy and there was an increased expression of cleaved caspase 3 in slices treated with combination therapy compared with untreated controls and virus alone. The purpose of the next experiment was to assess the following, lower concentrations of virus: $1 \times 10^5$ pfu/ml and $1 \times 10^6$ pfu/ml as these had demonstrated evidence of infection in treated organotypic cultures in previous experiments (see results chapter 1). The experiment was carried out in the same way that the first experiment was
performed (see methods). Results were assessed through the expression of green fluorescent protein on confocal microscopy and z-stack imaging (Figure 5.5). Results of treatment with vaccinia virus at $1 \times 10^6$ pfu/ml has not been shown.

In this experiment, we saw that there was evidence of expression of green fluorescent protein suggestive of vaccinia virus presence in tissue slices following treatment with vaccinia virus at $1 \times 10^5$ pfu/ml after 72 hours when compared to untreated controls. In combination with cavitational ultrasound and sulphur hexafluoride microbubbles there was a subjective increase in the expression of green fluorescent protein compared to virus treatment alone (Figure 5.5). As per the initial experiment, this was suggestive of an improved effect of virus when used in combination compared to its use alone. Due to limitations in the number of tissue slices that were obtained from this patient tumour, it was not possible to further validate the findings on confocal microscopy with immunohistochemical staining as was performed in our initial experiment.

![Figure 5.5 Z-stack imaging demonstrating green fluorescent protein expression in organotypic cultures treated with vaccinia virus and vaccinia virus plus ultrasound and sulphur hexafluoride microbubbles compared with untreated control. Images taken at x20 magnification. Liver sections were prepared from human tissue obtained at the time of surgical resection. Organotypic cultures created from 8mm punch biopsies were left untreated or treated with vaccinia virus expressing green fluorescent protein at a concentration of $1 \times 10^5$ pfu/ml in culture for 72 hours. Cultures were fixed in formalin at 72 hours. Fixed tissue was then counterstained with nuclear stain TOPRO®-3 and left for 30 minutes before being mounted on a slide with a coverslip. Images were obtained by confocal microscopy. Blue staining corresponds to TOPRO®-3.](image-url)
As there was evidence of viral presence in organotypic cultures at this lower concentration of virus, it was decided that further experiments would be carried out at this concentration of virus. As mentioned previously, the ability to use a lower concentration of virus whilst maintaining efficacy is desirable in the treatment of cancer in order to reduce side effects of treatment.

Analysis of the remaining experiments would take place with combined analysis of both confocal microscope z-stacked images as well as through the quantification of DAB staining in immunohistochemistry.

5.7.3 Evaluation of the treatment of colorectal liver metastases with vaccinia virus at $1 \times 10^5$ pfu/ml, cavitational ultrasound and sulphur hexafluoride microbubbles

Three subsequent experiments were performed in order to validate our findings of an improved treatment effect following combination therapy with vaccinia virus treatment compared with virus alone. Organotypic cultures were procured from punch biopsies obtained at the time of resection of colorectal liver metastases as described in methods. These were then exposed to treatments as previously described before being returned to media and left in culture conditions for 24 hours. These were then fixed in formalin before undergoing subsequent analysis through either confocal microscopy or through immunohistochemical staining.

Two of the three patient tumours treated showed evidence of successful infection by oncolytic vaccinia virus through the expression of green fluorescent protein following virus application to tissue slices (Figure 5.6). In one patient, there was no convincing evidence of viral infection on confocal microscopy (Figure 5.7).
Figure 5.6 Z-stack images from confocal microscopy demonstrating increased expression of green fluorescent protein on organotypic culture treated with vaccinia virus at $1 \times 10^5$ pfu/ml plus cavitational ultrasound and microbubbles in comparison with virus treatment alone and untreated controls. Images taken at x20 magnification. Liver sections were prepared from human tissue obtained at the time of surgical resection. Organotypic cultures created from 8mm punch biopsies were left untreated or treated with vaccinia virus expressing green fluorescent protein at a concentration of $1 \times 10^5$ pfu/ml and then left in culture conditions for 72 hours. Cultures were fixed in formalin at 72 hours. Fixed tissue was then counterstained with nuclear stain TOPRO®-3 and left for 30 minutes before being mounted on a slide with a coverslip. Images were obtained by confocal microscopy. Blue staining corresponds to TOPRO®-3.
To further evaluate these treated organotypic cultures immunohistochemical analysis was performed and quantification of DAB staining undertaken using the IHC profiler application for ImageJ software as described in methods.

To ensure that there was evidence of tumour tissue within the punch biopsy H&E staining was performed. In preliminary experiments performed with organotypic cultures we had confirmed through immunostaining for CK7, CK20 and CDX2 (panel of markers used to confirm liver metastases originate from the gastrointestinal tract) that tissue taken at the time of resection of pathological specimens was a colorectal liver metastasis. Results were also corroborated through the official histological reports for the resected specimens.

Figure 5.7 Z-stack images from confocal microscopy with no evidence of viral infection following combination treatment with vaccinia virus at $1 \times 10^5$ pfu/ml plus cavitational ultrasound and microbubbles. Images from viral treatment alone and untreated controls are not shown. Images taken at x20 magnification. Liver sections were prepared from human tissue obtained at the time of surgical resection. Organotypic cultures created from 8mm punch biopsies were left untreated or treated with vaccinia virus expressing green fluorescent protein at a concentration of $1 \times 10^5$ pfu/ml and then left in culture conditions for 72 hours. Cultures were fixed in formalin at 72 hours. Fixed tissue was then counterstained with nuclear stain TOPRO®-3 and left for 30 minutes before being mounted on a slide with a coverslip. Images were obtained by confocal microscopy. Blue staining corresponds to TOPRO®-3.
Figure 5.8 IHC images of organotypic cultures derived from colorectal liver metastasis in one patient following immunohistochemical staining for both H&E and cleaved caspase 3 for untreated control; cavitational ultrasound and microbubbles alone; vaccinia virus alone and vaccinia virus in combination with ultrasound and microbubbles. H&E images taken at x20 magnification giving an overview of the tissue slice and evidence of tumour material. Cleaved caspase 3 stained images taken at x40 magnification. Each image represents one of three fields of view used to quantify the positivity of DAB staining. H&E stained images show cellular content within each slice and evidence of tumour presence. Cleaved caspase 3 staining appears to be greatest in slice treated with virus in combination compared to other treatments (see Figure 9 for quantification of staining).
While H&E staining was being performed, staining for cleaved caspase 3 was undertaken. Cleaved caspase 3 is a marker of apoptotic cell death. We compared the staining of cleaved caspase 3 in untreated controls; cultures treated with media, ultrasound and microbubbles; and vaccinia virus alone, with combination treatment. This would enable us to make an assessment of virus activity through staining for apoptosis. Figure 5.8 gives a representative example of the images acquired at the time of analysis. Images for H&E staining were taken at x20 magnification to give an overall perspective of the tissue slices created. Images were taken at x40 magnification for cleaved caspase 3 staining for a more detailed assessment of staining. To ensure that the staining calculated for each slice was representative of staining within the whole slice rather than the area at which the x40 magnification image was taken the process was repeated at random 3 times within each culture. Positive staining was then quantified using the IHC profiler application for image J and an average score was then taken for each slice (Figure 5.9).

Figure 5.9 Percentage positive staining for cleaved caspase 3 in a colorectal liver metastasis comparing no treatment, cavitational ultrasound and microbubbles; virus alone or virus in combination with ultrasound and microbubbles. There was a statistically significant increase in positive staining for cleaved caspase 3 in slices treated with combination of vaccinia virus and cavitational ultrasound and sulphur hexafluoride microbubbles compared to all other treatments and controls. Treatment with vaccinia virus alone showed a statistically significant increase in staining for cleaved caspase 3 compared with untreated controls and slices exposed to cavitational ultrasound and sulphur hexafluoride microbubbles. Quantification of positive staining undertaken using IHC profiler application software for ImageJ. Statistical analysis using two-way ANOVA. Statistical level of significance: *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001
Analysis of DAB stained tissue slices for cleaved caspase 3 (figure 5.9) showed similar positive staining in the control groups (untreated and exposure to ultrasound and microbubbles alone) of 4.1% and 5.67% indicating that there was no increased apoptotic activity with exposure to cavitational ultrasound and sulphur hexafluoride microbubbles compared to tissue slices left untreated in culture conditions. There was an increase in positive staining for cleaved caspase 3 in organotypic cultures that had been treated with vaccinia virus alone with a score of 10.1%. This was a statistically significant increase in staining compared to both control groups (untreated vs VV 1x10^5 pfu/ml (p=0.03) and media, ultrasound and microbubbles vs VV 1x10^5 pfu/ml (p=0.03)). Positive staining for cleaved caspase 3 was highest in the organotypic cultures treated in combination with vaccinia virus, ultrasound and microbubbles with a score of 18.2%. This showed that staining for cleaved caspase 3 in these organotypic cultures was higher than controls (combination vs untreated (p=0.0002); combination vs media, ultrasound and microbubbles (p=0.0002)) and compared with treatment of slices with virus alone was also statistically significant (p=0.009). These results would suggest an increased effect of virus in combination with cavitational ultrasound and sulphur hexafluoride microbubbles compared to treatment with vaccinia virus alone.

In a second patient, similar results were seen. Analysis of DAB staining for cleaved caspase 3 (figure 5.10) showed similar positive staining in both the untreated control slice (2.03%) and those treated with cavitational ultrasound and sulphur hexafluoride microbubbles (2.70%). There was a slight increase in positive staining for cleaved caspase 3 with virus treatment alone (3.93%), however treatment of virus in combination with ultrasound and microbubbles showed the highest staining for cleaved caspase 3 (13.47%). In this patient, there was no statistical difference in cleaved caspase 3 positive staining between the control groups and virus treatment alone. In this repeat experiment combination treatment with vaccinia virus, cavitational ultrasound and sulphur hexafluoride microbubbles was found to cause a statistically significant increase in cleaved caspase 3 staining compared to other treatments (combination vs control (p=<0.0001); combination vs media, ultrasound and microbubbles (p=0.0002); combination vs virus alone (p=0.0002)).
In a subsequent experiment assessing the same treatments there was no evident infection of the tissue slice by oncolytic vaccinia virus in combination with ultrasound and microbubbles and by itself (Figure 5.7). Due to the workflow and the time taken to evaluate treated organotypic cultures using immunohistochemistry slices were often first assessed by confocal microscopy and z-stack imaging prior to immunohistochemical analysis due to the speed at which slices could be processed in this way. Review of H&E stained images of tumour from the patient in whom the treatment was unsuccessful, control tissue showed that this was a mucinous cystic tumour (Figure 5.11). Within each slice there was very little in the way of cellular material. There were also large gaps between cells which would have limited vaccinia virus spread given that it spreads from cell to cell. It was felt that this histological appearance was likely to account for results seen in this instance.

**Figure 5.10** Percentage positive staining for cleaved caspase 3 in another patient with colorectal liver metastasis, comparing no treatment; cavitational ultrasound and microbubbles; virus alone or virus in combination with ultrasound and microbubbles. There was a statistically significant increase in positive staining for cleaved caspase 3 in slices treated with combination of vaccinia virus and cavitational ultrasound and sulphur hexafluoride microbubbles compared to all other treatments and controls. There was no significant increase in staining for cleaved caspase 3 in slices treated with vaccinia virus alone compared with untreated controls and slices exposed to cavitational ultrasound and sulphur hexafluoride microbubbles. Quantification of positive staining undertaken using IHC profiler application software for ImageJ. Statistical analysis using two-way ANOVA. Statistical level of significance: *p<0.05; **p<0.01; ***p<0.001 and ****p<0.0001
Figure 5.11 Comparison of immunohistochemical staining for H&E in organotypic cultures from two patients that underwent combination treatment of vaccinia virus with cavitation ultrasound and sulphur hexafluoride microbubbles. Images were taken at x20 magnification. Image a) shows the histological appearance of the organotypic culture with large areas of mucin (demonstrated by the arrow) within the tumour. There is also scanty cellular material and no glandular architecture that is typically seen in colorectal liver metastases. Image b) shows the typical architecture of an organotypic culture derived from a colorectal liver metastasis with maintenance of the glandular architecture that is typically seen and a marked increase in cellular material in comparison with image a). Liver sections were prepared from human tissue obtained at the time of surgical resection and following a specific period of time fixed in formalin and then paraffin embedded. Slides were then prepared using 4µm slices before undergoing immunohistochemical staining for H&E. Staining was performed using DAB substrate (brown) for peroxidase.
5.8 Discussion

The aims of this chapter were to assess the use of cavitational ultrasound and sulphur hexafluoride microbubbles with virus in the treatment of colorectal liver metastases and to see if this improved infection of organotypic cultures. Having previously demonstrated that vaccinia virus successfully infected organotypic cultures in earlier experiments, we wanted to assess if there was any improvement in viral infection of cultures treated in this way. This could potentially represent a means of improving the effects of systemically administered vaccinia virus, which has so far been limited in current literature. Infection of tissue slices was assessed through the expression of green fluorescent protein in treated slices using confocal microscopy as well as through the assessment of apoptotic activity within treated tissue slices using immunohistochemical analysis. We also wanted to confirm that any effects observed within treated tissues, were not a consequence of exposure to ultrasound frequencies and the sulphur hexafluoride microbubbles alone.

There has so far been limited success following the intravenous administration of virus in trials despite it being generally well tolerated. This is reflected in the fact that the only oncolytic virotherapy currently approved for use by the U.S Food and Drug Administration is talimogene leherparepvec (T-VEC), a second-generation oncolytic herpes simplex virus. This has been approved for use in the treatment of advanced melanoma and is administered intra-tumorally. Whilst intra-tumoral administration of virus maximises initial delivery into the tumour, there is a reduction in hydraulic conductivity within the tumour due to high interstitial hydrostatic pressure as well as the high levels of extracellular matrix. Oncolytic virus particles can be lost quickly from the tumour due to circulatory clearance or through leakage along the tract of injection. If the success of the viral treatment is dose related, then this reduction in viral particles would suggest that intra-tumoral delivery of oncolytic virotherapy is an inefficient method of treatment. As some cancers are not amenable to intra-tumoral injection this method also limits the tumours that can be treated. It is
therefore desirable to administer this tumour-targeted treatment systemically to broaden the use of oncolytic virotherapy.

A study by Bazan-Peregrino et al. found that the combination of an oncolytic adenovirus in combination with cavitational ultrasound and sulphur hexafluoride microbubbles caused a 50-fold increase in tumour transgene expression in the treatment of breast cancer in a mouse model. This was assessed through the expression of luciferase using an in vivo imaging system to measure fluorescence in a flank mouse model. The sulphur hexafluoride microbubbles act as cavitation nuclei when exposed to certain ultrasound frequencies and these cause shockwaves and microstreaming within tissues with a resultant propulsion of oncolytic virus throughout the tumour.

Myers et al. combined oncolytic vaccinia virus with cavitational ultrasound and a different form of cavitation nuclei (polymeric cups) in a mouse flank model of colon carcinoma and hepatocellular carcinoma. In this study, the oncolytic virus used also expressed the luciferase transgene and so this expression was measured using an in vivo imaging system to assess fluorescence. This study found that there was a significant increase in viral transgene expression in tumours following exposure to virus, ultrasound and polymeric cups.

Both of these studies have demonstrated a statistically improved infection of tumour tissue using an in vivo model with different oncolytic virotherapies. This data is promising with regards to the optimisation of systemically administered oncolytic virotherapy. Given the success of this method in mouse models, it seemed appropriate to assess the effects of our oncolytic vaccinia virus in the organotypic cultures derived from human tumours. Being derived from human tumours, organotypic cultures are a more representative model with which to assess both oncolytic virotherapy as well as combining this treatment with cavitational ultrasound and sulphur hexafluoride microbubbles. Tumours derived from immortalised cell lines are subject to the genetic changes that take place in
these immortalised cell lines in culture over time making them less representative of patient tumours and often over-emphasising the effects of anti-cancer treatments.

Given that organotypic cultures are only 300µm thick and that the cavitation ultrasound and sulphur hexafluoride microbubbles had not been used in combination with tissue slices before it was not certain that the tissue may have become damaged by these treatments rather than the organotypic culture. Previous papers have proposed that damage to tissues can occur following exposure of microbubbles to cavitation ultrasound and so it was necessary to ensure that any effects observed in the tissue slice model was not a result of this. During the experiment, we utilised ultrasound frequencies that had previously been shown not to cause tissue damage. To demonstrate this in our experiments we compared the effects of organotypic cultures exposed to ultrasound frequency and sulphur hexafluoride microbubbles with those treated with virus as well as ultrasound and microbubbles. The effects were assessed through immunohistochemical staining for cleaved caspase 3, a marker of apoptotic cell death. Staining was quantified using the IHC profiler software available for Image J. We found that positive staining for cleaved caspase 3 was low in our experiments with positive staining less than 5% for both the organotypic cultures left untreated in culture conditions as well as those exposed to cavitation ultrasound and microbubbles. There was no statistical difference between the staining in these control groups. These findings confirmed that there was no effect on tissue seen following exposure to cavitation ultrasound and microbubbles. Furthermore, we saw a statistically significant increase in staining for cleaved caspase 3 in tissue slices when treated with oncolytic vaccinia virus as well as being exposed to cavitation ultrasound. This result suggests an improvement in tumour treatment with this method and further supports the use of combination therapy to improve the effects of oncolytic virotherapy in patients with colorectal liver metastases.

In one of the experiments performed there was no evidence of successful infection with oncolytic vaccinia virus. The morphology of the tissue was assessed through H&E staining and found that the histological appearance of the tumour was that of a colorectal liver metastasis, however this was
found to be a mucinous tumour with scanty cellular material when compared with the tissue obtained from previous positive experiments as highlighted in Figure 5.11. As vaccinia virus spreads from cell to cell \(^ {188}\), the lack of cellular material and large quantities of mucin may have dampened any potential treatment effect. This result highlights the importance of tumour morphology in treatment of tumours with oncolytic virotherapy. This variable morphology seen between the same types of tumour indicate that this treatment, as with all anti-cancer therapies is not a panacea and that there will always be variability from patient to patient. The effects in clinical practice will be variable with inter-patient tumour variation likely affecting virus spread. The result also highlights the limitations of animal models which are derived from immortalised cell lines that are more homogeneous. Results in these models can be far more consistent in pre-clinical trials before seeing a potential reduction in efficacy when it comes to clinical application in clinical trials.

In the successful experiments performed treatment with vaccinia virus alone was seen to have a greater effect through the expression of cleaved caspase 3 than untreated controls, whilst treatment with vaccinia virus and exposure to cavitational ultrasound and sulphur hexafluoride microbubbles resulted in a statistically significant improvement in treatment effect compared to treatment with virus alone. Previous studies assessing the combination of oncolytic virotherapy with ultrasound and various cavitation nuclei have shown an increased infection of up to one thousand-fold \(^ {204-206}\). We did not see an improvement as extensive as this with our results showing a two-fold increase in infection with virus plus exposure to ultrasound and microbubbles compared with virus treatment alone. This may be due to the lower concentration of virus used in our experiments compared to these other studies (vaccinia virus concentration \(1 \times 10^5\) pfu/ml in our experiments) as well as the brief period that the organotypic cultures were exposed to the virus for (20 seconds).
5.9 Conclusion

Overall, we found that the combined treatment of colorectal liver metastases with vaccinia virus and exposure to cavitational ultrasound and sulphur hexafluoride microbubbles improved the anti-cancer effects of this oncolytic virus. Combining this treatment with oncolytic virotherapy is a promising technique to improve the effect of the systemic administration of oncolytic vaccinia virus. The results from these preliminary experiments are small in number and so further repeat experiments would help to validate the initial findings of these experiments and support the use of this technique in the clinical setting. This is a promising finding for patients with colorectal liver metastases that are not amenable to surgical resection of their disease. These patients could have intravenous virotherapy administered with focused cavitational ultrasound over specific tumour sites, thereby promoting viral penetrance into tumour following systemic administration and potentially enhancing replication.
Chapter 6

General Discussion
Chapter 6 – General discussion

6.1 Oncolytic vaccinia virus in the treatment of colorectal cancer

Colorectal cancer is the fourth most common cancer in the developed world with approximately 50% of patients going on to develop metastatic disease. Only 20% of patients with colorectal liver metastases have disease that is initially considered to be surgically resectable. Although progress has been made in the development of effective agents in the treatment of metastatic colorectal cancer, survival remains stubbornly low. Patients not eligible to have surgical treatment will have a median survival of between 16-24 months. Given that surgical resection gives the greatest possibility of cure, the need for the development of further agents remains.

Oncolytic viruses are an attractive new treatment option in patients with metastatic colorectal cancer. It has long been recognised that viruses have the ability to kill cancer cells and recent clinical trials have shown evidence of therapeutic benefit in patients with multiple tumour types. The mechanism by which anti-tumour activity is mediated by oncolytic viruses is not completely understood. Two distinct mechanisms are thought to contribute to this; these are, selective replication within and lysis of tumour cells in situ and activation of systemic anti-tumour immunity. Vaccinia virus is a promising oncolytic virotherapy as it has a broad tumour tropism and is stable in the bloodstream enabling treatment of distant metastases. It also spreads rapidly within tumours, whilst having a large transgene-encoding capacity. Vaccinia virus also has an established safety profile in humans given its use in the smallpox vaccination process. The vaccinia virus JX-594, which has the immunomodulatory gene encoding for granulocyte monocyte colony-stimulating factor (GM-CSF) and β-galactosidase is the best studied vaccinia virus in cancer treatment and has been shown to be effective in the treatment of patients with colorectal liver metastases in phase I clinical trials.

In our experiments we found oncolytic vaccinia virus to be an effective treatment in colorectal cancer using both tissue culture and our developed organotypic culture system. There was a reduction in the
viability of three colorectal cancer cell lines with low MOIs of 0.001 and 0.01 respectively. This finding is encouraging as one of the limitations of oncolytic virotherapy is patient toxicity. To be able to treat patients with a low pfu of virus would potentially limit the side effects of any treatment. The mechanisms by which oncolytic vaccinia virus induces tumour cell death are poorly understood and several modes of cell death have been implicated to varying degrees. These include apoptosis, necrosis, necroptosis and autophagy. Vaccinia virus strain also is a factor in the type of cancer cell death that occurs. We have evaluated cell death pathways following infection of colorectal cancer cell lines with oncolytic vaccinia virus. The results of our experiments have provided evidence to suggest that cell death in colorectal cancer cell lines treated with oncolytic vaccinia virus was predominantly apoptotic. The same mode of cell death has also been reported in the treatment of melanoma cells with this virus. The type of cell death induced is important with regards to immunogenicity of the virus and the influence that it has on the tumour microenvironment.

Our oncolytic vaccinia virus induced parts of immunogenic cell death in all three colorectal cancer cell lines. Different parts of immunogenic cell death were induced in each cell line. In the HCT116 cell line there were increases in calreticulin and HMGB1 expression; there was an increase in FAS and HMGB1 in the SW620 cell line and there was an increase in CD80 and HMGB1 expression in the HT29 cell line. These increases were statistically significant. Increased expression of HMGB1 has also been seen in other studies using vaccinia virus. Two types of immunogenic cell death induction exist, of which oncolytic viruses appear to be type II inducers. In our colorectal cancer model vaccinia virus was not able to induce a full picture of immunogenic cell death. Interrogation of the organotypic culture system was unsuccessful for in the assessment for HMGB1. Further work is required in this system to assess these organotypic cultures for evidence of immunogenic cell death. Following oncolytic cell death tumour cells release viral PAMPs as well as danger associated molecular patterns (DAMPs) including heat shock proteins (HSPs), high mobility group box 1 proteins (HMGB1), calreticulin, adenosine triphosphate (ATP), and uric acid. They also release cytokines that promote the maturation of antigen presenting cells such as dendritic cells. These cytokines include type 1 interferons, tumour
necrosis factor-α (TNF-α), interferon gamma (IFN-γ) and interleukin-12 (IL-12)⁶⁸. These then activate CD4⁺ and CD8⁺ T cell responses⁶⁸. Other DAMPs, cytokines in supernatant or activated CD4⁺ and CD8⁺ T cells may be other targets to assess for ICD in organotypic cultures treated with this vaccinia virus.

6.2 Assessment of the pro-drug activation system with the fusion suicide gene (FCU1)

Most cancer therapies rely on the use of multiple agents in conjunction with one another. Similarly, it has been demonstrated that there is a superior effect when oncolytic virotherapy is used in combination with traditional therapies²¹². An example of this includes the treatment of patients with advanced hepatocellular carcinoma. JX-594 oncolytic vaccinia virus was combined with Sorafenib, which is a small-molecule molecule multikinase inhibitor with antiproliferative and antiangiogenic effects. The combination of these two agents was found to induce tumour necrosis in patients and limit disease progression, whilst use of the virus alone did not achieve such results¹²⁰.

For many years 5-FU has been the principle active agent to treat colorectal cancer and is still included in chemotherapy regimens such as FOLFOX and FOLFIRI for the treatment of colorectal liver metastases. One such way of incorporating this treatment with oncolytic virotherapy is through the use of pro-drug activation model which converts 5-FC to toxic 5-FU through the viral expression of the fusion suicide gene (FCU1). Vaccinia virus encoding the fusion suicide gene is a promising candidate in the use of patients with colorectal cancer and liver metastases. The ability of the virus to convert nontoxic fluorocytosine into its active metabolite 5-fluorouracil lends itself to the treatment of these cancers as this is one of the predominant chemotherapy agents used in the treatment of patients with colorectal cancer that has metastasised to the liver. This has been demonstrated in pre-clinical work in cell lines as well as in murine models¹³⁰,¹⁴². We have assessed the use of this vaccinia virus in our experiments, firstly in tissue culture and found that oncolytic vaccinia virus has a potent effect on the killing of the colorectal cancer cell lines HCT116, HT29 and SW620, with only low MOIs of 0.001 and 0.01 required to reduce cell proliferation by 50%. In our hands we were unable to show success of
pro-drug activation and an improved anti-cancer efficacy compared with virus treatment alone in tissue culture experiments. This is in contrast to the results published by Foloppe et al that saw a greater reduction of cell viability in colorectal cancer cells (WiDr, LoVo and A549) treated with virus and pro-drug compared with virus alone. The same study also found in a murine orthoptic mouse model for liver metastases that there was a greater reduction in tumour burden following the systemic administration of virus and oral administration of 5-FC compared to virus alone. Heinrich et al used the same virus and pro-drug activation model in melanoma cell lines. Whilst this study did find a reduction in cell viability in cells treated with virus and 5-FC compared to those treated with virus alone it was not a significant amount. Studies have shown that in the presence of chemotherapeutic agents such as 5-FU the replication of oncolytic viruses can be inhibited and this may be a limitation of the pro-drug activation model used with oncolytic virotherapy. In Herpes simplex virus this issue has been overcome through the use of a mutant HSV-1 missing one copy of its ICP0, ICP4, and ICP34.5 gene which resulted in enhanced viral replication. Despite our unsuccessful results in tissue culture we did demonstrate an effect of virus and pro-drug in the treatment of organotypic cultures when compared with treatment with virus alone. This was a solitary result in which we saw a significant increase in the expression of cleaved caspase 3 in the organotypic culture compared with virus treatment alone in one of two cases that were assessed. In combination with other published results from different groups using the pro-drug activation model with vaccinia virus the inconclusive results from this study does not advocate the use of this model in the treatment of colorectal liver metastases in combination with oncolytic virotherapy.
6.3 The organotypic culture model

Given the limitations of existing 2D and 3D models in vitro we wanted to develop and assess the feasibility of using an organotypic culture model for the treatment of oncolytic vaccinia virus. Following the workflow set out by Davies et al we achieved this. Following the recommendations set out, as well as our experience in creating these organotypic cultures we were able to refine our methods and produce a workflow for this technique. One of the key issues in this particular culture system is the viability of the organotypic cultures once created. This factor was critical in our work as we hoped to assess the effect of treatment with oncolytic vaccinia virus as well as a pro-drug activation model. Given that treatment of slices would have required some time to enable virus to infect tissue and replicate a short viability of less than 48 hours would have been unsuitable for assessment. Our initial work assessing the organotypic culture viability found that these were viable for a minimum of 72 hours in our hands and a maximum period of 120 hours (5 days). These results are in keeping with other groups’ work using differing methods of creating organotypic cultures as well as different tissues.

In actuality the use of these slices was an excellent modality for the assessment of our vaccinia virus as we were able to visualise infection and spread of virus through tissue using confocal microscopy whilst being able to assess for this and quantify viral presence and effect using IHC and staining for anti-vaccinia virus, anti-GFP and cleaved caspase 3 antibody. Furthermore, the excellent results that we saw were encouraging given the fact that these slices had been created from actual patient tumour without any manipulation. For this reason, organotypic cultures represent a complete 3D representation of the actual tumour microenvironment in vitro. These slices capture the architecture, heterogeneity and native cellular elements of the tumour of interest rather than surrounding tumour with artificial matrices.

Whilst organotypic cultures are not a panacea for the assessment of tumours ex-vivo and novel oncological therapies they do represent an adjunct that can aid in the assessment of these. That said,
the model does have its limitations, as with other systems. Critical to the process is the availability of patient tissue, as well as the successful processing of the tumour tissue which we found to be variable. We were fortunate to have excellent ties with our local hepatobiliary surgical department which enabled regular access to this. Further limitations included the heterogeneity of tumours in terms of size and cellular content as well as the expense of this system and the time involved from the start of processing to the end of assessment which often took several weeks once IHC staining and confocal microscopy had been completed.

Despite the limitations of this method, organotypic cultures represent a high content experimental platform for the interrogation of tumours in vitro. The organotypic culture model is a powerful technique that is an accurate representation of the tumour in vivo including its stroma and heterogeneous microenvironment, permitting the interrogation of tumours in the laboratory.

6.4 Use of cavitational ultrasound to improve the effects of oncolytic vaccinia virus

Oncolytic virotherapy is a powerful emerging tool in the treatment of cancer. Clinical trials have confirmed the therapeutic effects of oncolytic virotherapy in the treatment of multiple solid tumours \(^{66,122,161}\). A limitation of this novel treatment is the restricted ability of oncolytic viruses to reach and to penetrate target tumours following intravenous administration. This hurdle has limited their clinical utility with many oncolytic virotherapies reliant on intratumoral administration. Whilst this approach provides a localised anti-tumour effect and has the ability to stimulate the generation of systemic anti-tumour immunity it does not utilise the unique ability of viruses to infect and destroy metastatic tumour deposits.

For patients with metastatic disease such as those with colorectal liver metastases, intravenous administration of oncolytic virus is seen as the preferred method of administration as it provides access to all vascularised tumour sites within the body \(^{162}\). Current research in oncolytic virus delivery
has focused on limiting sequestration of virus in the liver and spleen, evading viral neutralisation in patient serum, targeting viruses to vascular endothelia cells lining tumour bloods vessels and selectively enhancing their permeability.

Vaccinia virus has biological characteristics that make it particularly encouraging for use as an oncolytic agent. These characteristics include its stability intravenously and its ability to reach target tumours in the blood, its rapid spread within tumour tissues, the ability for the virus to be engineered to encode large transgenes as well as its safe historical use in humans as a vaccine against smallpox. Moreover genetically modified vaccinia virus is able to selectively target tumour cells whilst sparing non-malignant cells, thereby reducing damage to healthy tissues. As well as its selective targeting of tumour cells its safety is further enhanced due to its site of replication in the cytoplasm of cells, which prevents the integration of viral DNA into host chromosomes and the passage of viral progeny into daughter cells. Cavitation ultrasound has been predominantly used on drug or gene therapy agents that do not have the capacity to replicate. It is potentially a strategy that could improve systemic oncolytic virotherapy. In combination with systemic oncolytic adenovirus treatment, cavitation ultrasound and sulphur hexafluoride microbubbles were found to significantly increase tumour transgene expression without causing tissue damage in a mouse model.

Our results showed that in combination with cavitation ultrasound and sulphur hexafluoride microbubbles there was the highest percentage of positive staining for cleaved caspase 3 in IHC compared with virus at $10^7$ PFU/ml alone. In these cultures, the percentage staining was 25.67% which represented a near 2-fold increase compared to treatment with vaccinia virus alone. Combination with vaccinia virus, ultrasound and microbubbles showed a statistically significant increase in positive staining for cleaved caspase 3 compared to all other treatments in our experiments. The combination of cavitation ultrasound and sulphur hexafluoride microbubbles also enabled us to use a lower concentration of vaccinia virus whilst having similar efficacy on the organotypic cultures as vaccinia virus at the highest concentration of virus. Once again combination treatment with virus resulted in
improved results compared with virus alone. These results echo the findings from previous published experiments, albeit to a lesser extent \textsuperscript{204,205}. This may be a reflection of the heterogeneity of tumour tissue or of the brief period of time that organotypic cultures were exposed to virus for and the lower concentrations of virus that we used in our experiments.

Overall, we have found the combination of oncolytic vaccinia virus with cavitational ultrasound to be a promising treatment modality. Further repeat experiments are required to confirm this association as our experiments are small in number and so further repeat experiments would help to validate our findings. We also did not assess the pro-drug activation model using this system and further experiments would be required to investigate this.

6.5 Future work

In this thesis we set out to establish an organotypic culture model in our laboratory and work to date has gone some way to achieving this. To further establish the model further work should involve increasing our experience with colorectal liver metastases, creating more tissue slices and establishing a standardised protocol that enables us to evaluate organotypic cultures with different viruses as well as in different solid tumours. It is important to note that a change in tissue and tumour site would also warrant full optimisation as we have done in the case of colorectal liver metastases.

Vaccinia virus was assessed for immunogenic cell death and was found to induce this partly in our work with colorectal cancer cell lines. Despite this we were unable to evaluate this in our organotypic culture model. Further work assessing ICD in the organotypic culture model could be performed by staining for different markers in IHC such as calreticulin, another option would be to assess for cytokines in the supernatants of treated slices compared with controls to assess for any difference following virotherapy. Another option could be to assess immunogenicity with differing
concentrations of virus, it may be that a lower dose could produce a better response in the organotypic culture model.

Our work combining oncolytic virotherapy using cavitational ultrasound and sulphur hexafluoride microbubbles has been promising in the optimisation of oncolytic virotherapy in solid tumours. The next step would be to assess this in vivo using a murine subcutaneous flank model. Oncolytic virus could be administered locally and systemically with or without the combination of cavitational ultrasound and microbubbles and the responses could be assessed. This could also be undertaken in a murine liver metastasis model too.

Finally, newer technologies are in development to optimise the effects of cavitational ultrasound. One such technique is to use polymeric nanocups rather than simple sulphur hexafluoride microbubbles to further enhance this effect. These provide the nuclei for instigation of sustained cavitational events within tumours as they have a longer half-life than microbubbles. In early studies this technology has been shown to increase virus activity in solid tumour up to 10,000-fold which is stark in comparison to the two-fold increase that we have seen.\textsuperscript{205}
References


cell maturation and interaction with cytotoxic T lymphocytes. *OncoTargets and therapy.* 2017;10:2389-2401.


