A phase I toxicity trial of the herpes simplex virus HSV1716 in patients with oral squamous cell carcinoma, with an *in vitro* investigation of the cytotoxic effects of HSV1716 alone and in combination with cisplatin in head and neck squamous cell carcinoma.

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Contents

1.2 Molecular genetics of head and neck cancer

1.2.1 Cytogenetics

1.2.2 Oncogenes

1.2.3 Tumour suppressor genes

1.2.4 Telomerase

1.3 Gene therapy in head and neck cancer

1.3.1 Principles of gene therapy

1.3.2 Gene delivery vectors

- A) Chemical and physical vectors
- B) Viral vectors
  - i) Retroviral vectors
  - ii) Adenoviral vectors
  - iii) Herpes simplex viral vectors

1.3.3 Gene therapy strategies in head and neck cancer

- A) P53
- B) Epidermal growth factor receptor
- C) Immunotherapy
- D) Gene directed enzyme prodrug therapy

1.4 Other novel biological and chemical therapies

1.5 Oncolytic viruses

1.5.1 Principles of oncolytic viral therapy

1.5.2 Oncolytic adenovirus

1.6 Herpes simplex virus

Chapter 2: Materials

Chapter 3: Methods

References

Declaration

I declare that this work is my own. All laboratory experiments were performed by me, Alastair Mace, except for immunohostochemistry experiments which were performed in the Pathology Department at Glasgow Royal Infirmary. The principal surgeon for all operations on trial patients was Mr D.S. Soutar, Canniesburn Unit, Glasgow Royal Infirmary, UK.

This work has not been previously submitted for higher degree.

Tables and Figures

Figures.

Tables.

Presentations, prize and publications

The following presentations and prize have been given and awarded from the research described in this thesis:

- Mace A.T.M, Ganly I, Brown S.M. Combination cytotoxic effects of HSV1716 and cisplatin against head and neck squamous carcinoma cells. Podium presentation at the **Scottish Otolaryngology Society** Winter Meeting.. Dunfermline, November 2003
- Mace A.T.M, Ganly I, McDonald D.G, Soutar D.S, Brown S.M. A phase I study of preoperative intratumoural injection of HSV1716 in oral squamous cell carcinoma. Podium presentation at the **American Head and Neck Society's 6th International Conference on Head and Neck Cancer**. Washington DC, August 2004
- Mace A.T.M, Ganly I, Brown S.M. Combination cytotoxic effects of HSV1716 and cisplatin against head and neck squamous carcinoma cells. Poster presentation at the **American Head and Neck Society's 6th International Conference on Head and Neck Cancer**. Washington DC, August 2004
- Mace A.T.M, Ganly I, Brown S.M. Combination cytotoxic effects of HSV1716 and cisplatin against head and neck squamous carcinoma cells.

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PAPERS:

The following paper has been published:

- Mace A.T.M, Ganly I, McDonald D.G, Soutar D.S, Brown S.M. A feasibility study of preoperative intratumoural injection of HSV-1 mutant, HSV1716, in patients with respectable oral squamous cell carcinoma. **Chief Scientist Office of Scotland,** December 2004.

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Abbreviations

5-FU	5-Fluorouracil
AST	Aspartate transaminase
ATG	Adenine-thymine-guanine
BHK	Baby hamster kidney
bp	Base pair
CD	Cytosine deaminase
CDK	Cyclin-cependent kinase
CHART	Continuous hyperfractionated accelerated radiotherapy
CMI	Cell mediated immunity
CP	Carboxypeptidase
CSF	Cerebrospinal fluid
DCIA	Deep circumflex iliac artery
dCK	Deoxycytidine kinase
DMSO	Dimethyl sulfoxide
DNA	Deoxy nucleic acid
dsDNA	Double-stranded deoxy-nucleic acid
ECACC	European collection of cell cultures
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay

EORTC	European Organisation for Research and Treatment of Cancer
ETMC 10%	Eagle's medium containing 1% carboxymethyl cellulose , 10% new-born calf serum and 10% tryptose phosphate broth
ETC_{10}	100 IU/ml penicillin/streptomycin
FSI	Farnesyl transferase inhibitors
GADD	Growth arrest and DNA damage protein
GDEPT	Gene directed enzyme pro-drug therapy
GMP	Good manufacturing practice
Gy	Gray
H&E	Haematoxylin and eosin
HBSS	Hank's Balanced Salt Solution
HLA	Human leucocyte antigen
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HSV	Herpes simplex virus
IARC	International Agency for Research on Cancer
IC50	Inhibitory concentration 50%
ICP	Infected cell protein
IE	Immediate-early
Ig	Immunoglobulin
IL	Immediate-late

IR_L	Internal repeat long
ISD	Information and statistics division
ISH	*In-situ* hybridisation
IU	International unit
LAT	Latency-associated transcripts
MACH-NC	Meta-analysis of Chemotherapy on Head and Neck Cancer
MHC	Major histocompatibility complex
ml	Millilitre
MMC	Mitomycin-C
MOI	Multiplicity of infection
mRNA	Messenger ribo nucleic acid
MyD116	Mouse myeloid differentiation protein
nM	Nanomolar
NR	Nitroreductase
OTC	Ornithine transcarbamylase
GTAC	Gene Therapy Advisory Committee
pfu	Plaque forming unit
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PNP	Purine nucleoside phosphorylase
QED	Quick and early diagnosis

RNA	Ribo nucleic acid
RNAi	Interference ribo nucleic acid
SCC	Squamous cell carcinoma
SCCRO	Squamous cell carcinoma-related oncogene
SCID	Severe combine immunodeficiency
siRNA	Small interfering ribo nucleic acid
TBE	Tris/borate buffer
TBS	Tris buffered saline
TGF-α	Transforming growth factor alpha
TK	Thymidine kinase
TR_S	Terminal repeat short
UICC	International Union Against Cancer
TNM	Tumour, Nodes, Metastasis
U_L	Unique long
U_S	Unique short
UV	Ultraviolet
vhs	Virion host shutoff
VP	Virion proteins
μM	Micromolar

Abstract

Head and neck squamous cell carcinoma (HNSCC) is a challenging disease which causes significant morbidity and mortality. Tobacco and alcohol are the prime aetiological factors and incidence rates are much higher among people from deprived areas. Too many patients present with advanced disease. Unfortunately the substantial advances in established therapies, including surgery, radiotherapy and chemotherapy, have not had a significant impact on patient survival. Treatment failure is a significant problem and there is a great need for the development of novel therapies.

Oncolytic viruses have been developed to selectively infect, replicate in and kill targeted cancer cells, while leaving healthy normal cells alone. HSV1716 is an avirulent HSV-1 mutant with a 759 bp deletion in both copies of the RL1 gene (MacLean et al, 1991). HSV1716 has been characterised as a selectively replication competent virus and a potential novel cancer therapy. HSV1716 has not been previously investigated in HNSCC.

The aims of this thesis were to determine whether HSV1716 would replicate in and kill head and neck squamous cell lines, to determine if the cytotoxic effects *in vitro* were enhanced when combined with the conventional chemotherapeutic agent cisplatin and to carry out a phase I clinical safety trial involving the preoperative intratumoural injection of HSV1716 in patients with oral squamous cell carcinoma.

A panel of 3 HNSCC cell lines were used, and multicycle growth experiments were performed to investigate the permissivity to infection. HSV1716 was found to be able to replicate in the HNSCC cells and, during MTS assay studies, was found to be

cytotoxic. However, the use of oncolytic viruses in isolation may not necessarily produce an efficacious effect comparable to, or better than currently available therapies. Combining agents with different mechanisms of cytotoxicity is an attractive approach and gives a number of potential advantages. It gives potential for using lower doses, maximising the therapeutic effect and minimising side effects. Combining therapies also gives potential for enhanced effects, through different mechanisms of cytotoxicity. These interactions can be additive or synergistic. Cytotoxicity studies were performed to evaluate the effect of combining HSV1716 with the conventional chemotherapy agent cisplatin in 3 head and neck cell lines. Preliminary studies established there to be no toxic interactions between the two agents. Isobologram analyses following the cytotoxicity studies identified enhanced cell kill, with additivity, when HSV1716 was combined with cisplatin. We do not know the relationship between the mechanisms of action of these 2 agents and this needs further investigation.

For this therapy to be useful in head and neck cancer it was necessary to investigate whether direct intratumoural injection of HSV1716 is tolerated well and is safe. We conducted a Phase I study involving 20 patients with oral squamous cell carcinoma to evaluate this. Patients received intratumoural injections of HSV1716, under local anaesthetic, at intervals between 24 hours and 14 days preoperatively. Each patient also received an injection of HSV1716 into the contralateral normal buccal mucosa. Doses ranged between 10^5 pfu $5x10^5$ pfu.

The therapy was found to be completely safe with no toxicity. The procedure was technically uncomplicated and well tolerated under local anaesthetic. HSV1716

injection did not cause any clinically apparent tissue reaction. There were no signs of increased inflammation or ulceration of the injected tumour or buccal mucosa. H&E slides of sections of tissue at the tumour injection site did not identify any unexpected inflammation or necrosis. H&E slides of sections of tissue of injected normal buccal mucosa revealed lymphocyte infiltrations and no necrosis. Two out of 20 patients injected with HSV1716 were seronegative for HSV antibodies prior to injection. Both seroconverted within 5 weeks of their injection, indicating an immune response. HSV DNA was detected by PCR in injected normal mucosa in 3 patients up to 72 hours post-injection. There was no infectious virus recovered from any normal mucosa samples and no positivity in immunohistochemistry or *in situ* hybridisation analysis. Overall HSV1716 injection of normal mucosa appears to have consistently caused an inflammatory reaction, but no necrosis and we have found no evidence of viral replication.

Overall we have shown *in vitro* that the selectively replication competent oncolytic virus, HSV1716, is able to replicate in and kill HNSCC cell lines *in vitro*. Cytotoxicity studies have shown that there is additive enhanced cell kill when HSV1716 is combined with the conventional chemotherapy agent cisplatin. Following completion of a Phase I toxicity study we have shown that HSV1716 can be injected into patients with oral SCC readily, comfortably and safely. There was no toxicity in any of the 20 enrolled patients. The lack of toxicity is encouraging and suggests higher doses could be used in future trials. There was however little evidence of biological activity in the tumour specimens analysed in the clinical trial and further investigations are warranted into the reasons for this. As has been seen

with other oncolytic viruses, the effective clinical application is not as straightforward as laboratory studies might have indicated. The principal problem areas involve optimising the delivery and distribution of HSV1716 into a dense heterogeneous SCC tumour cell matrix. Increasing our knowledge of the interactions between HSV1716, the HNSCC tumour cell and the immune system will help to optimise antitumour efficacy. This will maximise its ability to disseminate throughout a tumour mass and endure efficacy, despite encountering the immune response.

Current therapies for recurrent head and neck cancer, such as tumour debulking, further irradiation and chemotherapy have all produced poor responses of limited duration, and significant morbidity. HSV1716 has the potential to complement and improve conventional therapies.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Head and neck cancer is a clinically challenging disease. It encompasses a range of tumours, including squamous lesions of the upper aerodigestive tract, skin neoplasms and glandular neoplasms of the thyroid and major salivary glands. The majority of cases involve squamous lesions of the larynx and oral cavity. Squamous cell carcinoma (SCC) accounts for over 90% of oral malignancy, with the remainder including salivary gland neoplasms, sarcomas and lymphomas.

1.1.2 EPIDEMIOLOGY

More than 500,000 cases of head and neck squamous cell carcinoma (HNSCC) are diagnosed annually, and the UK incidence is over 10 per 100,000 population. Oral cancer accounts for 2-3% of all cancers in the UK, and currently there are over 300 new cases of oral cancer diagnosed in Scotland each year (ISD Scotland, 1999). It is an even bigger problem elsewhere in the world. In parts of India the incidence is 20 per 100,000 population, accounting for 40% of all malignancies.

Oral SCC is predominantly a disease of the elderly with 72% occurring between 55 and 75 years of age, and a mean age of 63 years (ISD Scotland, 1999). There is a higher incidence in males, although this ratio has decreased during the last 50 years. Unfortunately there is an increased incidence among younger age groups, and the incidence is very much higher among people in deprived areas. (Scottish Cancer Intelligence Unit, 2000) (Figure 1).

1.1.3 AETIOLOGY

Tobacco smoking (IARC, 2002) and alcohol consumption (IARC, 1988) are well documented as the most important aetiological factors in oral carcinoma.

However variations in global incidences highlight cultural, environmental and genetic factors. Whereas smoking tobacco and drinking alcohol are significant in

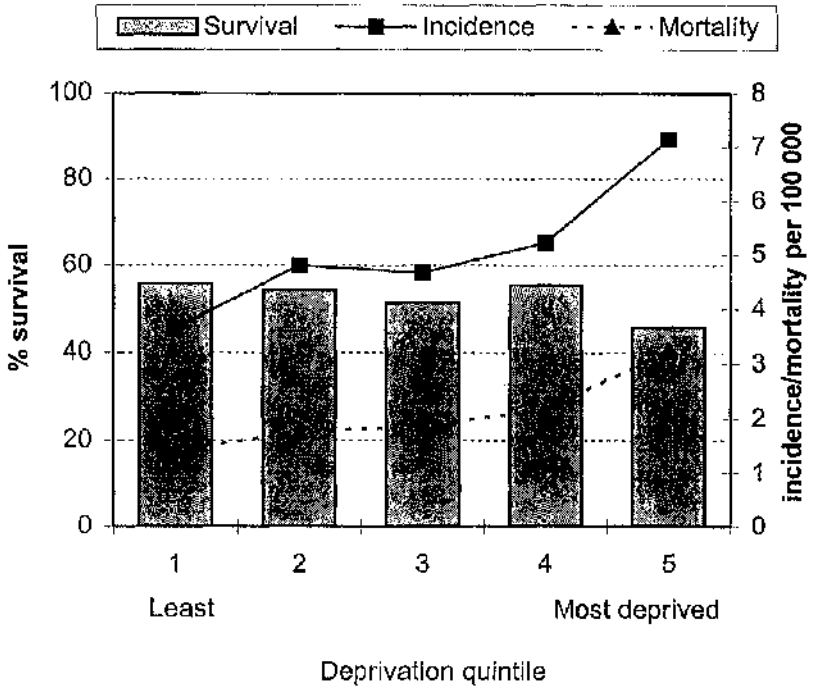


Figure 1. ISD data: Oral Cancer. Incidence, mortality and cause specific survival at 5-years by deprivation. Patients diagnosed 1991-1995.

the West, chewing betel or areca nuts, smoking bidis and smoking snuff are particularly important in the Indian subcontinent (Moore et al, 2000). Cigarette smoke contains over 30 tumorigenic agents including benzanthracene and benzopyrene. Heavy smokers, smoking greater than 20 cigarettes per day, are 6 times more likely to develop oral cancer than non-smokers. In patients who continue to smoke, there is a higher incidence of second primary tumours (10-30% of patients) with a 2-3% risk each year (Silverman et al, 1983). Chewing tobacco and snuff dipping are potent causes of oral SCC in the area of the mouth where the tobacco is held. N-Nitrosamines are the most important carcinogenic agents in chewing tobacco. Alcohol itself is not carcinogenic but it may disturb

the protective cellular lining of the oral cavity to allow access of co-carcinogens like tobacco. Evidence exists that there is a synergistic relationship in the effects of alcohol and tobacco (Saracci et al, 1987), although this is not always a consistent finding.

Unhealthy diets, vitamin deficiencies, particularly A, C and E, and poor dentition are recognised as risk factors for oral carcinoma. A study from Milan in North Italy found that of 17 indicator foods, six were associated with a reduction in risk of oral and pharyngeal cancers (La Vecchia et al, 1991). These were milk, meat, cheese, carrots, green vegetables and fruit. This risk reduction persisted after adjustment for smoking and alcohol consumption.

Anaemia is also associated with oral carcinoma, and several studies have demonstrated the adverse impact of anaemia upon locoregional tumour control and survival (Overgaard et al, 1989; Fein et al, 1995; Warde et al, 1998). The mechanism of action of the anaemia is not fully understood.

The role of infectious agents as aetiological factors remains unclear. There has been some evidence implicating HSV-1 in the aetiology of human oral carcinogenesis. Eglin et al, 1983 examined oral carcinoma specimens by in-situ hybridisation, for evidence of RNA complementary to HSV-1. A notable 66% of the carcinomas were positive. However the authors later discovered that the technique used detected areas of homology between the HSV genome and human cellular DNA, transcribed to excess in proliferating cells. Nevertheless, Kumari et al, 1987 demonstrated HSV-1 antigens in 71% of biopsy samples from oral carcinoma. HPV type 16 DNA has been detected in oral carcinoma biopsies (de Villiers et al, 1985; Shroyer et al, 1991). However Young et al, 1991, did not detect HPV DNA in any biopsies of epithelial dysplasia or SCC. They confirmed

the consistent and frequent finding of HPV in squamous cell papillomas, but the inconsistency of identifying HPV DNA in oral keratotic, premalignant, or cancerous lesions. Overall the roles, if any, that HSV or HPV play in oral carcinogenesis remain unclear.

1.1.4 CLINICAL PRESENTATION

Most head and neck cancers present with symptoms from the primary site. For oral carcinomas this may be a persistent ulcer, with or without associated pain, a persistent red or white mucosal plaque, odynophagia or dysphagia. The patient may have no symptoms at all and the diagnosis made during a routine dental check-up or medical examination. It is not uncommon to present only with an enlarged cervical lymph node, especially with certain "silent" sites such as the tongue base. Systemic metastases are uncommon at presentation. Excluding SCC of the lower lip the most frequent sites for oral SCC are tongue, floor of mouth and mandibular alveolus. It is postulated that these areas form a "sump" into which carcinogen containing saliva pools, and can act over an extended period of time.

1.1.5 PREVENTION AND SCREENING

Primary prevention is crucial in tackling head and neck cancer, and is the most effective way to reduce mortality. Stopping smoking requires combating the chemical and psychological addiction, and ultimately requires personal motivation. Nicotine replacement therapy has been shown to be effective in approximately 13% of smokers who seek advice (Silagy et al, 1994). Early detection of premalignant or early stage disease is also paramount. One good thing about the presentation of oral carcinomas is that they are usually directly visible and accessible. However, this is very often not the case for base of tongue

and more distal head and neck tumours. Increasing patient awareness of the symptoms attributable to oral cancer, and increasing primary care physicians' and dentists' knowledge of the symptoms, signs and indications for referral are fundamental. Unfortunately screening by dentists of a self-selecting population does not target the high-risk heavy smokers and drinkers from deprived areas. Screening patients using a mouth rinse, containing Toluidine blue, has been tested with some effect to identify oral mucosal lesions (Warnakulasuriya et al, 1996). The big problem remains in trying to determine which lesions are likely to progress to malignancy. Epithelial dysplasia is the most important predictive factor (Warnakulasuriya et al, 2000), but aberrant oncogenes (Werkmeister et al, 1999), and integrin expression (Hamidi et al, 2000) may also be significant.

Tobacco and alcohol are clearly defined as aetiological factors in HNSCC. However it remains unclear, for example, why only a fraction of smokers develop cancer. Genetic susceptibility may be significant and research is ongoing. Inheritable differences may exist in the efficiencies of the complex pathways of carcinogen metabolising systems, DNA repair systems, cell cycle controls or apoptosis pathways. Large family studies have demonstrated a three-to-eightfold increased risk of HNSCC in first-degree relatives of patients with HNSCC, indicating a genetic component. (Goldgar et al, 1994; Foulkes et al, 1995; Copper et al, 1995) Identifying high-risk individuals will be important in cancer prevention in the future.

There have been a number of trials using chemicals to suppress or reverse premalignant progression to invasive malignant disease. Agents used include retinoids (vitamin A, β-carotene and 13 cis-retinoic acid), N-acetyl cysteine and seleniums. Chemoprevention using safe non-invasive drugs is appealing, but no

evidence suggests these treatments are effective in routine clinical practice (Lodi et al, 2001).

1.1.6 REFERRAL AND DIAGNOSIS

In 1998 the Department of Health published a White Paper setting a target for all patients with suspected cancer to be seen by a specialist within 2 weeks of their general practitioner deciding that they need to be seen urgently (HMSO, 1998). Since then there have been an increasing number of one-stop clinics or quick and early diagnosis (QED) clinics for the management of head and neck lumps, dysphonia, dysphagia and lesions of the oral mucosa (Kendall et al, 1996). Unfortunately, at present, there is little public awareness of head and neck cancer and too many people present with advanced disease.

All patients are referred to a multidisciplinary team, including a surgeon, clinical oncologist, nurse counsellor, dietician and speech therapist. Patients are often in poor general health, have comorbidities and psychosocial problems, and all team members are essential for a holistic approach to patient management. Ideally a specialist pathologist and radiologist should also be part of the team and, in particular for the management of oral carcinoma, a prosthetic technician is invaluable.

Once referred to a specialist centre, the diagnosis is confirmed by biopsy of the primary site and fine needle aspiration of any enlarged cervical lymph nodes. A panendoscopy is required to fully clinically stage the primary lesion and exclude any synchronous lesions in the upper aerodigestive tract. In the management of oral carcinoma an orthopantomogram is essential to assess dentition and to look for signs of bone invasion. Some centres also obtain a computed tomography scan from the base of skull to the diaphragm, again to exclude synchronous lesions.

The overall incidence of second primaries is 10-20%, (McGarry et al, 1992; Schwartz et al, 1994).

1.1.7 CLASSIFICATION AND STAGING

Accurate classification of head and neck cancer is crucial, both clinically and pathologically. The UICC TNM classification is used universally, and describes the anatomic extent of the tumour (Table 1). In particular it relates to the primary site (T) and regional lymph nodes (N), and to whether metastases exist beyond the regional lymph nodes (M). The TNM classification is a dual system with a clinical classification (cTNM), which is usually used pre-treatment, and a post surgical histopathological classification (pTNM). The former is used in making a decision on treatment, and the latter is used for the estimation of prognosis and the possible selection of adjuvant treatment.

Table 1:

TNM CLASSIFICATION FOR ORAL SQUAMOUS CELL CARCINOMA

Tumour T	
TIS	Carcinoma in situ
T1	Tumour 2 cm or less in greatest dimension
T2	Tumour > 2 cm but no more than 4cm in greatest dimension
T3	Tumour > 4cm in greatest dimension
T4	Tumour with direct extension to bone, muscle, skin, neck
Nodes N	
N0	No evidence of regional lymph node involvement
N1	Metastasis to ipsilateral lymph node no more than 3 cm diameter
N2a	Metastasis to single ipsilateral lymph node >3cm & <6cm diameter
N2b	Metastases in multiple ipsilateral lymph nodes, none >6cm diameter
N2c	Metastases in bilateral/contralateral lymph nodes, none >6cm diameter
N3	Metastases in lymph nodes >6cm diameter
Distant Metastases M	
M0	No evidence of distant metastases
M1	Distant metastases present

Staging into groups I-IV is done according to the TNM classification (Table 2).

Table 2: ORAL CARCINOMA STAGES I-IV

Staging	
Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage III	T3 N0, T1-3 N1 and M0
Stage IV	T4 any N, T1-3 N2-3, any T and N M1

Unfortunately there is a significant amount of inaccurate TNM data recording. Accurate data recording and audit is crucial to evolve, compare and monitor the quality of cancer care. As a result the British Association of Head and Neck Oncologists have introduced National Minimum and Advisory Head and Neck Cancer data sheets. These will hopefully greatly improve the audit potential, especially when looking at the quality of life and functional outcomes from treatment.

1.1.8 MANAGEMENT

Management of head and neck cancer has to be considered in respect to both the primary tumour and the cervical lymph nodes. Surgery and radiotherapy offer equally good long term results in small early tumours. When looking at either form of treatment, the associated early and late morbidity, quality of life and loss of function have to be considered. In general, function is better after radiotherapy than after surgery, but treatment time for surgery is shorter. Many early oral carcinomas are easily accessible for resection and especially if there is no neck disease, a relatively short anaesthetic is required. Radiotherapy usually involves attending daily, Monday to Friday, for 4-6 weeks.

More advanced oral carcinoma is usually managed by surgical resection, if possible, and postoperative radiotherapy. Chemotherapy is included depending on the presence of poor prognostic markers. These include close or involved excision

margins, extranodal spread of tumour, multiple nodes and poorly differentiated pathology with perineural or perivascular spread.

A) Surgery

During the last 20-30 years there have been great advances in the surgical management of advanced disease. This has been aided by advances in anaesthesia to increase the safety of unhealthy patients undergoing long operations. Advances in the use of pedicled myocutaneous flaps and, more recently, with the use of free flaps, larger resection defects can be filled, while improving the patient's loss of function and cosmetic disfigurement. Three common free flaps used in the management of oral carcinoma are the radial forearm free flap (Figure 2), anterolateral thigh flap and deep circumflex iliac artery flap (Figure 3).

Surgery is usually the first line of management of cervical node disease. The nodes are divided into five anatomical levels (Figure 4).

Figure 2: RADIAL FOREARM FREE FLAP

(Cutaneous flap: Left forearm flap is shown with the radial vessels dissected)

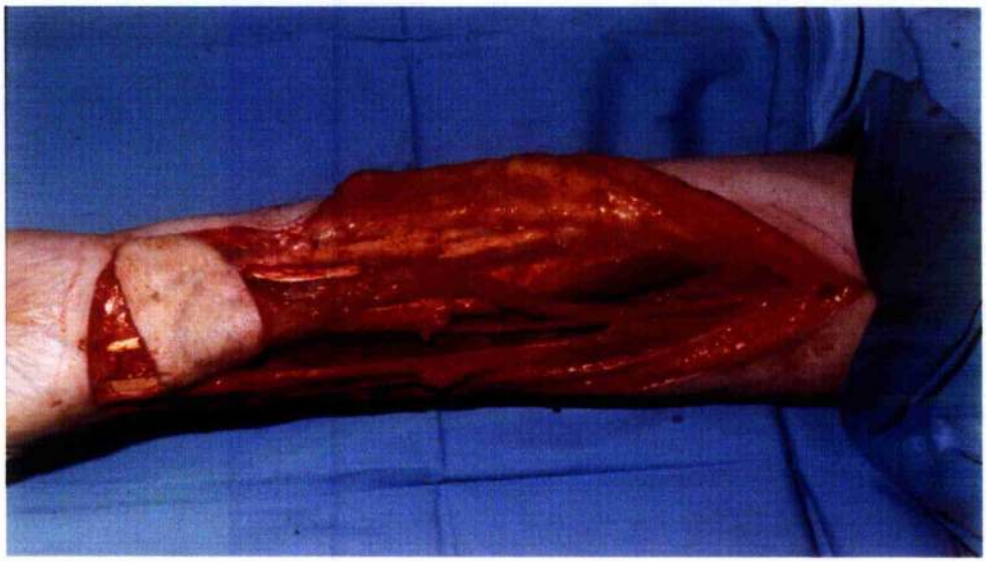


Figure 3:DEEP CIRCUMFLEX ILIAC ARTERY FREE FLAP

(Osseomyocutaneous flap: Skin paddle is shown overlying the left iliac crest)

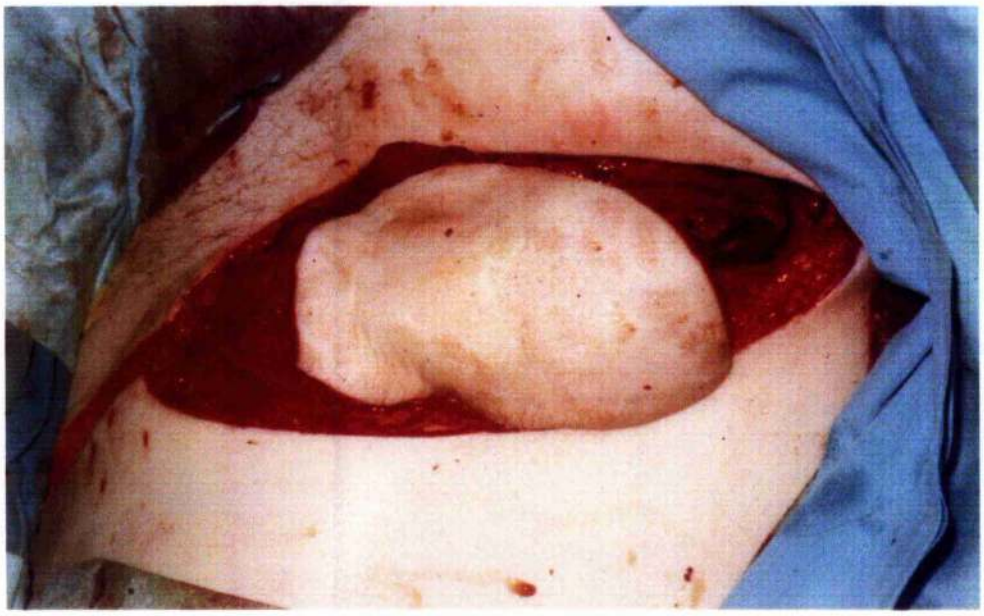
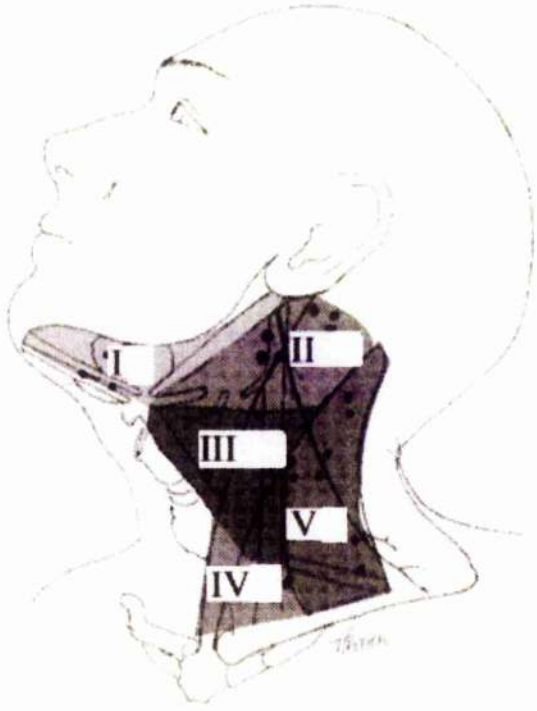


Figure 4: Diagram of the neck showing lymph node levels I-V

(Adapted from McMahon et al, 2000)



Neck dissections are graded into radical, modified radical or selective surgery, depending on the levels of nodes resected, and which "important" structures are resected en bloc.

Neck dissections

Radical Removes levels 1-5, sternomastoid muscle, internal jugular vein, accessory nerve

Modified Removes levels 1-5 but preserves:

Type 1— Accessory nerve

Type 2— Accessory nerve, internal jugular vein

Type 3— Accessory nerve, internal jugular vein, sternomastoid muscle

Selective Removal of only selected lymph node groups and preservation of other structures

Extended As classic procedure, but with the removal of additional lymph node groups or non-lymphatic structures such as overlying skin

In the clinical presence of nodal disease, some type of modified radical dissection is usually indicated. There has been a move away from performing radical surgery. Modified radical dissection has been shown to be oncologically safe in the N1 and N2 neck, while improving the significant morbidity, especially shoulder disability, associated with radical surgery (Buckley et al, 2001). Controversy remains over the potential indications for selective neck dissections. If there is no clinical evidence of nodal disease, but the expected incidence of node metastasis is greater than 20%, it is common practice to treat the neck (Table 3). The alternative is to wait and see. As yet there has not been a prospective randomised trial comparing the 2 approaches.

Unfortunately the advances in surgery have not been paralleled with increased patient survival. Research continues into the best regimens and indications for radiotherapy and chemotherapy, while constantly exploring novel therapies.

SITE	TX, T1, T2	T3, T4
Oral tongue	**22**	**62**
Floor of mouth	**20**	**49**
Tonsil fossae	**71**	**80**
Base of tongue	**69**	**79**
Nasopharynx	**88**	**86**
Supraglottis	**41**	**62**
Pyriform sinus	**34**	**77**
Glottis	**5**	**20**

Table 3: Incidence of nodal metastases by site and T stage of primary tumour (Lindberg, 1972)

B) RADIOTHERAPY

Radiotherapy has been used to treat head and neck cancer for decades, and it remains unclear what the optimum regimens are for patient groups. Trial data is confused by the inclusion of patients with varying subsites of disease. New regimens are compared to conventional therapy, which in itself has varying schedules throughout the UK and the rest of the world. Current regimens are not always based on scientific evidence, but on resource limitations. Demands on staff and machinery are usually the reason for delays in oncological therapies.

A typical regimen for conventional radiotherapy in the UK would involve a total dose of 66 Gray (Gy), given in 33 doses or fractions. Important factors in the efficacy of radiotherapy are the total dose, the number of fractions, the resulting dose per fraction and the overall treatment time. As with chemotherapy the therapeutic ratio between the effects on the tumour and on normal tissue in the radiation field are crucial. Strategies to improve the outcomes from radiotherapy have concentrated on altering radiation fraction schedules. Hyperfractionation

involves giving multiple smaller fractions, with an aim of decreasing the incidence of late radiation changes (Fowler, 1984). A higher overall dose can therefore be given without incurring excessive late damage, with the objective of improving tumour control. Late tissue damage can occur months to years after irradiation. The EORTC 2791 trial compared a hyperfractionated schedule of 80.5 GY given in 70 fractions twice per day, over 7 weeks, with a conventional dose of 70 Gy using 35-40 daily fractions (Horiot et al, 1992). Five-year local control was increased from 40% in the daily fractionation, to 59% in the hyperfractionated arm. The improvement in locoregional control was not associated with an increase in late tissue damage.

Accelerated fractionation involves reducing the overall treatment time while maintaining the same or a similar number of fractions as for conventional radiotherapy. This is thought to allow less time for tumour cell division during treatment, and accelerated repopulation due to exposure to radiation. This approach is associated with increased mucosal toxicity and dose reduction is necessary (Skladowski et al, 2000).

Accelerated Hyperfractionation aims to achieve both objectives of increasing locoregional control and decreasing late tissue damage. Saunders et al, 1991, compared continuous, hyperfractionated, accelerated radiotherapy (CHART) with conventional therapy in patients with head and neck and bronchial carcinoma. Locoregional control in the head and neck patients was 90% in the CHART group compared to 62% in the conventional treatment arm. Increased acute mucositis was significant in the CHART group, but late changes in the skin, mucosa, connective tissues and salivary glands were reduced. Bigger randomised multicentre trials followed. The EORTC 22851 trial corroborated the finding of

increased locoregional control with 13% increase after 5 years in the accelerated hyperfractionated group (Horiot et al, 1997). In this study patients in the experimental arm were given a rest period of 12-14 days in the middle of a 5 week regimen to allow recovery of the normal epithelium. However the increased acute and late morbidity was significant. Severe late toxicity occurred in 14% of patients in the accelerated fractionated group, including 2 cases of radiation induced myelitis. Dische et al, 1997 conducted a larger CHART trial involving 918 patients randomised between conventional therapy and an accelerated and hyperfractionated schedule of 54 Gy in 36 fractions over 12 days. There was no difference in locoregional control between the 2 arms, but reduced late morbidity in the CHART arm.

A more novel approach to radiotherapy involves intensity modulation. This aims to give an even distribution of radiation dose within a target tumour, which is naturally irregularly shaped. It aims to spare the surrounding normal tissue and so allows for escalation of the radiation dose (Nutting et al, 2000). The technique is complicated, time consuming and clinical trials are required to prove its benefit.

Overall, the evidence for the optimal external radiotherapy regimen remains inconclusive. There are too many variables between fractionation parameters, head and neck cancer subsites included in trials, the staging of the carcinomas included and tangibly significant molecular biological differences in the carcinomas. It remains unclear why a percentage of head and neck carcinomas at a particular subsite respond to radiotherapy and others do not. Unfortunately this means that some patients are enduring treatment side effects with no chance of benefit.

Brachytherapy is the implantation of radioactive sources directly into the tumour. High doses of radiation are delivered to the tumour while sparing healthy surrounding tissues. This technique can be used for the primary treatment of tumours, or for boosting to the primary tumour after locoregional external beam radiotherapy. In the latter, it is important that the external treatment and brachytherapy run close together to avoid any extension in the overall treatment time. It can also be used as salvage therapy in previously irradiated tumours where no further external radiotherapy can be given. There are a number of potential benefits for its use in head and neck cancer. Notably structure and function is preserved, and this is particularly relevant to tongue carcinomas where the destruction and disability from a glossectomy are considerable. Also avoiding or reducing external beam radiotherapy means nearby critical structures, including brain, eyes and spinal cord, are spared. Wound infection, breakdown and pain are the most common complications of this therapy.

C) CHEMOTHERAPY

Chemotherapy in the past has been reserved for palliative therapy for head and neck cancer patients. It is now a common component in the curative management of advanced (stage III and IV) HNSCC. The most commonly used combination agents are platinum-based cisplatin and 5-fuorouracil (5-FU). This combination has been shown to have significantly higher response rates than single agents alone, although there is no survival benefit and toxicity is increased (Forastiere et al, 1992; Jacobs et al, 1992). Newer drugs have been investigated to try and improve patient survival. Taxanes, including docetaxel and paclitaxel, have significant activity in head and neck cancer. Early trial response rates were high, at up to 40%, but acute toxicity was high too. Severe or life-threatening

granulocytopenia affected 91% of patients in one trial (Forastiere et al, 1998). New combination regimens pairing standard agents, such as cisplatin, with new-generation drugs have shown some promising results. But they have not shown superiority over cisplatin and 5FU. Toxic effects, including myelosuppression, have been significant.

For patients presenting with locally advanced HNSCC, standard treatment includes radical surgery and/or radiotherapy. Unfortunately the long-term survival of these patients remains poor. To try and improve the prognosis in these patients, chemotherapy has been investigated as a treatment in head and neck cancer for decades. Different approaches have been investigated, including administering the chemotherapy before definitive surgery and/or radiotherapy (neoadjuvant), during radiotherapy (concomitant), or following completion of surgery and/or radiotherapy (adjuvant).

Neoadjuvant therapy was investigated for many years. The main aims were to potentially allow for greater organ preservation, improve local and distant disease control, and improve survival. Many randomised trials were conducted, but most were too small to detect even a moderate effect on survival. The Meta-Analysis of Chemotherapy on Head and Neck Cancer (MACH-NC) analysed 63 trials of locoregional treatment with or without chemotherapy. They concluded there is no survival benefit associated with neoadjuvant therapy (Pignon et al, 2000). Other meta-analyses have drawn the same conclusions (El-Sayed et al, 1996; Browman, 1994). Neoadjuvant chemotherapy used with an aim of organ preservation remains investigational (The Department of Veteran Affairs Laryngeal Cancer Group, 1991; Lefebvre, 1996). Trials have compared radical surgery and radiotherapy with neoadjuvant chemotherapy and radiotherapy in responders; or

neoadjuvant chemotherapy, radiotherapy and salvage surgery in non-responders. Patterns of recurrence differed between the groups, with more local recurrences and less distant metastases in the chemotherapy group than in the surgery group. Jacobs et al, 1987, treated 30 patients with resectable stage III or IV, M0 head and neck cancers in the oral cavity, oropharynx, hypopharynx and larynx with three cycles of neoadjuvant cisplatin and 5FU. 40% of patients responded completely and were treated with radiotherapy alone, sparing surgery. The partial or non-responders underwent salvage surgery and postoperative radiotherapy. There was no significant difference in the relapse-free survival at 2 years. This indicates that patients with complete pathological responses to neoadjuvant chemotherapy could be spared surgery, with no compromise to their survival. A significant problem is not being able to predict a tumour's response.

Analyses of adjuvant therapy trials have not shown any survival benefit (Munro, 1995; Pignon et al, 2000). However the use of concomitant chemotherapy and radiotherapy offers the most promising data. Pignon et al, 2000, in their meta-analysis showed an absolute survival benefit of 8% at 2 and 5 years with concomitant therapy. Unfortunately this result came from a number of very heterogeneous trials involving only 11% of the patients analysed, and so needs corroboration. Browman et al, 2001, analysed 18 randomised controlled trials involving concomitant therapy, and showed a survival benefit of 11%, but with significant acute toxicity. Platinum-based regimens were used in nearly half of the patients, and were the most effective. Acute toxicity is increased by most regimens of simultaneous chemoradiotherapy, and the late tissue effects may be enhanced. This implies that regimens are not necessarily improving the therapeutic ratio.

1.1.9 PROGNOSIS

Overall the modifications of established cancer therapies, including surgery, radiotherapy and chemotherapy, have not had a significant impact on patient survival. Most patients with HNSCC present with advanced disease, with incidences of locoregional recurrence and distant metastases at 50% and 25% respectively. Five-year survival rates for stage III and IV disease range from 0% to 40%, depending on site and resectability status (Forastiere, 1994). Unfortunately the patient with recurrent or metastatic disease is often considered incurable. Treatment failure is a significant problem and there is a great need for the development of novel therapies.

1.2 MOLECULAR GENETICS OF HEAD AND NECK CANCER

The process of carcinogenesis is complex and involves many genetic mutations. At least 10 independent genetic alterations are required to convert a normal cell into a squamous carcinoma cell (Renan, 1993). During this transition the normal regulation of the cell cycle is lost. An important aim for investigating head and neck cancer genetics is to find markers to indicate the most effective type of treatment or prognostic indicators of patient survival. Understanding of the molecular genetics may also permit the development of new cancer therapies.

1.2.1 CYTOGENETICS

Cytogenetic characterisation of HNSCC identifies multiple complex chromosomal breakpoints, gains and losses. The most frequent changes usually involve deletions, with losses commonly affecting chromosomes 3p, 5q, 8p, 9p, and 18q (Van Dyke et al, 1994). Loss of 18q is associated with a poor prognosis and losses of 3p21 and 8p11 are associated with radioresistance. Breakpoints in 11q13 have been associated with radiosensitivity (Cowan et al, 1993). These markers could be

useful in predicting a tumour's response to radiotherapy. Califano et al, 1996, proposed a model for head and neck carcinogenesis (Figure 5).

The normal cell cycle is controlled in 2 main ways: a cascade of protein (CDK-cyclin complexes) phosphorylations that relay a cell from one stage to the next, and a set of checkpoints that monitor completion of critical events (Figure 6).

Figure 5: HUMAN CELL CYCLE

1.2.2 ONCOGENES

Naturally occurring proto-oncogenes encode proteins that regulate normal cell proliferation or apoptosis. If mutations occur they become oncogenes with resulting increased function or over-expression and unregulated cellular proliferation. Examples include cyclin D1, erbB, ras, and SCCRO. The proto-oncogene Cyclin D1 is a cell cycle regulator localised to chromosome 11q13, and is commonly amplified in HNSCC. This amplification has been correlated with a poor prognosis (Callender et al, 1994; Namazie et al, 2002). Over-expression is also associated with occult cervical metastases, early recurrence and shortened survival (Michalides et al, 1995; Capaccio et al, 2000).

Ras genes, including H-ras, K-ras and N-ras, are rarely mutated in HNSCC at a frequency of <5% in the Western world (Yarbrough et al, 1994; Matsuda et al, 1996). However some studies have indicated over-expression is involved in oral carcinoma (McDonald et al, 1994). Interestingly Hoa et al, 2002, found the level of K-ras protein expression to be a major determinant of HNSCC proliferation and the amplification of K-ras to contribute to tumour growth.

Epidermal growth factor receptor (EGFR), erb-B1, is part of the erb-B family of receptor tyrosine kinases.

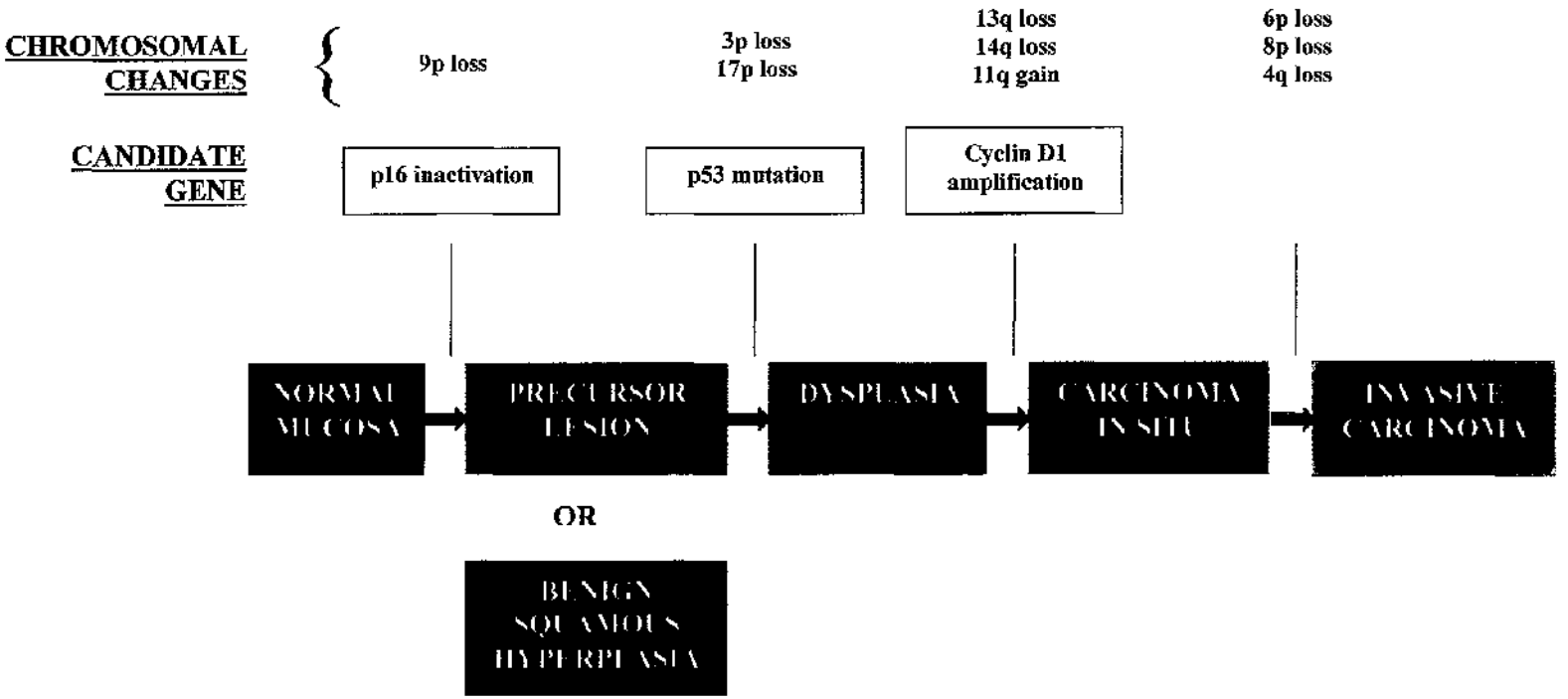
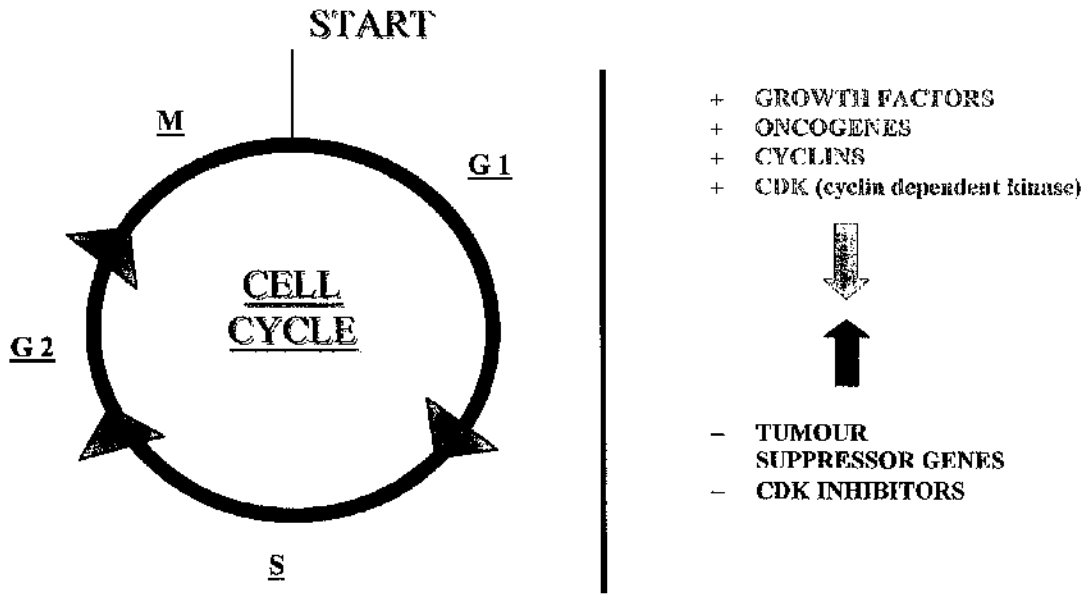


Figure 6: Genetic progression model for head and neck squamous cell carcinoma

(Adapted from Califano et al, 1996)

Figure 6: HUMAN CELL CYCLE



(The cell cycle consists of four ordered phases Gap 1 (G1), DNA synthesis (S), Gap 2 (G2) and DNA mitosis (M). Stimulatory (+) and inhibitory (-) proteins control the cycle. Genetic mutations in cancer cells cause defects in the cell cycle regulation.)

EGFR expression is reported in up to 90% of HNSCC, and is associated with a poor prognosis (Grandis et al, 1996; Ke et al, 1998). It has a complex role and signal transduction pathways contribute to the development of malignancies through various processes, including cell cycle progression, inhibition of apoptosis, angiogenesis and metastases.

Squamous cell carcinoma-related oncogene (SCCRO) is located on chromosome 3q. It is overexpressed in oral carcinoma, and this expression has been correlated

with aggressive clinical behaviour, including nodal metastases and poorer survival outcome (Estilo et al, 2003).

1.2.3 TUMOUR SUPPRESSOR GENES

Tumour suppressor genes encode either negative regulators of the cell cycle or positive regulators of apoptosis. Many are involved in DNA repair and mutations lead to loss of function and prevent maintenance of genomic stability. Examples include p53, Retinoblastoma (Rb), p16 and p21.

p53 is a tumour suppressor gene located on the short arm (p) of chromosome 17. Once activated the p53 protein can induce growth arrest as well as cell death (apoptosis). Mutations in the p53 gene are found in up to 40-50% on HNSCC (Greenblatt et al, 1994). Alcohol and tobacco are associated with these mutations, but there does not appear to be a clear correlation between mutation and poor prognosis (Boyle et al, 1993; Brennan et al, 1995). In tumour cells the enhancement of p53 expression has been demonstrated after exposure to anticancer agents, which suggests its role in triggering cellular responses to these drugs (Eastman, 1990). Further, loss of p53 function has been linked to resistance to chemotherapy, and mutant p53 could prevent cells from undergoing apoptosis induced by cytotoxic agents (Lowe et al, 1993, Lowe et al, 1994).

Retinoblastoma is an important tumour suppressor gene involved in controlling the cell cycle. Study results looking at the incidence of Rb abnormalities in HNSCC vary significantly, between 6% and 74%, and there does not appear to be a clear correlation between Rb mutation and poor prognosis (El-Nagger et al, 1999; Koontongkaew et al, 2000).

p16, p21 and p27 tumour suppressor genes act to modulate cell proliferation. Abnormalities of p16 are common in HNSCC, with low expression in most head

and neck cancers. Abnormal p16 is associated with decreased survival, increased recurrences, tumour progression and nodal metastases (Jares et al, 1997; Danahey et al, 1999; Bova et al, 1999). Expression of p21 and p27 is variable in HNSCC, and there is no clear relationship between p21 staining and clinical outcome. The presence of p27 has been correlated with improved survival (Pruneri et al, 1999; Tamura et al, 2001).

1.2.4 TELOMERASE

Telomeres are tandem repeats of DNA associated with specific proteins, and function to cap the ends of chromosomes and maintain their integrity. In doing so they prevent chromosomes from fusing together and stops their ends being processed in the same way as broken DNA ends. Telomerase is a cellular ribonucleoprotein reverse transcriptase responsible for replenishing the telomeric DNA, as telomeres shorten with each cell division. Telomerase activity is completely repressed in most somatic cells, and so telomere length is not adequately maintained throughout life. Cell mortality ensues this replicative senescence. Telomerase activation is an important event in the conversion of a normal cell to a malignant cell. This results in an immortal phenotype with loss of replicative senescence. This state can arise early in HNSCC progression, but generally is more common in advanced disease (McGregor et al, 1997; Edington et al, 1995). Mao et al, 1996, showed telomerase expression in 90% of primary HNSCC and 100% of pre-malignant lesions.

1.3 GENE THERAPY IN HEAD AND NECK CANCER

There are several approaches being investigated in the development of novel therapies for head and neck cancer including gene therapy, antibody therapy and viral oncolytic therapy.

1.3.1 PRINCIPLES OF GENE THERAPY

The aim of cancer gene therapy is to introduce new genetic material into target cells, for a therapeutic effect, without toxicity to non-target tissues. This includes the transfer of new genetic material as well as the manipulation of existing genetic material. Two key factors in its success are the mode of delivery and the transfection efficiency.

Transfer of genes may either be *in vivo,* in which the DNA and vector are directly introduced into the body, or ex vivo, in which cells are removed from the body, transfected with DNA and then reintroduced into the patient. The accessibility of oral cancer lesions readily allows the direct, *in vivo*, injection of genetic material. Gene therapy has the potential to treat primary, recurrent or inoperable disease, by injecting surgically resected margins or injecting the tumour directly. Systemic therapy is most desirable to target metastatic disease, although it presents significant problems.

Our increasing knowledge of cancer molecular genetics gives direction to the different strategies utilised in gene therapy. These include adding a tumour suppressor gene (gene addition therapy); deletion of a defective gene (gene excision therapy); down-regulation of the expression of genes that stimulate tumour growth (antisense RNA); enhancement of immune surveillance (immunotherapy); activation of prodrugs that have a chemotherapeutic effect ("suicide" gene therapy or gene directed enzyme pro-drug therapy, GDEPT); introduction of viruses that destroy tumour cells as part of the virus replication cycle; delivery of a drug resistance gene to normal tissue for protection from chemotherapy and the introduction of genes to inhibit tumour angiogenesis.

1.3.2 GENE DELIVERY VECTORS

A) CHEMICAL AND PHYSICAL VECTORS

The therapeutic gene is transferred into the tumour cells using chemical, physical or viral vectors. Chemical transfection introduces DNA by calcium phosphate, lipid or DNA/protein complexes. Lipid vectors are generated by a combination of plasmid DNA and a lipid solution that result in the formation of a liposome. Liposomes, as with protein complexes, are rapidly cleared from the body via the immune system, thus this mode has limited use clinically due to transfection inefficiency. Physical methods of DNA transfection include electroporation, microinjection and the use of ballistic particles. During electroporation high-voltage electric impulses can be delivered into a tumour, cell membrane permeability is increased with enhanced uptake of naked DNA, for example, after injection. Allegretti et al, 2001, demonstrated that electroporation was safe and effective in 14 patients with squamous cell carcinoma. Particle bombardment has been studied to deliver genes to the oral mucosa in pre-clinical models, although it is limited by transfection inefficiency (Shillitoe et al, 1998). Overall nonviral vectors have advantages in terms of simplicity of use, ease of large-scale production and lack of specific immune responses, however transfection efficiency is a problem.

B) VIRAL VECTORS

Viral vectors commonly used in cancer gene therapy are retroviruses (including lentiviruses), adenoviruses and herpesviruses. Viruses are obligate intra-cellular parasites and tend to be very efficient at transfecting their own DNA into host cells. This DNA is expressed to form new viral particles. The aim in developing a

viral vector is to eliminate the pathogenicity of a specific virus, while retaining the efficiency of gene transfer and expression.

i) RETROVIRAL VECTORS

Retroviruses are RNA viruses that undergo reverse transcription after infecting a dividing cell, thereby producing double-stranded DNA. This DNA integrates stably into the host genome, thus passing copies of the genes to all specific generations of cells. Transfection is efficient and gene expression is permanent, which is advantageous in situations where long-term expression of the transgene is required. This is pertinent to inherited conditions such as cystic fibrosis, however permanent expression raises long-term safety concerns. Another concern is the random DNA integration, which may be harmful if germ cell transfection occurs. The use of retroviruses is limited in solid tumours due to the low production titre that can be produced, thus reduced transduction efficiency.

ii) ADENOVIRAL VECTORS

Adenoviruses are double-stranded DNA viruses that infect a cell, lose their protein coat, and transfer DNA into the nucleus, where it is transcribed. This DNA does not integrate into the host genome, and so its effects are transient. To generate a defective adenovirus for gene transfer application, certain genes involved in viral gene expression and replication can be removed. The transgene can then be introduced into the vacated site. Adenoviruses have a larger genome than retroviruses, allowing more regions of DNA to be removed with transgenes of up to 36 kb inserted. The advantage of adenoviral vectors is that most cells are susceptible to infection, regardless of their position in the cell cycle. Also transduction efficiency is enhanced due to the production of relatively higher titres. Since exposure to adenovirus in the community is common, approximately

90% of humans have already formed antibodies against the virus. Pre-existing antibodies can limit the effectiveness, particularly on second exposure to the vector (Mack et al, 1997). Another disadvantage of adenoviral vectors is that they continue to express other viral products, which are recognised by the immune system. This can lead to an inflammatory response and short-term gene expression.

iii) HERPES SIMPLEX VIRAL VECTORS

Most herpes simplex virus vectors are developed from strains of herpes simplex virus type 1 (see section 1.6). The double-stranded DNA virus infects cells, replicates within the cells and causes cell lysis thereby infecting surrounding cells. Genes can be transferred rapidly and efficiently. HSV has several proteins that are expressed early in infection, and these in turn activate expression from the other HSV promoters. Inactivation of one or more of these immediate early proteins, ICP0, ICP4, ICP22, ICP27 and ICP47 results in a replication defective vector, except in a complementing cell line (Marconi et al, 1996; Wu et al, 1996; Zhu et al, 1996).

HSV vectors have the advantages of being able to infect non-dividing cells and establish latency in some cell types. Also many HSV proteins are nonessential for viral replication and can therefore be deleted to permit the insertion of large exogenous coding regions into the viral genome.

1.3.3 GENE THERAPY STRATEGIES IN HEAD AND NECK CANCER

A) P53

There have been a variety of gene therapy strategies for oral carcinoma. The most extensively studied mutations in oral cancer are those of p53. p53 plays a role in cell cycle regulation and in apoptosis. *In vitro* and *in vivo* studies using p53 gene

transfer into HNSCC, via a replication deficient adenoviral vector, showed suppression of tumour growth by apoptosis (Liu et al, 1994; Liu et al, 1995). The over-expression of the transferred wild type p53 becomes dominant over its mutant gene, resulting in programmed cell death. Clayman et al, 1998, conducted a clinical trial using adenoviral-p53 in patients with advanced HNSCC. Results showed this approach to be safe, well tolerated and some signs of activity were observed. However the viral transduction appeared to be low. A further *in vitro* study showed that the retinoid, All-trans-retinoic acid, given 72 hours pre-viral therapy, enhanced the adenoviral-p53 expression and synergistically induced apoptosis in HNSCC (Nakashima et al, 2001).

B) EPIDERMAL GROWTH FACTOR RECEPTOR

RNA that is complimentary to a strand of DNA expressing a gene can inhibit gene expression. This "antisense" RNA can prevent the activity of oncogenes, including EGFR and its ligand, transforming growth factor alpha (TGF-α). Pre-clinical studies using a liposome mediated gene transfer of plasmids capable of expressing antisense EGFR or TGF-α in HNSCC, have shown tumour growth inhibition, suppression of protein expression and increased apoptosis (He et al, 1998; Endo et al, 2000). A Phase I study is under way to determine the safety of liposome-mediated intratumoural EGFR antisense gene therapy, at the University of Pittsburgh, USA. The successes of this approach depend on introducing sufficient quantities of antisense molecules to downregulate the target gene, and inhibit tumour growth. A potentially more efficient method involves using small interfering RNAs (siRNA), which mediate mRNA degradation in the process of RNA interference (RNAi) (Bertrand et al, 2002). RNAi is the degradation of

cellular RNAs by gene specific double stranded RNA. The increased efficiency is due to enhanced resistance of siRNAs to nuclease degradation.

C) IMMUNOTHERAPY

Immunotherapy is used to enhance the patient's immune response to a tumour or enhance the immunogenic potential of tumour cells. Unsuccessful attempts have been made to enhance the patient's immune response with the non-specific immunostimulant Bacillus Calmette-Guerin (Papac et al, 1978; Taylor et al, 1983)). However OK-432, a non-specific cytokine inducer prepared from human streptococcus pyogenes, has been shown to cause tumour regression (Kitahara et al, 1996).

Pre-clinical studies have been successful in enhancing the immunogenic potential of tumours, by transfecting tumours with cytokines such as the interleukins IL-2, IL-4 and IL-12; tumour necrosis factor-alpha (TNF-α) or the growth factor granulocyte macrophage colony-stimulating factor (GM-CSF) (Boscia et al ,1988; Whiteside et al, 1996, Sacchi et al, 1990). The use of combined IL-2 and IL-12 non-viral gene therapy in a HNSCC murine model resulted in significant anti-tumour effects, most likely due to increased activation of cytolytic T-lymphocyte and natural killer cells (Li et al, 2001).

Tumour cells frequently have low levels of class I major histocompatibility complex (MHC) proteins, which limits the ability of these cells to present antigens to cytotoxic T cells. Lang et al, 1999, have shown that there is impairment of T cell activation in head and neck cancer patients and this is partly due to B7 expression. If tumour cells are transduced with low levels of class I MHC alloantigens they become less oncogenic (Tanaka et al, 1985; Wallich et al, 1985; Plautz et al, 1993). These pre-clinical findings led to the development of

Allovectin-7 and a phase I clinical trial (Gleich et al, 1998). Allovectin-7 is a drug designed to produce expression of HLA-B7, a class I MHC protein, in patients with head and neck cancer. It contains a cationic lipid complexed to a respiratory syncitial virus promoter and plasmid DNA that encodes the HLA-B7 heavy chain. Patients with advanced HNSCC received intratumoural injections of Allovectin-7, with no toxic effects and tumour regression in 4 out of 9 patients. Similar results were found in a larger multi-centre trial, and an efficacy trial is under way (Gleich et al, 2001).

D) GENE-DIRECTED ENZYME PRODRUG THERAPY

Gene-directed enzyme prodrug therapy (GDEPT), also known as "suicide "gene therapy, is a technique that involves physical delivery into a cell of a gene whose protein product enables a non-toxic prodrug to be activated into an active cytotoxic drug. This process relies on a bystander effect, as expression of the foreign enzymes does not occur in all cells of a targeted tumour. There are different theories explaining the cytotoxicity in untransfected cells including gap junctional communication, local inflammation, haemorrhagic necrosis and the production of Fas and FasL, both members of the tumour necrosis factor family (Elshami et al, 1996; Floeth et al, 2001; Hall et al, 2002). The success of this approach depends on the design of the gene therapy vectors, the chemistry of the prodrugs and their toxic metabolites and the means of delivery specifically to target cells. This is in addition to the efficiency of the bystander effect. The enzymes used fall into two categories. The first comprises foreign enzymes of non-mammalian origin, with or without human homologues. Examples include viral thymidine kinase (TK), bacterial cytosine deaminase (CD), carboxypeptidase G2 (CPG2), purine nuceoside phosphorylase (PNP), and nitroreductase (NR). The

second category comprises enzymes of human origin that are absent or are expressed only at low concentrations in tumour cells. Examples include deoxycytidine kinase (dCK) and cytochrome P450. Enzymes from the first group have the potential of eliciting an immune response, unlike dCK or cytochrome P450. However use of the second group may result in some prodrug activation in normal cells. A common example of GDEPT is the combination of HSV-thymidine kinase (HSV-TK) and the nucleoside analogue ganciclovir. Ganciclovir is an antiviral drug that is phosphorylated by HSV-TK and then by cellular kinases to produce ganciclovir triphosphate, which disrupts DNA synthesis during S phase resulting in cell death (Matthews et al, 1988). This approach selectively targets actively dividing cancer cells. The vectors used can be chemical, physical or viral, but there has been relatively little research using GDEPT in HNSCC. An *in vivo* study using adenovirus mediated gene transfer of the HSV-TK gene did show tumour regression in a murine SCC model (O'Malley et al, 1995; Sewell et al, 1997).

1.4 OTHER NOVEL BIOLOGICAL AND CHEMICAL THERAPIES

Other novel therapies include anti-EGFR monoclonal antibodies, EGFR tyrosine kinase inhibitors and farnesyl kinase inhibitors to target ras genes. Cetuximab (IMC-C225) is an anti-EGFR monoclonal antibody that has been shown to be safe in phase I clinical trials (Baselga et al, 2000; Shin et al, 2001). A xenograft model has shown cetuximab to enhance the antitumour efficacy of chemotherapy agents such as cisplatin and doxorubicin (Shin et al, 2001, Baselga et al, 1993). A phase II trial showed cetuximab to be well tolerated when combined with platinum based chemotherapy in patients with recurrent or metastatic HNSCC. The tumour response rate was 10% (Baselga et al, 2005)

ZD1839 is a selective EGFR tyrosine kinase inhibitor. Pre-clinical studies have shown it to inhibit cellular proliferation in SCC cell lines, and enhance cisplatin-induced apoptosis and radiosensitivity in oral SCC cell lines (Magne et al, 2002). A phase I trial showed ZD1839 to be well tolerated with some antitumour activity observed (Baselga et al, 2002).

Farnesyl transferase inhibitors (FTIs) are a class of compounds that inhibit a critical step in the expression of mutated ras genes (Sepp-Lorenzino et al, 1995). Several novel FTIs have been developed including R115777, SCH66336 and L-778,123. Pre-clinical studies have shown that these agents have potential activity in various solid tumours, including HNSCC (Chun et al, 2003). The exact mechanism of action appears unclear, as *in vivo* studies have shown antitumour activity in the absence of ras mutations (Sepp-lorenzino et al, 1995; End et al, 2001). Farnesylated proteins other than ras may contribute to the action of FTIs. A toxicity study demonstrated the FTI, L-778,123, to be well tolerated when combined with HNSCC. Some anti-tumour response was observed without an increase in radiotherapy toxicity (Hahn et al, 2002).

1.5 ONCOLYTIC VIRUSES

1.5.1 PRINCIPLES OF ONCOLYTIC VIRAL THERAPY

For several decades viruses have been investigated as a means of killing tumour cells (Wheelock et al, 1964; Asada, 1974). Smith et al, 1956, described the use of adenoviruses in the treatment of cancer where tumour necrosis was shown following direct intratumoural injection in patients with cervical cancer. Our greater understanding of molecular genetics during the last 15 years has enabled the design of far more effective oncolytic viruses. Martuza et al, 1991, first

demonstrated the potential utility of genetically engineered replication-selective viruses for cancer treatment, treating gliomas in nude mice.

The perfect oncolytic virus is one that selectively infects, replicates and kills targeted cancer cells, while leaving healthy normal cells alone. During this process the virus replicates to levels that are many logs higher than the input dose. Research has concentrated on a number of different oncolytic viruses, particularly adenovirus and HSV in head and neck cancer. Some are naturally attenuated viral strains, including some strains of reovirus or vesicular stomatitis virus that infect or replicate more effectively in cancer cells. Adeno-associated virus has been shown to selectively induce apoptosis in cells that lack active p53 (Raj et al, 2001). Other viruses, such as HSV-1 and adenovirus, are genetically modified to produce oncolytic effects. A significant disadvantage of current gene therapy vectors is the poor tumour transduction, despite any bystander effects. Using a replication competent virus that selectively replicates in tumour cells overcomes this problem.

The selectivity for cancer cells can occur either during infection or during replication. Viruses can be engineered, for example, by altering their surface proteins to recognise cellular receptors specific to tumours. Alternatively the viral genes required for efficient replication can be modified, so that the virus only replicates in cells that have disruptions in normal homeostatic pathways. This could involve tumour suppressor gene defects or oncogenic pathways.

1.5.2 ONCOLYTIC ADENOVIRUS

A common genetic defect in HNSCC involves the p53 tumour-suppressor pathway, which can be targeted using adenovirus. Cellular DNA damage results

in p53 induced cell-growth arrest or apoptosis. This same cellular stress response results from viral infection, which eliminates propagation of the virus. The E1B55kD adenoviral protein has been thought to block p53 activity, prevent apoptosis and allow adenoviral replication. Adenovirus lacking E1B55kD, *dl*1520 (ONYX-015), was successfully engineered to selectively replicate in and lyse cancer cells that have defects in p53 function (Bischoff et al, 1996). The exact mechanism of action remains unclear, as altering a viral gene can affect several cell functions. Deleting E1B55kD provides the virus with some tumour specificity, but it also affects other phases of the viral cycle that reduce the efficacy of viral replication and oncolytic activity (Harada et al, 1999). Consequently several viral gene products might have to be altered to improve replicative selectivity.

ONYX-015 was the first genetically engineered, replication-selective virus to be used in humans. Safety was of paramount concern and a staged clinical research and development approach was designed to increase systemic exposure to the virus sequentially. A number of clinical trials with a variety of tumour types have been completed, initially starting with intratumoural injection (Ganly et al, 2000) and then progressing to intraperitoneal (Vasey et al, 2002), intra-arterial/hepatic artery (Reid et al, 2001) and finally intravenous administration (Nemunaitis et al, 2001) in patients with metastatic carcinoma. The virus was well tolerated at doses up to $1x10^{12}$ pfu, with flu-like symptoms being the most common toxicities. Not surprisingly these symptoms were greatest in patients receiving intravascular treatment. Neutralising antibodies increased in all patients, regardless of dose, route of administration or tumour type. Having established safety, two phase II trials were conducted to assess the efficacy of ONYX-015 in head and neck

cancer (Kirn et al, 1998; Nemunaitis et al, 2000). 40 patients were recruited and tumours were treated aggressively with repeated injections, but 87% did not objectively respond. Also, there was no correlation between evidence of antitumoural activity and neutralising antibody levels, at baseline or following treatment.

Oncolytic viruses have great potential as combination therapy with standard chemotherapy drugs. Agents that act by different mechanisms should make the emergence of resistant disease less likely. Also the toxicities associated with these agents would be different, thus allowing safe combination treatment. The most encouraging clinical results using ONYX-015 in head and neck cancer have come from trials combining it with standard chemotherapy. A phase II trial of a combination of intratumoural ONYX-015 injection with cisplatin and 5-fluorouracil in patients with recurrent HNSCC concluded an objective response in 63% of patients (Khuri et al, 2000). Also, the length of time to tumour progression for the injected tumours was improved. It is notable that there was no correlation between tumour response and baseline tumour size, baseline neutralising antibody titre, p53 gene status or prior treatment. 46% of patients reported grade 3 (severe) and 14% grade 4 (life-threatening) adverse events. The most common adverse reaction was injection site pain. A phase III trial combining ONYX-015, cisplatin and 5-fluorouracil was started; however in June 2003 all ONYX-015 programmes were discontinued in order to redirect resources towards a different line of research.

1.6 HERPES SIMPLEX VIRUS

Eight distinct herpes viruses have been identified (Table 4). They are all characterised by typical herpes particle morphology. The genome is typically

large, consisting of a single molecule of double stranded DNA (dsDNA), ranging in size between 120 and 250 kb. Also they have the ability, following productive infection, to produce disease as well as enter a latent phase in some host cells. This latent phase allows survival of the viral genome throughout the lifetime of the infected individual and the ability to re-enter the productive phase.

Table 4: HERPES SIMPLEX VIRUSES

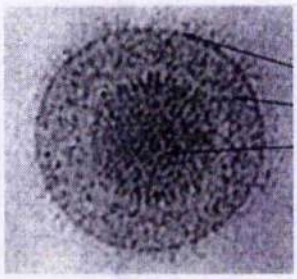
HUMAN HERPESVIRUSES	ABBREVIATION	ASSOCIATED CLINICAL ILLNESS
Herpes Simplex Virus Type-1	HSV-1	Cold sores
Herpes Simplex Virus Type-2	HSV-2	Genital ulcers
Varicella-Zoster virus	VZV	Chicken pox/Shingles
Epstein Barr Virus	EBV	Glandular fever
Cytomegalovirus	HCMV	Congenital anomalies
Human Herpesvirus 6	HHV-6	Fever/fits/rash in infants
Human Herpesvirus 7	HHV-7	Fever/fits/rash in infants
Kaposi's sarcoma Herpesvirus	KSHV,HHV-8	AIDS related Kaposi's Sarcoma

1.6.1 HSV-1 VIRION AND GENOME

HSV-1 is a human neurotropic virus. The morphological structure of the infectious virus particle, the virion, is characterised by a central icosahedral capsid, containing the core dsDNA genome. More than 50% of the HSV genes are known to code for proteins that make up the virion structure (Figure 7). The capsid is surrounded by the tegument, which in turn is surrounded by a protein-containing lipid bilayer, the envelope. The tegument contains proteins involved in the induction of viral gene expression and the process of host cell synthesis shut off immediately post infection. The envelope is composed primarily of lipids

derived from the host cell membrane, into which are inserted the HSV glycoproteins. Membrane glycoproteins mediate HSV entry into the cell, cell-to-cell spread, cell fusion and immune evasion.

Figure 7: HSV-1 VIRION



ENVELOPE
TEGUMENT
CAPSID

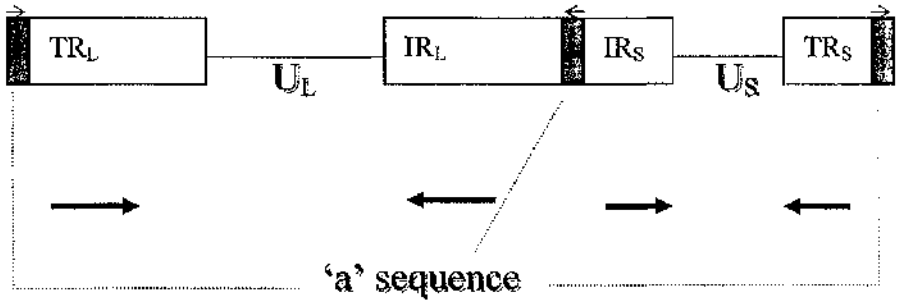
Electron Micrograph of a herpes simplex type 1 (HSV-1) virion, frozen in vitreous ice. The dsDNA genome is enclosed within the capsid.

(Department of Virology, Glasgow University)

The HSV-1 genome is a linear double stranded DNA duplex, 152 kb in length (Figure 8). There are two unique regions, long & short (termed U_L & U_S), which are linked in either orientation by internal repeat sequences (IR_L & IR_S). At the non-linker end of the unique regions are terminal repeats (TR_L & TR_S). The DNA sequencing of the HSV-1 genome was completed in Glasgow, Scotland in 1988 (McGeoch et al, 1988). There are up to 81 genes, of which about half are not essential for growth in cell culture. Once these non-essential genes have been deleted, 40-50 kb of foreign DNA can be accommodated within the virus. Most genes encoding proteins are located in the long or short regions, and they are named according to their location within L or S. For example, the US6 gene encodes glycoprotein D, a membrane glycoprotein involved in virus entry. Three main classes of HSV-1 genes have been identified, namely the immediate-early (IE or alpha) genes, early (E or beta) genes & late (L or gamma) genes. The 'a'

sequence is present as a direct repeat at the genomic termini and as an inverted repeat at the L-S junction.

Figure 8: HSV-1 VIRAL GENOME



TR_L	Long terminal repeat
IR_L	Long internal repeat
IR_S	Short internal repeat
TR_S	Short terminal repeat
UL	Unique long region
US	Unique short region

1.6.2 HSV-1 REPLICATION

The HSV lytic cycle encompasses the primary infection of host cells and viral DNA replication in epithelial cells. During the course of its normal life cycle HSV must infect and replicate in at least two cell types, epithelial and neuronal.

HSV binds to the cell surface of host cells using glycoproteins. A number of glycoproteins are then required to aid penetration of the virus, including gB, gD, gH and gL (Cai et al, 1988; Ligas et al, 1988; Forrester et al, 1992). The virus penetrates the cell by fusion of the virus envelope with the cell plasma membrane (Shukla et al, 2001). Virus capsids are then transported to the nuclear pore and DNA is released into the nucleus to allow gene expression (Batterson et al, 1983). Replication is facilitated by the preferential production of viral proteins, at the expense of host cell gene expression (Zelus et al, 1996). HSV inhibits host gene

transcription and RNA processing, and destabilises host and viral mRNAs (Schek et al, 1985). Within 3 hours post-infection, HSV-DNA replication is detected in the nucleus (Roizman et al, 1964).

Infected cell proteins (ICP), virion proteins (VP) and Virion molecular weight (Vmw) are the major nomenclatures for HSV proteins. Following infection of cells, lytic replication is regulated by a coordinated sequence of gene transcription. Vmw65, a tegument structural protein, activates immediate-early (IE) genes (ICP0, ICP4, ICP22, ICP27, ICP47), which transactivate the production of early (E) genes. The early genes encode proteins for nucleotide metabolism and DNA replication. Late genes are activated by the early genes, and code for structural proteins.

Newly synthesised DNA is cleaved into unit-length molecules and packaged into virions. Capsids containing DNA attach to the nuclear surface of the inner nuclear membrane, at sites where viral glycoproteins and tegument proteins have accumulated, and are rapidly enveloped. The virions accumulate in the endoplasmic reticulum and are transported into the extracellular space. Controversy remains over the exact intricacies of virus maturation and egress. Following egress, mature virions are capable of infecting neighbouring cells, either via the extracellular space or through cell-to-cell spread.

There are a number of protective host cell responses to HSV infection, which need to be overcome for successful viral replication and pathogenesis. A number of viral proteins, including virion host shut off (vhs) and ICP34.5, are integral in blocking these innate host cell reactions. Infected cells try to inhibit viral replication and, following the induction of a stress response, pro-apoptotic

pathways are activated to modulate virus spread. Another cellular response is the activation of protein kinase R.

A tegument protein, virion host shutoff (vhs), induces degradation of cellular and viral RNA early in infection (Read et al, 1983; Kwong et al, 1988; Krikorian et al, 1991). The immediate early protein, ICP 27, blocks splicing of cellular mRNA. This reinforces the shutoff of cellular protein synthesis, initiated by vhs. Viral proteins gD, gJ and protein kinase US3 play important roles in blocking programmed cell death induced by exogenous agents or by viral products.

Activation of protein kinase leads to phosphorylation of the α subunit of the translation initiating factor eIF-2, and total protein synthesis shutoff, thereby preventing viral replication. HSV-1 has evolved a mechanism to evade this, by precluding host cell protein synthesis shutoff (Chou et al; 1992). ICP 34.5 binds to protein phosphatase 1 and redirects it to dephosphorylate eIF-2α, insuring uninterrupted synthesis of viral proteins (Chou et al, 1995; He et al, 1997). This pathway has only been demonstrated *in vitro* in one neuroblastoma cell line SK-N-SH.

1.6.3 LATENCY AND REACTIVATION

HSV has two unique biological properties that influence human disease. These are the capacity to invade and replicate in the central nervous system, and the capacity to establish a latent infection. Latency is the persistence of the virus in a host cell in a non-infectious form. Following infection of nerve endings HSV-1 is transported to the nuclei of sensory ganglia by retrograde movement (Stevens et al, 1971). In most infected sensory neurones, the viral genome remains in an episomal state for the entire life of the individual. In contrast to the lytic pathway, viral gene expression is almost completely repressed during latency, and so

neuronal lysis is prevented. This reduced gene expression helps to diminish recognition of infected cells by the immune system. Transcription therefore only occurs from a single area of the genome and results in a family of RNA molecules, latency-associated transcripts (LATs). The LAT locus is located in the long internal and terminal repeats (Perng et al, 1996). The exact mechanisms whereby HSV establishes latency, along with the integrated roles of LATs remain unclear.

Reactivation of HSV from ganglia results in the appearance of infectious virus at the site of the initial infection. It can occur following local or systemic non-specific stimuli including physical or emotional stress, pyrexia, UV light, systemic illness, tissue damage and immunosuppression. A number of different viral gene products, including thymidine kinase, are thought to be involved in this process, but the exact pathways remain unclear (Coen et al, 1989).

1.6.4 HSV-1 HISTOPATHOLOGY

Histopathological characteristics of a primary or recurrent HSV infection reflect virus-mediated cellular death and associated inflammatory response. A vesicular fluid forms between the epidermal and dermal layers, and contains large quantities of virus along with cellular debris and inflammatory cells. Shallow ulcers replace the vesicles when mucous membranes are infected. There is usually an intense inflammatory reaction in the dermis.

HSV infected cells lose intact plasma membranes and form multinucleated giant cells. Characteristically the cells balloon with membrane modifications and loss of matrix binding proteins on the cell surface. Also cytoskeletal destabilisation, nucleolar alterations and chromatin margination, aggregation or damage occur (Avitabile et al, 1995; Heeg et al, 1986; Roizman et al, 1996). This viral

destruction of cells is generally believed to occur through a necrotic route. It is well documented that HSV-1 blocks apoptosis. This programmed cell death is characterised by morphological and biochemical changes that include cell shrinkage, membrane blebbing, nuclear condensation and fragmentation of chromosomal DNA (Aubert et al, 1999; Aubert et al, 1999 Galvan et al, 1999; Koyama et al, 1997; Zachos et al, 2001; Zhou et al, 2000).

1.6.5 HSV-1 PATHOGENESIS AND CLINICAL FEATURES

The pathogenesis of human HSV depends on close contact between a seronegative individual, and one who is actively excreting HSV. The virus must come in contact with mucosal surfaces or broken skin for infection to be initiated. A virion or capsid is transported retrograde by neurones to the dorsal root ganglia where, latency is established. Once latency is established, a reactivation stimulus can cause the virus to again appear as skin vesicles or mucosal ulcers. Infection with HSV-1 usually occurs in the oropharyngeal mucosa, as the trigeminal ganglion becomes colonised and harbours latent virus.

HSV infection in a healthy immunocompetent individual results in non-specific and specific immune responses. An inflammatory reaction occurs, and parallels viral replication. Specific immune responses in humans are delayed, and develop 7-10 days post infection. Cytotoxic T lymphocytes, $CD8^+$ cells, and helper T lymphocytes, $CD4^+$ cells have been shown to be important in providing protection from an HSV viral challenge, and to reduce viral replication. $CD8^+$ cells are important in resolving cutaneous disease (Leung et al, 1984). Neutralising antibodies generally appear 2-6 weeks after infection and persist for the lifetime of the host. Humoural immunity does not prevent either recurrences or exogenous

reinfection, whereby an individual is reinfected with a different strain of HSV (Timbury, 1991).

Primary HSV-1 infection in humans is usually asymptomatic. The incubation period ranges from 2-12 days, with a mean of approximately 4 days. Neutralising antibodies peak at approximately 3 weeks post infection. Symptomatic primary infection often presents with intraoral gingival lesions and sometimes pharyngitis with associated pyrexia, malaise, cervical lymphadenopathy and odynophagia. Recurrent infections typically present with lip vesicles which progress to pustular or ulcerative lesions. Other cutaneous HSV-1 infections occur including eczema herpeticum and herpetic whitlow. HSV infections can also affect the eye causing conjunctivitis and keratitis.

The most serious HSV infection is encephalitis. The mortality if untreated exceeds 70%, and only 2.5% of patients who survive regain normal neurological function (Ward et al 1994). No clinical signs are pathognomonic for HSV encephalitis. The diagnosis should be considered in any ill patient with fluctuating levels of consciousness, pyrexia, abnormal CSF profile and focal neurological signs, in the absence of other causes. Prompt neurodiagnostic procedures, along with supportive and systemic treatment are imperative.

1.6.6 HSV-1 TREATMENT

Acyclovir (9-[2-hydroxyethoxymethyl] guanine), a synthetic acyclic purine nucleoside analogue, is the standard treatment for HSV infections. Viral thymidine kinase converts acyclovir to acyclovir monophosphate. The host cell enzymes convert the monophosphate to the diphosphate and then to the active compound, acyclovir triphosphate. This inhibits HSV DNA replication. Viral thymidine kinase has a much greater affinity for acyclovir triphosphate than does

mammalian thymidine kinase. Consequently acyclovir triphosphate only accumulates in virus-infected cells.

1.7 HSV1716: ONCOLYTIC HERPES SIMPLEX VIRUS

HSV1716 is an avirulent HSV-1 mutant with a 759 bp deletion in both copies of the RL1 gene (MacLean et al, 1991). The long repeat regions of the wild type HSV-1 genome contain the diploid RL1 gene, whose protein product ICP34.5 is a major determinant of pathogenicity (Ackermann et al, 1986; Chou et al, 1990, MacLean et al, 1991, McKie et al, 1994). HSV1716 was first isolated following a spontaneous mutation of HSV 17^{+}, while trying to identify the precise regions of the HSV genome involved in pathogenicity. An avirulent mutant HSV1714 was found to have a deletion in both copies of the Bam HI fragment of the long repeat region. This is in addition to deletions in the four XbaI sites of the unique long region and a thymidine kinase negative phenotype. The long repeat deletion of HSV1714 was introduced into a totally wild type genome (17^{+}) to produce HSV1716, which is avirulent and is as efficient as wild type HSV in thymidine kinase synthesis. Thymidine kinase is essential for acyclovir antiviral medication efficacy. The deletion in HSV1716 removes most of the RL1 gene, including the initiating ATG, and the mutant fails to make ICP34.5 (McKay et al, 1993).

HSV1716 has been characterised as a selectively replication competent virus and a potential novel cancer therapy. It fails to replicate in neurones of the central nervous system or peripheral nervous system (MacLean et al, 1991; McKie et al, 1998; Robertson et al, 1992). Also it does not replicate in terminally differentiated cells or cause encephalitis following intracerebral inoculation of mice (MacLean et al, 1991). However its replication in many tissue culture lines matches that of the parental wild type HSV-1 strain 17. In general, dividing cells support

replication of HSV1716 and non-dividing cells cannot (Brown et al, 1994). These findings highlight the potential for targeting tumour cells, with an aim of oncolysis and sparing of the surrounding normal tissue

1.7.1 HSV1716 REPLICATION

A) ICP 34.5

The mechanisms whereby HSV1716 selectively replicates in and lyses rapidly dividing cells are not fully understood. ICP 34.5-cellular interactions have been shown *in vitro* to be cell type and cell state, or differentiation state, dependent (Brown et al, 1994). For example, HSV1716 does not replicate in confluent mouse fibroblast (3T6) cells, and the lack of ICP 34.5 results in a defect in maturation and egress of virus from infected cells (Brown et al, 1994). As described in section 1.6.2, ICP 34.5, in certain cells, interacts with protein phosphatase 1 to preclude host cell protein synthesis shutoff. Also, a different ICP 34.5 null mutant has been shown to cause shutdown of protein synthesis, before completion of the viral replicative cycle, in the human neuroblastoma cell line SK-N-SH (Chou et al, 1995). However, further work with HSV1716 has shown it to cause tumour cell kill by lytic replication. Premature host cell shutoff via the PKR pathway is not induced *in vivo* by HSV1716 in most cells, irrespective of type or state (McKie et al, 1996; Markovitz et al, 1997; Harland et al, 2003). Other viral-host cell interactions are therefore involved.

B) PROLIFERATING CELL NUCLEAR ANTIGEN

Following HSV-1 infection a cell cycle control protein, proliferating cell nuclear antigen (PCNA), is recruited to sites of DNA repair in the cell nuclei. Here it forms a complex, directly or indirectly, with ICP 34.5 (Brown et al, 1997). PCNA has important functions in DNA repair and replication (Prelich et al, 1987; Shivji

et al, 1992). ICP 34.5 concentrates *in vivo* in the cell nucleus with PCNA at early stages in the infection, and subsequently accumulates in the cytoplasm. The ICP 34.5-PCNA complex allows HSV DNA replication to continue. This is particularly pertinent to nondividing cells and HSV encephalitis. The intricacies are not fully understood as other cell-cycle regulation proteins are involved. PCNA is regulated by a variety of proteins and it is believed that this regulation may act as a switch between the processes of DNA repair and replication (Harland et al, 2003).

A 63-amino acid region of ICP 34.5 shares significant homology with two cell-cycle regulation proteins; mouse myeloid differentiation protein (MyD116) and growth arrest and DNA damage protein 34(GADD 34) (Lord et al, 1990; Fornace et al, 1989; McGeoch and Barnett, 1991). Interestingly the same region shares significant homology with part of an African swine fever virus protein, which is also a determinant of virulence (Sussman et al, 1992). The functions of both MyD116 and GADD 34 are not fully understood but may be involved in blocking growth and DNA replication after damage, and the genes may act as tumour suppressor genes. The ability of ICP 34.5 to preclude premature shutoff of protein synthesis, in SK-N-SH neuroblastoma cells, is preserved when there is in-frame substitution of the homologous domain of ICP 34.5 with the corresponding domain of MyD116 (He et al, 1996). *In vivo* PCNA complexes with the region of MyD116 that shares homology with ICP 34.5 (Brown et al, 1997). The homologous regions of MyD116 and GADD 34 could also regulate the DNA replication mode of PCNA.

Overall the pathways are complex and not fully understood. ICP 34.5 is certainly a multifunctional protein, which can overcome a block in protein synthesis

induced as an antiviral defence mechanism in certain cells. Its primary function *in vivo*, relevant to the HSV1716 phenotype, is its interaction with PCNA to overcome the block in DNA replication in nondividing cells. PCNA levels are high in tumour cells, and its correlation with disease stage and poor prognosis in head and neck cancer is well documented (Sarac et al, 1998; Liu et al, 2003; Krecicki et al, 1999). Due to the high PCNA levels ICP34.5 is not needed for HSV replication, ergo HSV1716 replication.

1.7.2 *IN VIVO* STUDIES OF HSV1716

Having characterised HSV1716 as a potential novel cancer therapy, studies were performed to establish a safety and efficacy profile. Early *in vivo* experiments showed intracerebral inoculation of wild type HSV 17^{+}, at a dose of less than 10 pfu to cause encephalitis in immune competent BALB/c mice. In comparison, HSV1716 doses of 10^{7} pfu resulted in no evidence of viral replication or encephalitis (MacLean et al, 1991). Another study confirmed avirulence following intracerebral inoculation of HSV1716 in immunocompromised SCID mice (Valyi-Nagy et al, 1994). The safety profile was supported further by a study using G207, a different ICP 34.5 mutant of HSV-1, which confirmed avirulence on intratumoural inoculation of gliomas in a murine model (Mineta et al, 1995).

HSV1716 was shown to be effective in selective lytic replication in a variety of tumour types, prior to progressing to clinical trials. Improved survival was seen following intratumoural injection of medulloblastoma tumours in a murine model (Lasner et al, 1996). Significant tumour regression and improved survival was seen following HSV1716 intratumoural injection of melanoma tumour deposits grown in the brains of immunocompetent C57B mice (Randazzo et al, 1995). Viral replication was confirmed, and seen to be restricted to tumour cells and not

to involve the surrounding brain tissue. Similar findings were seen when intracerebral deposits of human embryonal carcinoma cells were injected with HSV1716 (Kesari et al, 1995). Effective oncolysis of malignant mesothelioma, with tumour regression and improved survival, was seen when intraperitoneal deposits were injected with HSV1716 (Kucharczuk et al, 1997). A murine model involving subcutaneous human melanoma was also successful in showing selective replication and efficient oncolysis (Randazzo et al, 1997). Immunohistochemistry confirmed the viral replication to be restricted to the tumour cells.

HSV1716 had been characterised as a selectively replication competent oncolytic virus, with an established safety profile. Showing efficacy against a variety of tumour types had expanded the potential scope of HSV1716 as a novel cancer therapy. With this competent profile it was possible to progress to human clinical trials using HSV1716.

1.7.3 CLINICAL STUDIES OF HSV1716

A) FIRST MALIGNANT GLIOMA PHASE I TRIAL

The first clinical trial involving HSV1716 was a phase I toxicity study in patients with glioblastoma multiforme (Rampling et al, 2000). The main objective of the study was to demonstrate safety at a dose at which therapeutic activity was possible. Glioblastoma is a very aggressive disease that is universally fatal and has a median survival of approximately 9 months.

In planning the study, a difficult decision had to be addressed regarding the dosages of virus to be administered. In the development of conventional cytotoxic chemical agents, a safe starting dose is usually derived from animal experiments. Dose escalation protocols are designed with mandatory toxicity end-points. This

approach cannot be applied in the same way to a replication competent biological agent. Following inoculation the final titre of virus could be several orders of magnitude higher than the input dose. Biological therapies may produce non-specific and sporadic toxicities, which are not clearly dose related. The adverse effects of other gene therapy agents have sometimes been unexpected with regard to the preclinical models, and in one instance fatal (Raper et al, 2002; Lehrman, 1999). In 1999 an 18-year-old man died of multi-organ failure during a gene therapy trial. He had received liver-directed gene transfer to correct partial ornithine transcarbamylase (OTC) using an adenovirus vector. Unpredictable side effects and immunological effects give justification for the prudent approach of the Gene Therapy Advisory Committee (GTAC), which oversees gene therapy trials in the UK.

As HSV1716 had the potential to replicate in tumours, it was estimated that 20% of infectious particles could result in a productive infection and each infected cell could give a burst size of 100 pfu. It was then decided that the maximum dose in the first trial, legislated by GTAC, would be 10^5 pfu.

Patients recruited into the study had recurrent high-grade gliomas and had exhausted all other conventional therapies, including surgery, radiotherapy and chemotherapy. HSV1716 was injected into the tumours by stereotactic injection. The first three patients were injected with HSV1716 at a low dose of 10^3 pfu, followed by three at 10^4 pfu and three at 10^5 pfu Patients were observed in the hospital and followed up in the clinic.

The study demonstrated that HSV1716 could be injected intratumourally in immunocompromised patients with glioblastoma, in doses up to 10^5 pfu with no evidence of associated toxicity. Eight patients were seropositive for HSV and

there were no significant changes in their IgM or IgG antibody levels. One patient was seronegative and did not seroconvert. Post-injection tissue was obtained from three of the patients during subsequent tumour resection and from two of the patients at post-mortem. There was no evidence of encephalitis and tissue cultures were negative for HSV. Immunohistochemical analysis showed no significant immunoreactivity of tumour cells or adjacent brain tissue for HSV-1 using monoclonal antibodies. There was no evidence of HSV1716 or wild type genomes by PCR. Tissue cultures were negative.

The study was successful in establishing an early safety profile. It had not been designed as an efficacy model, however the outcomes of four of the patients are notable (Table 5). At the time of viral injection these patients had a life expectancy in the region of 5-6 months.

Table 5: FIRST GLIOMA TRIAL - PATIENT OUTCOMES (Updated to October 2005)

PATIENT	AGE	SEX	SURVIVAL POST INJECTION (MONTHS)
1	22	M	94* (PATIENT STILL ALIVE)
2	48	M	10
3	62	M	3
4	34	M	26
5	41	M	6
6	63	M	6
7	37	M	87* (PATIENT STILL ALIVE)
8	65	F	38
9	56	F	2

B) SECOND MALIGNANT GLIOMA PHASE I TRIAL

The second clinical trial with HSV1716 was a proof of principle study (Papanastassiou et al, 2002). In the first study there was no formal analysis to assess viral replication. A primary objective in the second study was to demonstrate that the virus survives and replicates following intratumoural injection.

Twelve patients with recurrent and newly diagnosed high-grade glioma were recruited. They received an intratumoural injection of 10^5 pfu of HSV1716. Four to nine days post-inoculation the tumours were removed and assayed for evidence of viral replication. Nine patients received radiotherapy or chemotherapy after surgery.

No patients had any viral associated adverse reactions. In two patients, infectious HSV1716 was recovered from the injection site at titres in excess of the input dose. The same two patients were seronegative for HSV-1 pre-injection. They both seroconverted and initial changes in specific IgG were detected at 19-21 days post-injection.

HSV DNA was detected by PCR at the sites of inoculation in 10 patients and at distal tumour sites in four. A tumour biopsy taken at post mortem from one of the patients, 251 days post-injection, was PCR positive for HSV DNA. Immunohistochemistry analysis of tumour tissue for HSV antigen was positive from two patients.

The trial was successful and gave further support to the safety profile of HSV1716. It was able to conclude that HSV1716 replicates in high-grade glioma without causing toxicity in both HSV-seropositive and HSV-seronegative

patients. The survival of patients post-inoculation was variable, with a maximum of 26 months.

C) THIRD MALIGNANT GLIOMA PHASE I TRIAL

One further phase I study was conducted involving patients with high-grade glioma (Harrow et al, 2004). Twelve patients with recurrent or newly diagnosed disease were recruited and underwent maximal tumour resections. HSV1716, at a dose of 10^5 pfu, was then injected into the resulting tumour cavity with the intent of infecting residual tumour cells and testing safety when injected into brain adjacent to tumour.

There was no clinical evidence of toxicity associated with the administration of HSV1716. Longitudinal follow-up allowed assessment of overall survival compared to that of similar patients not treated with HSV1716. One patient remains alive and clinically stable at 47 months post surgery and HSV1716 injection. In the latter patient, radiological imaging has demonstrated a reduction of residual tumour following surgery and HSV1716 inoculation. This is despite no subsequent medical intervention. See Table 6 for summary of patient outcomes.

These three trials have established a safety profile for the intratumoural injection of HSV1716 in patients with high-grade glioma. In addition there have been promising signs of viral replication and patient survival. A European wide Phase III trial has been approved by the regulators (EMEA, MHRA, GTAC) and will commence in March 2006..

D) MELANOMA PHASE I TRIAL

A phase I trial was conducted involving melanoma patients (MacKie et al, 2001). Five patients were recruited and received intratumoural injections of subcutaneous nodules of metastatic melanoma. Two patients each received one injection, two

Table 6: THIRD GLIOMA TRIAL - PATIENT OUTCOMES (Updated to October 2005)

PATIENT	AGE	SEX	SURVIVAL POST INJECTION (MONTHS)
1	61	M	37
2	43	M	6
3	43	M	9
4	40	M	15
5	49	M	8
6	58	F	3
7	41	M	32
8	53	M	11
9	40	M	14
10	33	F	47* (PATIENT STILL ALIVE)
11	66	M	12
12	55	M	11

received two injections and one received four injections of HSV1716, at a dose of 10^3 pfu. Biopsies were taken at various intervals.

There were no adverse reactions to the HSV1716 injections. All patients were HSV seropositive and there were no significant changes in their IgM and IgG titres post-inoculation.

In one patient, flattening of previously palpable tumour nodules was seen 21 days after two direct injections of HSV1716. In all three patients who received two or more injections there was microscopic evidence of tumour necrosis.

Immunohistochemistry analysis showed evidence of viral replication within the limits of the tumour mass. There was no antigen staining in the adjacent normal connective tissue and the basal layer of the epidermis. This latter result suggested that the normal melanocytes were antigen-free. There was no evidence of infectious virus in any of the samples.

This trial strengthened the safety profile of HSV1716. It demonstrated that HSV1716 replicates and cause necrosis in melanoma cells. The fact that all patients were seropositive for HSV indicates that the replication competency of HSV1716 is not blocked by previous exposure to HSV.

1.8 OTHER ONCOLYTIC HERPES SIMPLEX VIRUSES

1.8.1 G207

G207 is a different mutant of HSV-1. It is a replication competent oncolytic virus and laboratory studies have demonstrated cytotoxicity against HNSCC. G207 was derived from HSV-1 strain F. It has multiple mutations including deletions at both loci of the ICP34.5 gene and insertion of a LacZ reporter gene into the ICP 6 gene (Mineta et al, 1995). G207 has a positive thymidine kinase phenotype. The ICP6 gene encodes the large subunit of HSV ribonucleotide reductase, and loss of its expression decreases the ability of G207 virus to proliferate in nondividing cells. The multiple mutations of G207 are thought to minimise the chance of reversion to wild-type virus, but the tumour is less replication competent in tumour cells.

In vitro and *in vivo* studies showed G207 to infect and cause efficient lysis of HNSCC (Carew et al, 1999; Chahlavi et al, 1999). Animal studies involved direct intratumoural injections into murine flank tumours as well as selective intra-arterial perfusion of oral cavity tumours. All experiments showed inhibition of tumour growth.

A safety profile has been established for G207 following intratumoural injections of patients with high-grade glioma (Markert et al, 2000). Doses between 10^6 pfu and 3 x 10^9 pfu were injected. These high doses are required due to the low replication efficacy. No adverse reactions specific to G207 were observed. There have been no clinical trials involving G207 and head and neck cancer patients.

1.8.2 NV1020

NV1020 is another multi-mutated HSV-1 oncolytic virus, which has shown cytotoxicity to HNSCC *in vitro* and *in vivo*. It has deletions of one copy of ICP 34.5 and the virulence-associated virion gene, UL 56. It also has a deletion in the thymidine kinase locus. As NV1020 was originally designed as a potential HSV vaccine, a fragment of HSV-2 DNA is inserted into the UL/S junction. This junction also contains an exogenous copy of the HSV-1 thymidine kinase gene under control of the strong ICP 4 promoter, in addition to a duplication of the UL5/6 sequences.

In vitro studies showed NV1020 to be highly cytotoxic to five human HNSCC lines (Wong et al, 2001). Tumour regression was seen following injections into flank tumours in a murine model. Biopsies from tumours identified areas of necrosis 24 hours post-injection.

A phase I clinical trial involving hepatic artery administration of NV1020 in patients with intrahepatic colorectal metastases has been performed (Kirn et al, 2001). The published results are awaited. No clinical trials have been performed involving head and neck cancer patients.

1.9 COMBINING THERAPIES FOR HEAD AND NECK CANCER

Combining therapies gives a number of potential advantages. For certain stages of a range of cancers, combinations of radiotherapy and chemotherapy, surgery and radiotherapy or surgery and chemotherapy have proved to be more effective.

Chemotherapy regimens usually involve a combination of agents. Using a combination of agents gives potential for using lower doses, maximising the therapeutic effect and minimising side effects. Combining therapies also gives potential for enhanced effects, and this can be additive or synergistic. Synergism

occurs when the effects of the combined agents are equal to or greater than the sum of the effects of the agents in isolation. A phase II clinical trial of intratumoural ONYX-015 inoculation in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer demonstrated tumour selective augmentation of chemotherapy efficacy by ONYX-015 (Khuri et al, 2000). Patients were recruited with more than one head and neck tumour mass, allowing one mass to be free from viral injection and to act as an internal control. The trial was unable to conclude a survival advantage.

Unfortunately randomised trials using combination chemotherapy in head and neck cancer failed to show improved survival compared with single agent therapy (see section 1.1.8 C). This emphasises the need for the development of novel therapies. These therapies could potentially be used in combination with conventional chemotherapy agents.

In vivo cytotoxicity studies involving G207 combined with cisplatin showed additive tumouricidal effects in one of two established human HNSCC murine flank tumours. There was no enhanced kill in the other tumour model, and it was noted that the cell type involved was less sensitive to cisplatin. The G207 and cisplatin appeared to be acting independently.

In vitro and *in vivo* studies with non-small cell lung cancer have shown synergistic effects between HSV1716 and mitomycin C (Toyoizumi et al, 1999). The synergistic dose of mitomycin-C neither augmented nor inhibited viral replication *in vitro*. Additive effects were found combining HSV1716 with *cis*-platinum II, methotrexate or doxorubicin. No laboratory studies have been performed looking at the effects of HSV1716 on HNSCC in isolation or in combination with conventional chemotherapeutic agents.

1.10 AIMS OF RESEARCH STUDY

The aims of this research study were:

1. To determine whether HSV1716 would replicate in and kill head and neck squamous cell lines.
2. To determine if the cytotoxic effects *in vitro* were enhanced when combined with the conventional chemotherapeutic agent cisplatin.
3. To carry out a phase I clinical safety trial involving the preoperative intratumoural injection of HSV1716 in patients with oral squamous cell carcinoma.

CHAPTER 2

MATERIALS

MATERIALS

2.1 CELLS

Baby hamster kidney: 21 clone 13 (BHK) cells (MacPherson and Stoker, 1961) and mouse embryo fibroblast (3T6) cells (Todaro and Green, 1963) were obtained from the European collection of Cell Cultures (ECACC).

HNSCC cell Lines UM-SCC 14C (Grenman et al, 1991), UM-SCC 22A and UM-SCC 22B (Carey et al, 1983) were kindly provided by Professor Guus van Dongen, VU University Medical Centre, Amsterdam.

2.2 Cell culture media

BHK21/C13 cells were grown in BHK-21 medium (Invitrogen Ltd, Paisley, UK) supplemented with 10% newborn-calf serum (Invitrogen Ltd); 10% (v/v) tryptose phosphate broth; 10,000 IU/ml penicillin (Invitrogen Ltd); 10,000 UG/ml streptomycin (Invitrogen Ltd) and 250 UG/ml amphotericin B(Invitrogen Ltd).

3T6 cells were grown in Dulbecco's modified eagles medium (Invitrogen Ltd) supplemented with 10% foetal calf serum (Invitrogen Ltd); 10,000 IU/ml penicillin; 10,000 UG/ml streptomycin and 250 UG/ml amphotericin B.

All HNSCC cell lines were grown in Dulbecco's modified eagles medium supplemented with 10% heat-inactivated foetal calf serum (30 minutes at 54°C); 10,000 IU/ml penicillin; 10,000 UG/ml streptomycin; 250 UG/ml amphotericin B and 100 nM non-essential amino acids (Sigma, Dorset, UK).

Eagle's medium containing 1% carboxymethyl cellulose , 10% new-born calf serum and 10% tryptose phosphate broth, (ETMC 10%),was used during the titration of virus stocks.

2.3 VIRUSES

Wild-type HSV-1 strain 17^+ (Brown et al., 1973) and HSV1716 (MacLean, A., et al., 1991) were used for all laboratory studies. HSV1716, GMP grade, was prepared by Q1 Biotech, Glasgow, and used for clinical studies.

2.4 SOLUTIONS

2.4.1 AGAROSE GEL REAGENT

TBE: 0.04M Tris, 0.14% acetic acid (v/v), 2mM EDTA (pH 8.0)

2.5 CHEMICALS

All chemicals were of AnalaR grade and were obtained form BDH Chemicals Ltd, Poole, Dorset or Sigma Chemicals Ltd, Poole, Dorset. Exceptions were:

APS and Temed – supplied from Bio-Rad laboratories.

Histoclear – supplied by Fisher Chemicals

Aqueous mounting medium – Dako®, Faramount

Platinum® P*fx* DNA polymerase, $MgSO_4$ – Invitrogen

2.6 CYTOTOXICITY ASSAYS

Cell viability assays (MTS) were measured using CellTiter 96 Aq_{ueous} One Solution Cell Proliferation Assay (Promega, Southampton, UK). Readings were taken using a Dynatech MR5000 96 well plate reader.

2.7 HSV ANTIBODY DETECTION KIT

HSV IgM and IgG detection was performed using Elisa Kit, Virotech, Russelsheim, Germany.

2.8 HUMAN BLOOD AND TISSUE DNA EXTRACTION KIT

DNA was extracted from clinical trial patients' blood and tissue biopsies using Nucleon® genomic DNA extraction kit (Amersham Biosciences, Buckinghamshire, UK).

2.9 IN-SITU HYBRIDISATION KIT

In-situ hybridisation was performed using Rembrandt® *in situ* hybridisation kit (Panpath, Amsterdam, Holland).

2.10 ANTIBODIES

In immunohistochemistry experiments, the primary monoclonal antibody to the HSV-1 strain Stoker was obtained from Abcam Limited, UK.

2.11 EQUIPMENT AND PLASTICWARE

Invitrogen Ltd, Life Technologies Ltd, Paisley

Nunc 1 ml cryotubes

Nunc 12 well tissue culture plates

Nunc 25 cm^2, 75 cm^2, 175 cm^2 tissue culture flasks

Tissue culture dishes (35mm, 60 mm)

Fisher Scientific UK, Loughborough, Leicestershire

Corning Incorporated 850 cm^2 tissue culture roller bottle

Greiner Bio-One Ltd, Stonehouse, Gloucestershire

Eppendorf tubes

Universal bottles

Bijoux bottles

96 well plates

Sterile pipettes

2.12 OTHER MATERIALS

2.12.1 CENTRIFUGES

Volumes <1.5ml, up to 13,000rpm:	Micro-centrifuge
Volumes 1.5ml-45ml, up to 13,000rpm:	Beckman Centrifuge

2.12.2 MICROSCOPE

Images of cells were captured under a DMLB Fluorescent microscope using a DC 200 digital camera (Leica) and Leica IM 500 software.

CHAPTER 3

METHODS

3. METHODS

3.1 IN VITRO STUDIES

3.1.1 BHK21/C13 Cells

BHK21/C13 and 3T6 cells were grown in 850 cm^2 tissue culture roller bottles containing 125 ml ETC10 medium at 37^0C for 3 days in an atmosphere of 95% air and 5% CO_2. Washing the monolayers twice with HBSS and trypsin-EDTA, and resuspending the detached cells in 20 ml of the appropriate medium harvested the cells. The yield from a confluent roller bottle is approximately $1x10^8$ cells, which would give 100 confluent monolayers on 60mm diameter tissue culture dishes after an overnight incubation at 37^0C.

3.1.2 HEAD AND NECK SQUAMOUS CARCINOMA CELLS

HNSCC cell lines were grown in 175 cm^2 tissue culture flasks and harvested under the same conditions. The yield of cells was determined as described by Freshney (1987) using an improved Neubauer counting chamber (Weber, England).

3.1.3 PASSAGING OF CELLS

T-175 flasks or roller bottles with 80-100% confluent monolayers were opened in a category 2 hood. The media was decanted and 22.5 ml of Hanks Balanced Salt Solution (HBSS) was poured over each monolayer and decanted after 30 seconds. Trypsin, stored in 2.5ml aliquots, was thawed from - 20°C and mixed with 22.5ml Hanks Balanced Salt Solution (HBSS) to yield 10% (v/v) trypsin/HBSS solution in 25ml. One volume of Trypsin/HBSS solution was added to each monolayer and 90% removed after 30 seconds. The cells detached and this was aided by incubation at 37°C. The cells were resuspended in 10-20ml of the appropriate growth medium and used to seed further flasks or roller bottles.

3.1.4 CRYOPRESERVATION OF CELLS

Confluent cell monolayers were harvested as described in section 3.2. The suspension containing cells for storage was pipetted into a universal container and spun at 2000rpm (Beckman centrifuge) for 10 minutes at 4^0C. The supernatant was decanted and the pellet resuspended in 1ml of appropriate growth medium containing 10% DMSO (Sigma). This suspension was pipetted into 1.5 ml cryo-vials and stored overnight at -70^0C. The cells were moved to a liquid nitrogen freezer for long-term storage.

3.1.5 TITRATION OF VIRUS STOCKS

The amount of infectious virus within a stock was quantitated as described by Brown and MacLean, 1997. Virus stocks were serially diluted 10-fold in ETC_{10}. 100µl aliquots were added to 85% confluent monolayers of BHK21/C13 cells on 60mm petri dishes, from which the medium had been removed. The plates were incubated at 37°C for 1 hour, to allow adsorption of the virus onto the cells. The plates were washed twice with PBS, before overlaying with 5 ml ETMC 10 % and incubated at 31°C for 3 days. Monolayers were fixed and stained with Giemsa stain at room temperature for 2 hours. After washing, plaques were counted on a dissection microscope and virus titres calculated as pfu/ml.

3.1.6 MULTICYCLE GROWTH EXPERIMENTS

The growth kinetics of HSV1716 in HNSCC cell lines, UM-SCC 14C, UM-SCC 22A and UM-SCC 22B, were determined by performing multicycle growth experiments. For each HNSCC cell line, BHK21/C13 and 3T6 cells were also infected with HSV 1716, at a low MOI of 0.1 pfu per cell, and monitored over 72 hours. A parallel experiment was also run infecting the same cells with wild type HSV strain 17^+.

Cells were initially seeded in 35 mm tissue culture plates at a density of 2 x 10^6 cells per plate, in 2 ml of the appropriate cell media. Following an overnight incubation at 37^0C and removal of the media, the cells were inoculated with HSV1716 or wild type HSV strain 17^+. The viruses were diluted to 2 x 10^6 p.f.u/ml and 100µl added to each plate (0.1 p.f.u/cell). The virus absorbed during a 1 hour incubation at 37^0C. Unabsorbed virus was washed off with 2 ml of 10% newborn calf serum/PBS. The plates were overlaid with 2 ml of the appropriate media and this marked 0 hours on the time-scale.

Virus samples were harvested at 0, 6, 24, 48 and 72 hours, by scraping the cell monolayers into the medium and transferring the suspension into a bijoux bottle. The samples were sonicated for 5 minutes and stored at -70^0C until they were titrated.

The virus samples were titrated as described previously, and titres calculated at each time-point. The titres were plotted on a log graph scale with pfu/10^6 cells on the y-axis and time (hours) on the x-axis.

3.1.7 MTS CYTOTOXICITY ASSAY

In vitro cytotoxicity was determined using a Celltitre 96® Aqueous One Solution Cell Proliferation Assay (Promega) in accordance with the manufacturer's instructions. This solution contains an MTS tetrazolium compund, an analogue of MTT, which is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium. The production of formazan is proportional to the number of living cells; therefore the intensity of the produced colour indicates the viability of the cells.

Cell suspensions were prepared from 80% confluent T-175 flasks as described in section 3.1.3. Cells were seeded in 96-well plates at densities between 4-6 x 10^3

cells per well, depending on the cell type, in 100 µl volumes. All the perimeter wells were kept free and filled with sterile water to aid humidification during the experiments. After overnight incubation at 37°C, the media was removed from all wells and serial concentrations of HSV 1716 and/or cisplatin were added in 100 µl volumes. Six wells were prepared for each concentration. In all experiments, six control wells were prepared with cells and medium alone.

After 72 hours incubation at 37°C, all wells were aspirated dry and 100 µl of fresh medium added. Six control wells were also prepared with 100 µl of media alone. 20 µl of MTS was then added to each well. Optical density readings were taken at 480nm after 2-4 hours incubation at 37°C.

3.2 PHASE I CLINICAL TRIAL

3.2.1 ENROLMENT CRITERIA

A total of 20 patients with injectable oral squamous cell carcinoma were entered into the trial. Patients were recruited from the multi-disciplinary head and neck clinic at the Canniesburn Unit, Glasgow Royal Infirmary.

Eligibility requirements included histologically confirmed oral squamous cell carcinoma, which was amenable to direct injection under local anaesthetic and appropriately treatable by complete surgical resection.

All patients had a Karnofsky Performance status of greater than or equal to 70% (Table 7), and were between 18 and 75 years of age. All patients had adequate haematological, renal and hepatic function. The maximum allowed blood creatinine was 1.5 mg/dL; maximum allowed aspartate transaminase (AST) and alanine transaminase was 2.5 fold the upper limit of normal; minimum allowed haemoglobin was 9 g/dL; minimum allowed white cell count of 3000/µl (neutrophils 1500/µl) and minimum platelet count of 100,000/µl.

Patients with any active acute infection or who were HIV positive, pregnant or had received previous treatment with viral therapy were excluded from the trial.

All patients gave written informed consent. The UK Gene Therapy Advisory Committee (GTAC), and the Medicines Control Agency of the Department of Health and North Glasgow University NHS Trust approved the protocol.

Table 7: Karnofsky Performauce Status Scale

GRADE	KARNOFSKY DEFINITIONS
100	Normal; no complaint; no evidence of disease
90	Able to carry on normal activity; minor signs of symptoms of disease
80	Normal activity with effort; some sign or symptom of disease
70	Cares for self; unable to carry on normal activity or do active work
60	Requires occasional assistance but is able to care for most personal needs
50	Requires considerable assistance and frequent medical care
40	Disabled; requires special care and assistance
30	Severely disabled; hospitalisation is indicated, although death is not imminent
20	Very sick; hospitalisation is necessary; active support treatment is necessary
10	Moribund; fatal processes progressing rapidly

3.2.2 HSV 1716

HSV 1716 is an oncolytic deletion mutant of HSV-1 strain 17^{+}. It has deletions of both copies of the RL1 gene and is unable to make the virulence factor ICP 34.5. HSV 1716 is avirulent.

Q1 Biotech (Glasgow, UK) produced purified, sterile lots of HSV1716. The virus was tested for titre, sterility and safety and is Good Manufacturing Practice (GMP) grade. HSV 1716 is stored long-term at -196^{0}C in a liquid nitrogen freezer. Vials were packaged in dry ice, in the short interval between removal from the freezer and patient injection.

3.2.3 TREATMENT PROTOCOL

Pre-treatment evaluation included complete blood cell count with differential, coagulation screen, routine biochemistry profile, immunology screen, urinalysis, chest x-ray and electrocardiogram. All patients had an initial examination under anaesthesia during which the tumour was clinically staged, and a core biopsy taken to confirm the diagnosis of squamous cell carcinoma.

Once all the eligibility criteria had been met, the patients were allotted to one of 4 groups, starting with group A. The injection protocols are shown in Table 8.

The patients were admitted to the normal head and neck surgical ward and fully informed and written consent was given. The patients were then prepared for the viral injections.

With the patient lying in a semi-recumbent position, the tumour and contralateral side of the mouth were re-examined under illuminated direct vision. An area of tumour was chosen for injection, which was most accessible to injection and would be easily identifiable following surgical resection. The tumour and the normal buccal mucosa on the contralateral side of the mouth were sprayed 5 times

Table 8: Patient groups and injection protocols

	NO. OF PATIENTS	HSV 1716 DOSE	TIME INTERVAL BETWEEN INJECTION & SURGICAL RESECTION
GROUP A	5	10^5 PFU	72 HOURS
GROUP B	5	$5X10^5$ PFU	72 HOURS
GROUP C	5	$5X10^5$ PFU	24 HOURS
GROUP D	5	$5X10^5$ PFU	14 DAYS

with lignocaine, each spray delivering 10mg of lignocaine base. 5 minutes later the virus was thawed and the required dose aspirated into a syringe in a 0.5 ml

volume. A blue 23-gauge needle was introduced into the target tumour, and the syringe initially aspirated. This determined if the needle was in a blood vessel and avoided direct intravascular injection. The virus was injected while the needle was slowly withdrawn in order to distribute the volume equally along the needle tract. The same dose was then injected into a chosen site of buccal mucosa on the contralateral side of the mouth. This area was tattooed circumferentially with 4 dots of Chinese ink to aid identification at the time of surgery.

Each patient was observed on the ward for the first 24 hours post-injection, to identify any symptom or sign of an adverse reaction. Toxicity was assessed using the National Cancer Institute Toxicity Criteria, Version 2.

A staff nurse reviewed the patient hourly during the first 4 hours and recordings were taken, including temperature, heart rate and blood pressure. 4-hourly review and recordings were taken during the remaining period. Blood samples were taken for detection of HSV (DNA and infectious HSV) and antibody levels at intervals post-viral injection and postoperatively for at least 4-6 weeks.

The following medications were available in the ward for use in the case of medical emergency: 100mg hydrocortisone for intravenous injection; adrenaline (1:1000) for subcutaneous injection and 10 mg chlorpheniramine for intravenous injection.

Group C patients were then taken to theatre for surgical resection, 24 hours post-injection, and groups A, B and D patients were given the opportunity to go home and return on the day before theatre, depending on clinical and social circumstances. All patients were reviewed again prior to theatre, and any new symptoms, clinical signs, or changes at the sites of viral injections were noted.

In theatre, all patients had *en bloc* resections of their tumours, and some required lymph node neck dissections and/or reconstructive surgery. Biopsies were also taken at the site of viral injection into the contralateral buccal mucosa (approximately 2cm x 1cm).

As soon as the tumour was resected a 5 mm punch biopsy was taken at the site of injection, and half snap frozen in liquid nitrogen for PCR and detection of infectious HSV, with the other half fixed in formalin for immunohistochemistry and *in-situ* hybridisation. In larger tumours, 2 adjacent biopsies were taken along with a biopsy distant from the injection site, to obtain more tissue and aid detection of viral spread. Half the buccal mucosa biopsy was also snap frozen, and half fixed in formalin. All formalin fixed tissue was sent to the Pathology Department, Glasgow Royal Infirmary, along with the tumour resection, for sectioning, staging and reporting by Professor MacDonald, Consultant Pathologist.

Following discharge, patients were followed up in the multi-disciplinary head and neck clinic. Patients were initially seen 1-2 times per month, and then this was extended to 3 monthly appointments during the first 12 months. Any patients requiring postoperative radiotherapy and/or chemotherapy were treated at the Beatson Oncology Centre, Glasgow. All patients will be followed up regularly for at least the first 5 years post-treatment.

3.2.4 BLOOD: RED BLOOD CELL AND SERUM EXTRACTION

Blood samples, in their pink-topped potassium EDTA tubes, were centrifuged at 2000 rpm in a bench-top Beckman-type centrifuge for 5 minutes at room temperature. 200µl from the upper serum layer was stored at -70^0C for detection

of infectious HSV. The remainder of the serum was stored at -20^0C for HSV antibody screening and archiving.

The red blood cells were stored at -20^0C in preparation for DNA extraction and archiving.

3.2.5 DETECTION OF SERUM HSV-1 IgG AND IgM ANTIBODIES

Detection of human serum IgG and IgM antibodies against HSV-1 was performed using an ELISA kit (Virotech). The antibodies formed immune complexes with the antigen coated on the microtitre test strips. All experiments, positive and negative controls, cut-off controls and patient sera, were performed in duplicate. Unbound immunoglobulins were removed by washing processes with PBS. The enzyme conjugate attaches to the immune complex, and unbound conjugate was removed by washing processes. After adding the substrate solution, tetramethylbenzidine, a blue dye was produced by the bound enzyme, peroxidase. The colour changed to yellow when the citrate stopping solution was added. The colour change was read using an ELISA plate spectrophotometer, set at a wavelength of 450 nm and reference length of 620 nm, to give a measure extinction (OD) value.

The OD values of the controls enabled quantification of a semi-quantitative determination of specific IgG and IgM antibodies. Their quantity was expressed in Virotech units (VE).

Calculation of VE units:

$$VE\ (positive\ control) = \frac{OD\ positive\ control}{OD\ cut\text{-}off\ control} \times 10$$

$$VE\ (patient\ serum) = \frac{OD\ patient\ serum}{OD\ cut\text{-}off\ control} \times 10$$

3.2.6 DNA EXTRACTION FROM RED BLOOD CELLS

DNA was extracted from human red blood samples using a Nucleon® genomic DNA extraction kit, in a designated laboratory to minimise contamination. 0.5 ml samples of red blood cells were prepared and lysed using the appropriate kit reagents. Following deproteination, the DNA extraction was performed using Nucleon® resin, without chloroform. The DNA was precipitated out using isopropanol, and washed with 70% ethanol. Each DNA extract was resuspended in 50µl of nuclease free water.

3.2.7 DNA EXTRACTION FROM HUMAN TISSUE

DNA from human tissue biopsies was extracted using the same Nucleon® kit and protocol as for red blood cell samples. In addition, prior to cell preparation and lysis, the tissue samples were homogenised in eppendorf tubes using an electric cordless homogeniser. Also, during cell lysis, the samples were incubated at 37°C overnight, rather than for 10 minutes.

3.2.8 HSV POLYMERASE CHAIN REACTION (PCR): BLOOD AND TISSUE SAMPLES

All reactions were set up in an ultraviolet cabinet in a laboratory solely used for human sample DNA extraction and PCR, to minimise contamination.

DNA extracted from BHK cells was used as controls. HS 13 (ACG ACG ACG TCC GAC GGC GA) and HS 14 (GTG CTG GTG CTG GAC GAC AC) primers were used, which detect the UL42 locus of HSV (Puchhammer-Stockl et al 1990), and were made up as 1nM/µl stocks in sterile, deionised water.

PCR reagents used per reaction were: 1.5µl 10 mM deoxynucleotide mix; 5 µl (10x) Pfx amplification buffer; 0.5 µl Platinum® Pfx DNA polymerase; 1 µl 50

mM Mg_2SO_4; 1µl 0.1 nM dilution of HS 13; 1µl 0.1 nM dilution of HS 14; 1 µl DNA template; all made up to 50µl total volume in sterile deionised water.

PCR conditions were 94^{0}C for 2 minutes; 32 cycles x (94^{0}C for 15 seconds, 72^{0}C for 1 minute, 72^{0}C for 1 minute); 72 C for 2 minutes followed by a 4^{0}C incubation.

After PCR, 20 µl of each reaction was electrophoresed on a 1% agarose gel and observed for a 278-bp band indicative of HSV. 100bp and 1Kb DNA markers of known concentration were run alongside the DNA samples to enable confirmation of the fragment size.

3.2.9 AGAROSE GEL ELECTROPHORESIS

PCR analysis used 100 ml gels of 1% agarose boiled in 1x TBE buffer. Once cooled to 75^{0}C, 5µl of ethidium bromide was added and the solution poured onto a sealed plate, with a Teflon-coated well-forming comb (12-18 teeth) positioned.

Once set, the gel was placed in a gel tank containing 1xTBE and the samples added and electrophoresed at 80-100 V for 45-75 minutes. The gels were visualized using a short wave ultraviolet lamp and photographed on Polaroid film.

3.2.10 HSV IMMUNOHISTOCHEMISTRY

Immunohistochemistry for HSV was performed on paraffin sections using a rabbit polyclonal antibody to HSV-1 (DAKO), dilution 1:100. Anti-rabbit secondary antibody (DAKO) was used at a 1:50 dilution. The regional centre for immunohistochemistry at Glasgow Royal infirmary performed the experiments.

3.2.11 HSV *IN-SITU* HYBRIDISATION (ISH): TISSUE SAMPLES

HSV DNA was detected using the Rembrandt® *in situ* hybridisation kit.

5μm paraffin sections of tissue biopsies were cut in additive-free distilled water at 55^{0}C. The sections were collected on organosilane coated glass slides and baked at 56-60^{0}C in a dry air oven for 2-16 hours.

Experiments were performed with negative and positive control slides, the latter being cultured HSV-1 infected human embryonic lung fibroblast cells. Prior to ISH the slides were dewaxed in fresh histoclear for 2 x 10 minutes. The slides were then placed in 100% ethanol for 5 minutes and air dried for 10 minutes.

A proteolytic treatment solution was prepared by mixing 50μl of the kit pepsin digestion reagent to 5ml 1M HCl. 400μl of the solution was added to each slide and incubated at 37^{0}C for 30 minutes.

Following dehydration of the slides in graded ethanol washes, the hybridisation was performed. 20μl of the negative or positive digoxigenin labelled DNA probe was added to sections and a coverslip positioned over the section. Incubating the slides on a 95^{0}C hotplate for exactly 5 minutes denatured the sections. They were then put in a humidified chamber and incubated overnight at 37^{0}C.

The slides were submerged in TBS to remove the coverslips and washed in formamide.

For detection and staining, alkaline phosphatase conjugated αDIG was added to sections and incubated for 30 minutes at 37^{0}C. Following a TBS wash, NBT/BCIP substrate was added to each section and incubated in the dark at 37^{0}C for 15 minutes. Following a wash in distilled water, the slides were mounted with an aqueous mounting medium.

3.2.12 DETECTION OF INFECTIOUS HSV1716: SERUM AND TISSUE SAMPLES

Serum: ETC_{10} medium was removed from confluent monolayers of BHK21/C13 cells on 60mm diameter tissue culture dishes. 200µl of serum was added to each plate and overlaid immediately with ETMC 10% to avoid toxicity to the cells.

Tissue: Samples were homogenised in eppendorf tubes using an electric cordless homogeniser, and suspended in 1ml of ETC_{10}. Following bath-sonication for 5 minutes, the tubes were spun in a Beckman-type bench-top centrifuge at 2000 rpm for 5 minutes. The supernatant was decanted from each tube and 500µl was added to confluent monolayers of BHK21/C13 cells on 60mm diameter tissue culture dishes, following removal of the ETC_{10} medium. The dishes were left for 30 minutes at 37^0C before adding 4.5ml of ETMC 10%.

All dishes were incubated at 31^0C for 3 days, and observed daily for signs of viral infection.

CHAPTER 4

RESULTS

4.1 CHARACTERISATION OF THE CYTOTOXIC EFFECTS *IN VITRO* OF HSV1716 AND HSV1716 PLUS CISPLATIN AGAINST HEAD AND NECK SQUAMOUS CARCINOMA CELLS

4.1.1 INTRODUCTION

HSV1716 is a deletion mutant of HSV-1, which is selectively replication competent. It fails to make the virulence factor ICP34.5 (MacKay et al, 1993). HSV1716 does not replicate in terminally differentiated cells or cause encephalitis following intracranial inoculation of animals or humans (MacLean et al, 1991; Rampling et al, 2000; Papanastassiou et al, 2002; Harrow et al, 2004). Cytotoxic effects of ICP34.5 null mutants of HSV-1 have been seen against a number of tumour types including glioblastoma, medulloblastoma, mesothelioma and melanoma (Mineta et al, 1995; Lasner et al, 1996; Kucharczuk et al, 1997; Randazzo et al, 1997). HSV1716 infection of head and neck squamous cell carcinoma cells has not been studied before.

Three HNSCC cell lines were chosen to work with, UM-SCC-14C, UM-SCC-22A and UM-SCC-22B (Grenman et al, 1991, Carey et al, 1983). Professor Guus van Dongen, VU University Medical Centre, Amsterdam kindly provided all the cells. The characteristics of the cells are shown in Table 9. 22A and 22B, a lymph node metastasis from the same patient. It was necessary to use cells that did not require the use of feeder cell layers. If virus infected epithelial feeders, any results attained would be invalid.

Cisplatin is a platinum based chemotherapy agent, which is commonly used in head and neck cancer. It enters the cell by passive diffusion, where it is hydrolysed to its active form due to the low chloride concentration inside the cell. It binds to proteins and nucleic acids causing DNA damage and cell apoptosis.

Table 9: HNSCC CELL LINES

(Donor information and specimen site. 22A and 22B cells are from the same patient))

CELL LINE NAME	AGE (YRS)	GENDER	TNM	STAGE	SPECIMEN SITE
UM-SCC-14C	58	FEMALE	T1N0M0	I	FLOOR OF MOUTH
UM-SCC-22A	58	FEMALE	T2N1M0	II	HYPOPHARYNX
UM-SCC-22B	58	FEMALE	T2N1M0	II	HYPOPHARYNX

Combining cancer therapies can potentially produce additive or synergistic effects, along with decreased side effects and overall, a better outcome. The potential side effects, including encephalitis, of HSV1716 do not overlap with cisplatin. Also, cross-resistance is theoretically unlikely due to the radically different mechanisms of action of viral and chemotherapeutic agents.

HSV1716 has an attractive profile for investigating as a novel head and neck cancer therapy, and could potentially be used in combination with conventional chemotherapy agents. Initial studies established the permissivity of HNSCC to HSV1716, and the cytotoxic effects of HSV1716 and cisplatin in isolation. The potential for enhanced cytotoxic effects by combining the two agents was then investigated.

4.1.2 GROWTH KINETICS OF HSV1716 IN HNSCC CELL LINES

Initial experiments aimed to establish permissivity of HNSCC cell lines to HSV1716. Studies have shown that PCNA levels are high in head and neck cancer cells and this high level of PCNA expression is associated with advanced disease

and poor prognosis (Sarac et al, 1998; Liu et al, 2003; Krecicki et al, 1999). This gave expectations of HSV1716 being able to replicate in the three cell lines.

Multicycle replication kinetics of HSV1716 and wild type HSV, strain 17^+, were examined by infecting UM-SCC-14C, UM-SCC-22A and UM-SCC-22B at a low multiplicity of infection (MOI). The cells were infected with the viruses at an MOI of 0.1 pfu/cell and the cells/virus harvested at 0, 6, 24, 48 and 72 hours post-infection. The samples were sonicated and progeny virus titrated in BHK21/C13 monolayers.

Figure 9 shows the growth kinetics of HSV1716 and HSV strain 17^+ in confluent UM-SCC-14C, BHK and 3T6 cells. BHK and 3T6 cells were used as controls as both are known to be fully permissive for HSV strain 17^+, but 3T6 cells are only permissive for HSV strain 17^+ and are non-permissive for HSV1716. The graph shows predictable results for the control cells and UM-SCC-14C cells are seen to be fully permissive for HSV strain 17^+ and semi-permissive for HSV1716.

Figure 10 shows the growth kinetics of HSV1716 and HSV wild type, strain 17^+, in confluent UM-SCC-22A, BHK and 3T6 cells. The control cells show predictable results and UM-SCC-22A cells are seen to be fully permissive for HSV strain 17^+ and semi-permissive for HSV1716.

Figure 11 shows the growth kinetics of HSV1716 and HSV strain 17^+ in confluent UM-SCC-22B, BHK and 3T6 cells. The control cells show predictable results and the UM-SCC-22B cells are seen to be semi-permissive for HSV strain 17^+ and HSV1716.

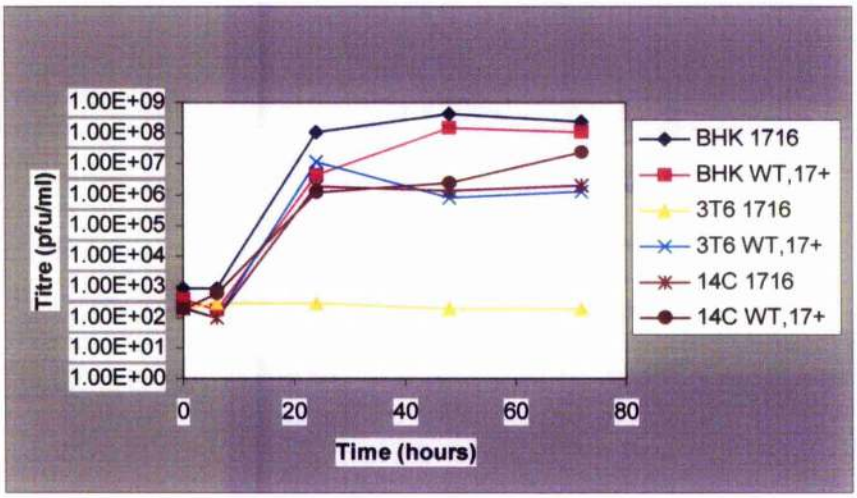


Figure 9. MULTICYCLE GROWTH KINETICS OF HSV1716 AND HSV WILD TYPE, STRAIN 17+, IN UM-SCC-14C, BHK AND 3T6 CELLS

UM-SCC-14C, BHK and 3T6 cells were infected with HSV1716 or HSV-1, strain 17+, at an MOI of O.1 pfu/cell. Infected cells were harvested at 0, 6, 24, 48 and 72 hours post-infection and progeny virus titrated on BHK21/C13 cells. Graph produced with Microsoft (MS) Excel computer software.

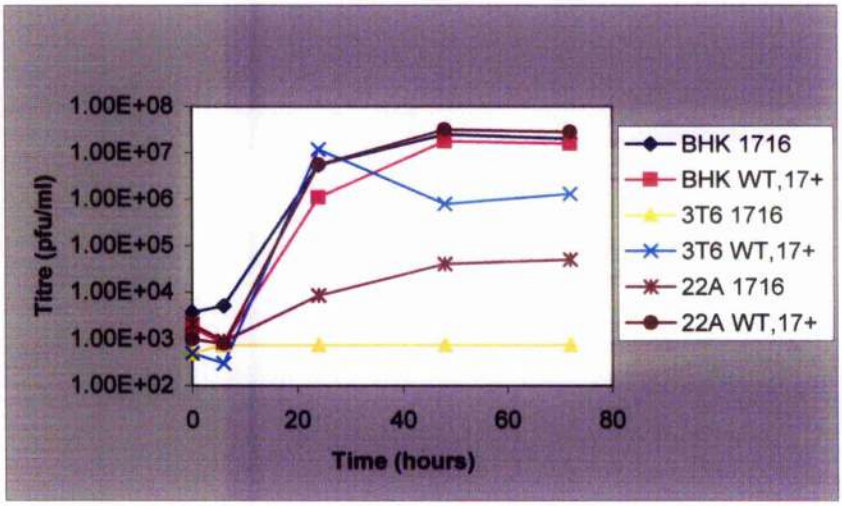


Figure 10. MULTICYCLE GROWTH KINETICS OF HSV1716 AND HSV WILD TYPE, STRAIN 17+, IN UM-SCC-22A, BHK AND 3T6 CELLS

UM-SCC-22A, BHK and 3T6 cells were infected with HSV1716 or HSV-1, strain 17+, at an MOI of O.1 pfu/cell. Infected cells were harvested at 0, 6, 24, 48 and 72 hours post-infection and progeny virus titrated on BHK21/C13 cells Graph produced with MS computer software.

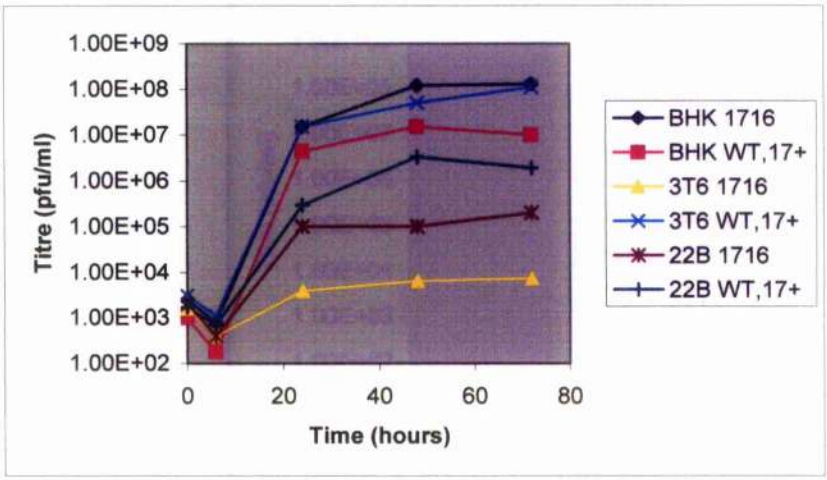


Figure 11. MULTICYCLE GROWTH KINETICS OF HSV1716 AND HSV WILD TYPE, STRAIN 17+, IN UM-SCC-22B, BHK AND 3T6 CELLS

UM-SCC-22B, BHK and 3T6 cells were infected with HSV1716 or HSV-1, strain 17+, at an MOI of O.1 pfu/cell. Infected cells were harvested at 0, 6, 24, 48 and 72 hours post-infection and progeny virus titrated on BHK21/C13 cells. Graph produced with MS Excel computer software.

Thus all three HNSCC cell lines have been shown similar trends by being permissive for replication of HSV1716, but the level of replication is less than that demonstrable in fully permissive BHK cells, and more than that demonstrable in confluent non-permissive 3T6 cells. The intermediate level of replication is a good result for an agent to study in combination therapy experiments. It would be more difficult to look for evidence of enhanced cytotoxicity in combination experiments if the cells were fully permissive for HSV1716 replication. Potential add on effects of cisplatin would be masked.

4.1.3 INTERACTIONS BETWEEN HSV1716 AND CISPLATIN

Before proceeding with cytotoxicity experiments it was essential to make sure that there were no toxic interactions between the two agents. Titrations of a stock of |HSV1716 were performed. Three titrations were performed adding serial dilutions of HSV1716 alone to 85% confluent BHK cells on 60mm petri dishes. The virus was added immediately in one experiment and after incubations for 1 hour at room temperature and 1 hour at 37^0C in the other two experiments respectively. Three further experiments were performed applying the same temporal and temperature condition but 10μM cisplatin was added to each virus sample. This was a higher dose of cisplatin than would be used in the cytotoxicity experiments. The results of the titrations experiments are shown in Table 10. Similar trends are seen in the final titre values as all values are to the same power of 10^9.and would hardly be detectable on a logarithmic scaled graph as in figures 9-11. This indicates that a concentration of 10 μM cisplatin is not detrimental to HSV1716 infectivity.

Table 10: HSV1716 titrations with and without 10μM cisplatin

	CONDITIONS	HSV1716 TITRE (pfu/ml)
HSV1716 ALONE	VIRUS ADDED IMMEDIATELY	3.5×10^9
HSV1716 ALONE	1 HOUR AT ROOM TEMP.	1.9×10^9
HSV1716 ALONE	1 HOUR AT 37^0C	2.1×10^9
HSV1716 + CISPLATIN	VIRUS ADDED IMMEDIATELY	1.9×10^9
HSV1716 + CISPLATIN	1 HOUR AT ROOM TEMP.	1.9×10^9
HSV1716 + CISPLATIN	1 HOUR AT 37^0C	2.6×10^9

A multicycle growth experiment was repeated to establish any interactions between HSV1716 and cisplatin that would affect the growth kinetics of the virus in HNSCC cell lines. UM-SCC-14C and BHK cells were infected with HSV1716 at an MOI of 0.1 pfu/cell. In parallel experiments the same cells were infected with HSV1716 and 1.0μM cisplatin. This cisplatin concentration would be used in cytotoxicity experiments. All cells/virus +/- cisplatin were harvested at 0, 6, 24, 48 and 72 hours post infection. The samples were sonicated and progeny virus titrated on BHK21/C13 monolayers.

Figure 12 shows the growth kinetics of HSV1716 in confluent UM-SCC-22A and BHK cells, with and with out the presence of 1.0μM cisplatin. The cisplatin does not affect the final viral titres and is not detrimental to HSV1716 infectivity.

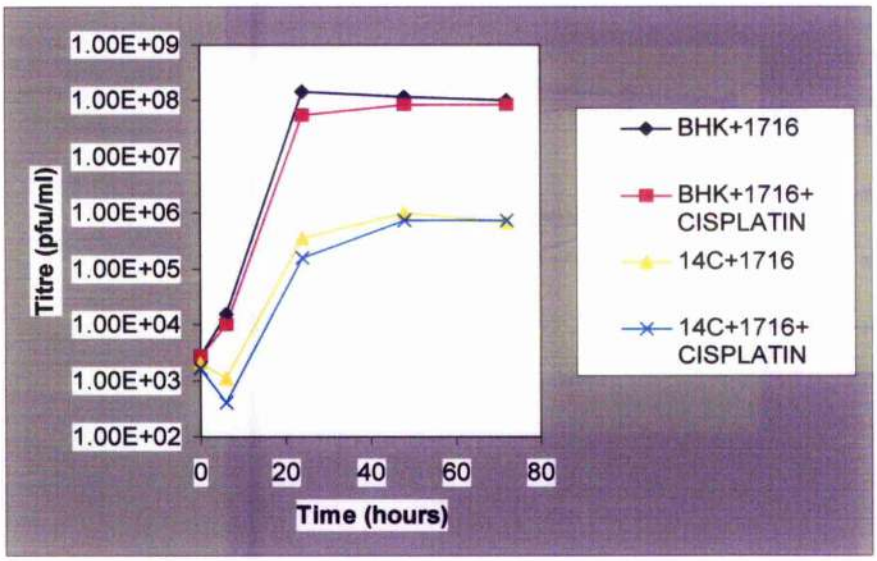


Figure 12. MULTICYCLE GROWTH KINETICS OF HSV1716 IN UM-SCC-14C AND BHK CELLS, WITH AND WITHOUT THE PRESENCE OF CISPLATIN

UM-SCC-14C and BHK cells were infected with HSV1716 at a MOI of O.1 pfu/cell, with and without the presence of 1.0μM cisplatin. Infected cells were harvested at 0, 6, 24, 48 and 72 hours post-infection and progeny virus titrated on BHK21/C13 cells. Graph produced with MS Excel computer software.

4.1.4 HSV1716 CYTOTOXICITY IN HNSCC CELL LINES

HSV1716 cytotoxicity in HNSCC cell lines was studied using a colorimetric MTS tetrazolium assay (Promega). The assay measures dehydrogenase enzyme activity found in metabolically active cells. The intensity of the colour change produced by the bioreduction of tetrazolium to formazan is proportional to the number of living cells. Before proceeding with cytotoxicity experiments it was necessary to confirm a linear relationship between viable cell numbers and colorimetric MTS optical density readings, taken at 480nm. A standard curve was produced using UM-SCC-22B cells. Cells were seeded in a 96 well plate at densities between 375 cells per well and 12,000 cells per well in 100 µl volumes of appropriate media. Each cell density was performed in six replicates to reduce errors when averaging the results. The plate was incubated at 37^0C for 20 hours. All wells were aspirated dry and replaced with 100 µl of fresh media. Six additional wells were set up with 100 µl of fresh media. 20 µl of MTS mixture was added to each well and the plates incubated for 2½ hours. Optical readings were taken and the value for each cell density taken as an average over six wells. The readings from the wells containing media alone were also averaged. Corrected readings were then calculated by subtracting the averaged media reading from the averaged reading for each cell density. The results were plotted on a graph (Figure 13).

The graph shows a linear relationship and an R^2 value of 0.9864. This result has significance for cytotoxicity experiments when plotting MTS absorbance readings against different concentrations of a cytotoxic agent. A fifty per cent reduction in MTS absorbance reading indicates a 50% reduction in viable cells.

An in vitro model was then designed to study the cytotoxic effects of HSV1716 on HNSCC cell lines. Many problems were encountered. All three cell lines were found

STANDARD CURVE: UM-SCC-22B

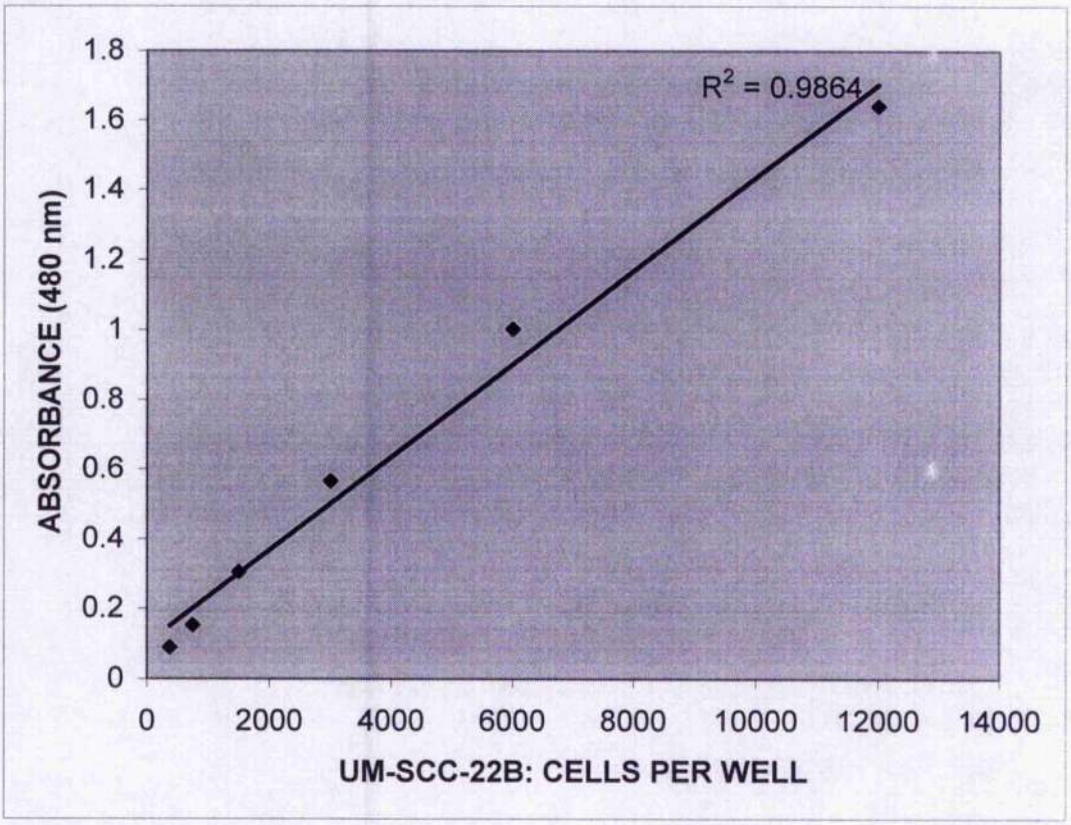


Figure 13: Standard curve of UM-SCC-22B cell absorbance at 480nm versus cell concentration. The R^2 value is the fraction of the variance in the data that is explained by regression. At 0.9864 the regression is approaching unity and so the absorbance is directly proportional to the number of cells. Graph produce with MS Excel computer software.

to be difficult to work with and at times their behaviour was unpredictable. They were prone to going into growth arrest at any time and this did not correlate with the number of times they had been passaged.

A cytotoxicity model was needed to produce dose response curves to determine IC50 values, the concentration of cytotoxic agent required to inhibit the cell number by 50%. It was first necessary to determine optimal initial cell seeding densities. Cells were to be exposed to different MOIs of HSV1716 and the results compared to cells exposed only to media. Absorbance readings were to be taken 72 hours after exposure to the cytotoxic agent, which is standard for this type of experiment. Ideally the cells should grow exponentially throughout the experiment and the control cells, exposed only to media, should reach 90-100% confluence at 72 hours.

Optimal seeding densities were found to be 4000 cells per well for UM-SCC-14C and 6000 cells per well for UM-SCC-22Aand UM-SCC-22B. It was also found to be important to seed cells taken from 70-80% confluent T-175 flasks. In this state the cells were already growing exponentially and at a full spectrum of stages in the cell cycle. If cells were seeded from fully confluent flasks, their growth in 96 well plates was unpredictable and initially very slow.

Having established optimal cell seeding densities, experiments were carried out to determine the IC50 of HSV1716 for each cell line. After the seeded cells were incubated at 37^0C overnight the media was removed and serial concentrations of HSV1716 were added in 100 µl volumes. Each viral concentration was performed in six replicates to reduce errors. A control lane of six wells was prepared, with cells exposed only to media. After 72 hours incubation at 37^0C, all wells were aspirated dry and 100 µl of fresh media added to avoid errors when taking optical density readings. A further control lane was prepared with six wells, each containing 100 µl

of fresh media. 20 µl of MTS reagent was added to each well and the plates incubated for 2½ hours. Optical readings were taken and the value for each viral MOI and control lanes was calculated as an average over six wells. The experiments were repeated many times until satisfactory consistency in the results was achieved. Particular problems that had to be addressed were the different rates of cell growth and cross-contamination while pipetting into wells and the accuracies of the cell counting and cell seeding. When seeding 4000 cells into a well a realistic error of 1000 cells is a notable 25% error. Dose response curves were produced for each cell line and IC50 values calculated accordingly. All experiments were repeated until consistency was achieved. HSV1716 has not been used before with HNSCC so there was no published data with which to compare IC50 values.

4.1.5 CALCULATION OF IC50 VALUE

Adjusted absorbance values were calculated by subtracting the average absorbance value from wells containing media alone. These adjusted absorbance values were plotted against viral MOI (Figure 14).

Dose response curves for UM-SCC-14C, UM-SCC-22A and UM-SCC-22B with HSV1716 are shown in Figures 15, 16 and 17. The HSV1716 IC50 values for the three HNSCC cell lines are shown in Table 11.

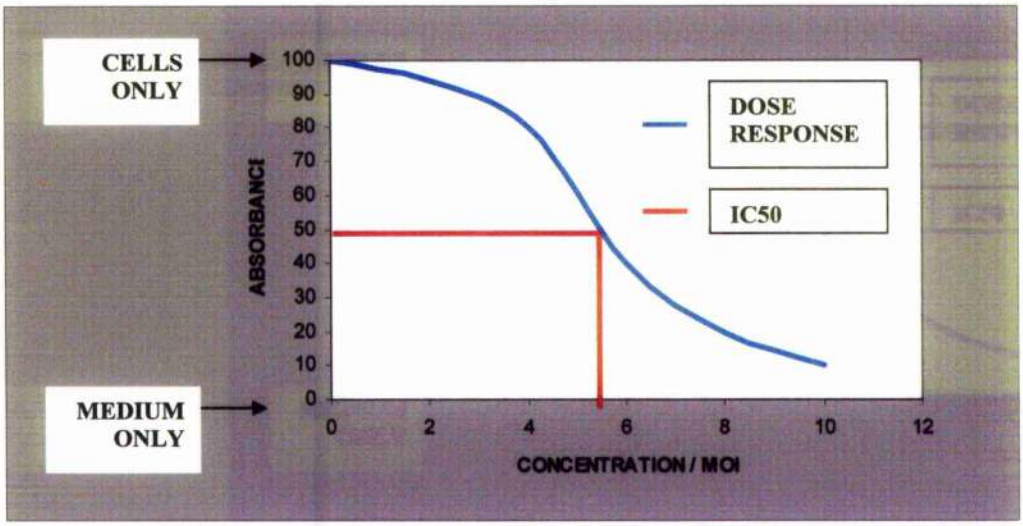


Figure 14: METHOD OF DETERMINING IC50 USING A DOSE RESPONSE CURVE. The IC50 is the x-axis intercept of the curve at the point determined by a 50% reduction in absorbance and therefore cell viability.

HNSCC CELL LINE DOSE RESPONSE CURVES WITH HSV1716

Figure 15: UM-SCC-14C DOSE RESPONSE CURVE WITH HSV1716

UM-SCC-14C cells were infected with serial concentrations of HSV1716. Each viral concentration was performed in 6 replicates to reduce errors. Optical readings were taken at 72 hours. Graph produced with MS Excel computer software.

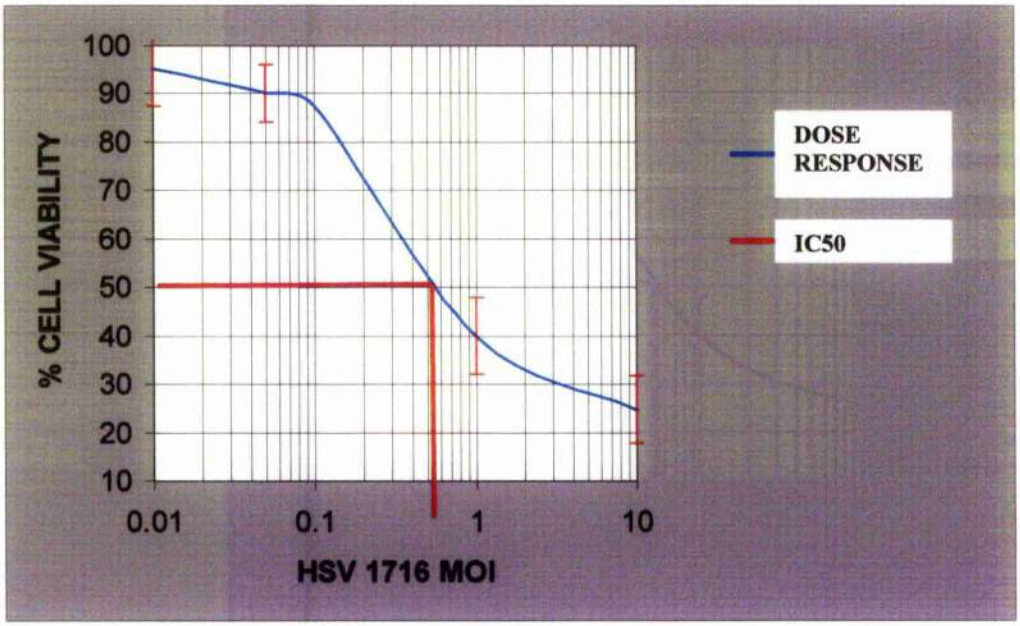


Figure 16: UM-SCC-22A DOSE RESPONSE CURVE WITH HSV1716

UM-SCC-22A cells were infected with serial concentrations of HSV1716. Each viral concentration was performed in 6 replicates to reduce errors. Optical readings were taken at 72 hours. Graph produced with MS Excel computer software.

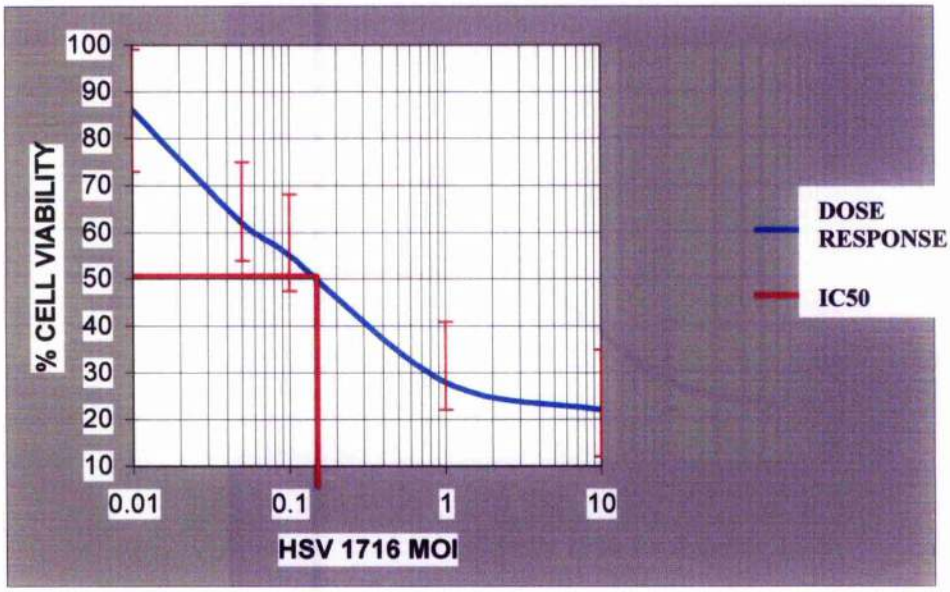


Figure 17: UM-SCC-22B DOSE RESPONSE CURVE WITH HSV1716

UM-SCC-22B cells were infected with serial concentrations of HSV1716. Each viral concentration was performed in 6 replicates to reduce errors. Optical readings were taken at 72 hours. Graph produced with MS Excel computer software.

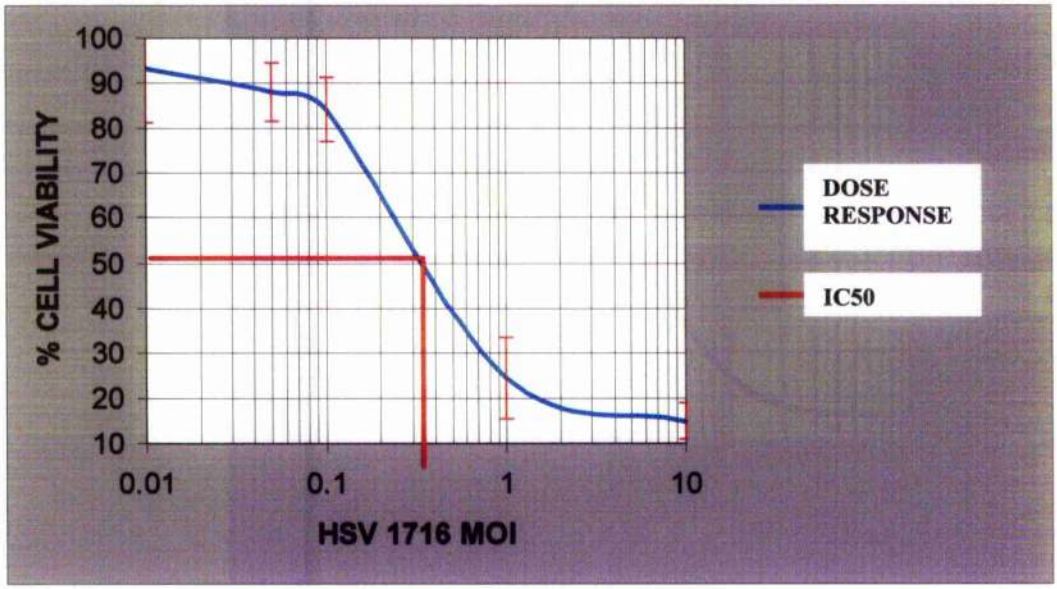


Table 11: HSV1716 IC50 VALUES FOR HNSCC CELL LINES (72 HOURS)

CELL LINE	UM-SCC-14C	UM-SCC-22A	UM-SCC-22B
IC50 MOI(pfu/cell)	0.55 +/- 0.12	0.15 +/- 0.039	0.35 +/- 0.074

4.1.6 CISPLATIN CYTOTOXICITY IN HNSCC CELL LINES

The same MTS assay *in vitro* model was use to study the cytotoxic effects of cisplatin alone on HNSCC cell lines. The cells were seeded in 96 well plates and after 24 hours incubation at 37^0C the cells were exposed to a range of concentrations of cisplatin between 0.5 μM and 5 μM. Each cisplatin concentration was performed in six replicates to reduce errors. The same protocol was adhered to as for HSV1716. Optical readings were taken and the value for each cisplatin concentration and control lanes calculated as an average over six wells. Dose response curves were produced for each cell line and IC50 values calculated accordingly. Dose response curves for cisplatin with UM-SCC-14C, UM-SCC-22A and UM-SCC-22B are shown in Figures 18, 19 and 20. All experiments were repeated until consistency was achieved. The cisplatin IC50 values for the three HNSCC cell lines are shown in Table 12. Previously published cisplatin IC50 data is also included (Welters et al, 1997)

The IC50 values are consistently higher in comparison to the results of Welters et al, however the trend and the error margins are similar. The differences can be explained by the use of different culture media ingredients, and more importantly the performance of a different cytotoxicity experiment. They used a semi-automated proliferation assay with sulforhodamine B and the cells were incubated for 72 hours at 37^0C prior to cisplatin exposure.

Figure 18: UM-SCC-14C DOSE RESPONSE CURVE WITH CISPLATIN

UM-SCC-14C cells were infected with serial concentrations of cisplatin. Each cisplatin concentration was performed in 6 replicates to reduce errors. Optical readings were taken at 72 hours. Graph produced with MS Excel computer software.

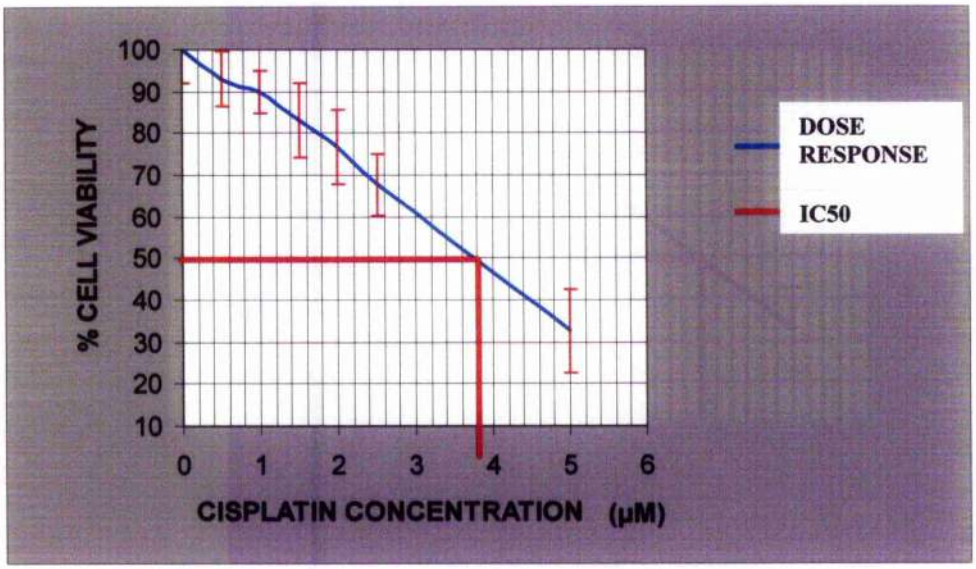


Figure 19: UM-SCC-22A DOSE RESPONSE CURVE WITH CISPLATIN

UM-SCC-22A cells were infected with serial concentrations of cisplatin. Each cisplatin concentration was performed in 6 replicates to reduce errors. Optical readings were taken at 72 hours. Graph produced with MS Excel computer software.

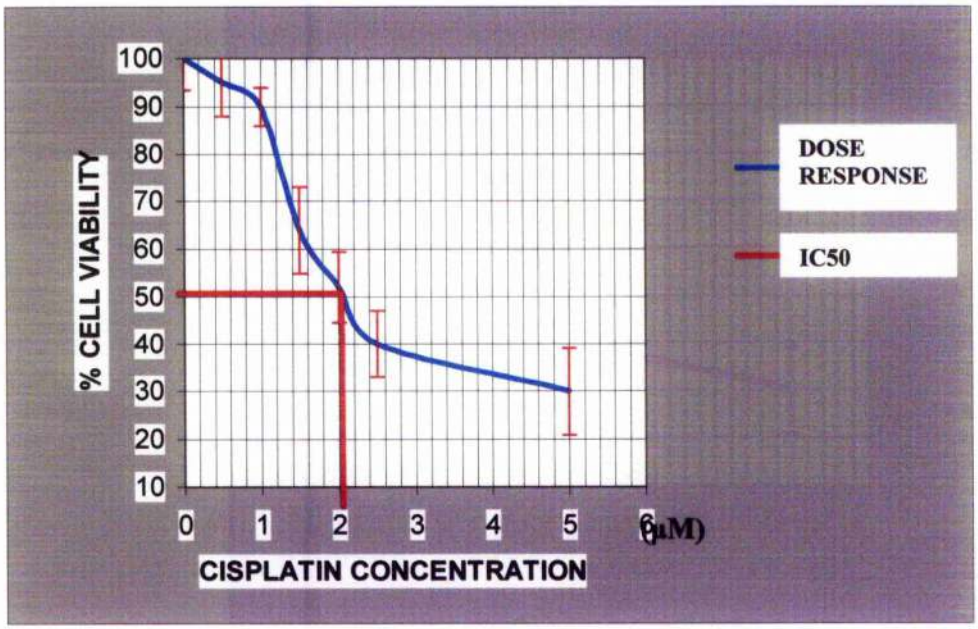


Figure 20: UM-SCC-22B DOSE RESPONSE CURVE WITH CISPLATIN

UM-SCC-14C cells were infected with serial concentrations of cisplatin. Each cisplatin concentration was performed in 6 replicates to reduce errors. Optical readings were taken at 72 hours. Graph produced with MS Excel computer software.

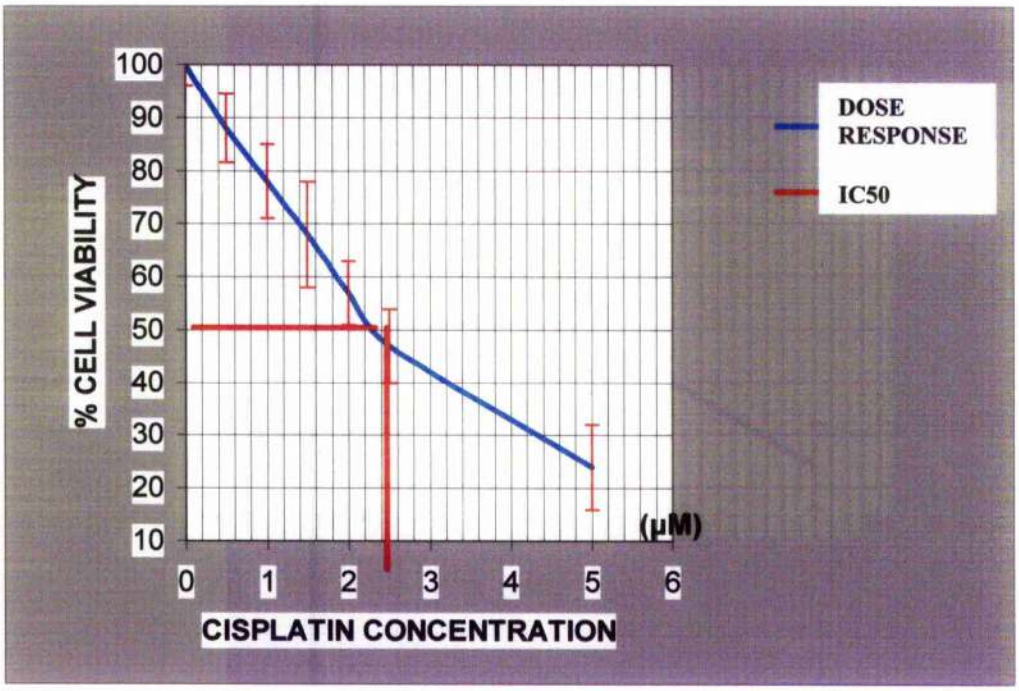


Table 12: CISPLATIN IC50 VALUES FOR HNSCC CELL LINES

(72 HOURS)

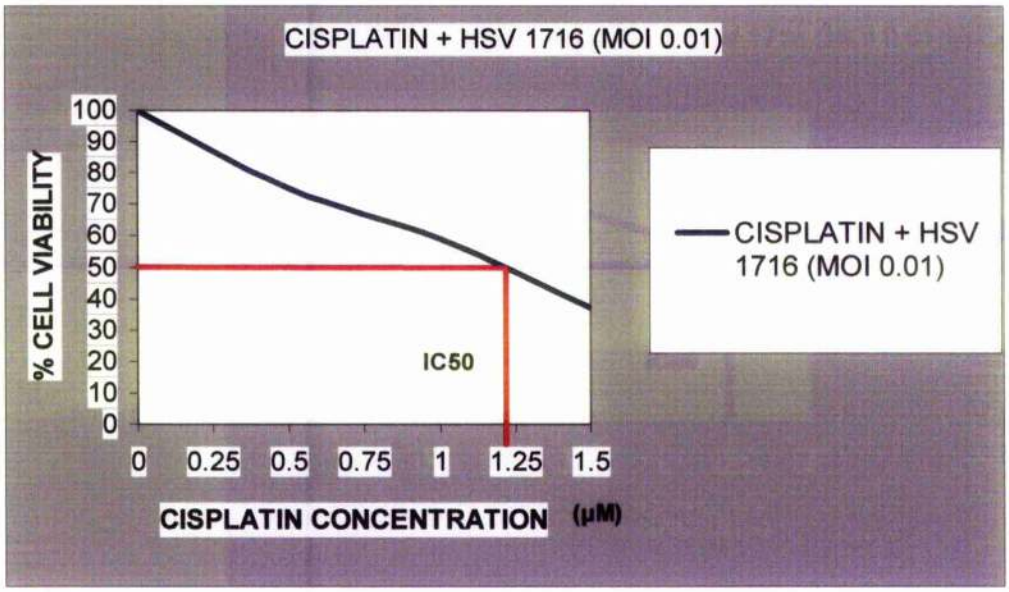
CELL LINE	UM-SCC-14C	UM-SCC-22A	UM-SCC-22B
IC50 (µM)	3.8 +/- 0.8	2.0 +/- 0.5	2.4 +/- 0.4
IC50 (µM) (Welters)	2.7 +/- 0.7	1.3 +/- 0.3	1.2 +/- 0.3

4.1.7 HSV1716 CYTOTOXICITY IN COMBINATION WITH CISPLATIN IN HNSCC CELL LINES

Having established the cytotoxicity profiles of HSV1716 and cisplatin in isolation, it was then possible to perform combination experiments. By combining the agents at doses less than their respective IC50 values it would be possible to look for signs of enhanced cytotoxic effects.

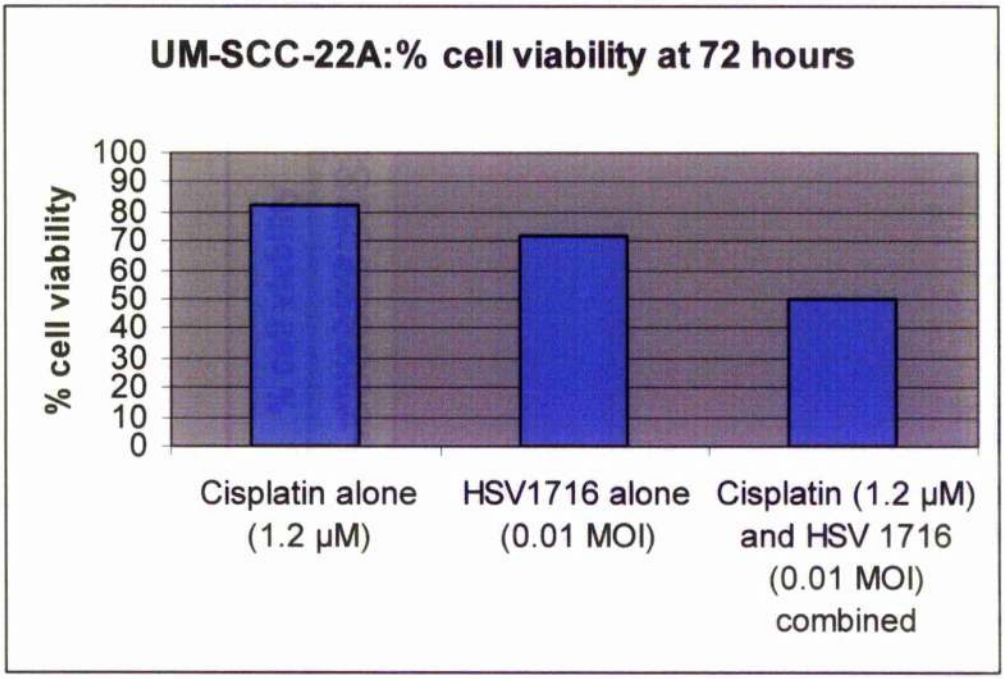
MTS assay experiments were performed with the same protocol and cell seeding densities as with each agent in isolation. 0.5 μM, 1.0 μM and 1.5 μM doses of cisplatin were each combined with doses of HSV1716 at MOI 0.01 pfu/cell, MOI 0.05 pfu/cell and MOI 0.1 pfu/cell. All experiments were carried out over 72 hours to produce dose response curves and corresponding IC50 values. Figure 21 shows an example of how the IC50 was calculated for each combination.

Figure 21: UM-SCC-22A: HSV1716 (MOI 0.01)+CISPLATIN DOSE RESPONSE CURVE. UM-SCC-22A cells were infected with HSV1716 (MOI 0.01) and serial concentrations of cisplatin. Each combination of virus and cisplatin was performed in 6 replicates to reduce errors. Optical readings were taken at 72 hours. Graph produced with MS Excel computer software



HSV1716 MOI 0.01 was combined with different doses of cisplatin and the solid blue line shows the effects on cell viability after 72 hours. The IC50 was calculated for this combination of doses and is shown by the red line intersecting the x-axis at 1.2 μM. Therefore a dose of HSV1716 at 0.01 MOI requires 1.2 μM of cisplatin to kill 50% of the cells. Figure 22 compares the % cell viability at 72 hours of cells exposed to 1.2 μM of cisplatin alone, HSV1716 alone at 0.01 MOI and a combination of the agents at the same doses.

Figure 22: UM-SCC-22A: % cell viability at 72 hours following exposure to HSV1716 (0.01 MOI) and cisplatin (1.2 μM) alone and in combination. Optical readings were taken at 72 hours.



IC50 values were calculated for each dose combination with each cell line and the results are shown in Table 13.

Figure 22 and Table 13 indicate signs of enhanced cytotoxicity when HSV1716 and cisplatin are added in combination. It is not possible to qualify this and also

Table 13: COMBINATION IC50 VALUES (72 HOURS)

	COMBINATION IC50 VALUES	
HNSCC CELL LINE	HSV1716 MOI	CISPLATIN μM
UM-SCC-14C	0.01	3.2
UM-SCC-14C	0.05	2.5
UM-SCC-14C	0.1	2.3
UM-SCC-22A	0.01	1.2
UM-SCC-22A	0.05	0.9
UM-SCC-22A	0.1	0.6
UM-SCC-22B	0.01	1.6
UM-SCC-22B	0.05	1.2
UM-SCC-22B	0.1	0.7

distinguish between antagonism, additivity or synergy without further analysis of the data. It is not possible to simply add the effects of the agents used in isolation. This is only possible if two agents follow closely to first-order kinetics, which would produce linear dose response curves. Synergy, additivity and antagonism would be deemed to be present if the effect of the combined agents were, respectively, more than, equal to or less than the sum of the effects of the constituents.

The combined cytotoxic effects of HSV1716 and cisplatin, which do not follow first-order kinetics, can be evaluated using an isobologram analysis (Kano et al, 1988, Kano et al, 1992). This method forms 3 isoeffect curves (Mode I, IIa and IIb) on an isobologram graph and the outermost boundaries of the 3 lines forms an envelope of additivity (Figure 23). The area to the left of the envelope is the zone of synergy, and the area to the right is the zone of antagonism. The combination IC50 doses from

Table 11 can be plotted on the graph and conclusions made regarding antagonism, additivity and synergy between the two agents.

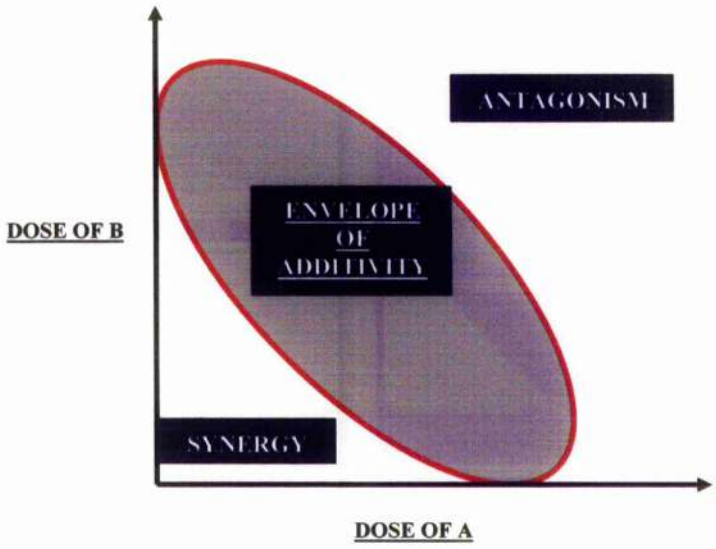


Figure 23: Isobologram graph. An envelope of additivity is constructed from the dose response curves of 2 agents (A and B). Combination IC50 data points can fall into 3 areas with corresponding classification of the 2 agents interactions as antagonistic, additive or synergistic.

The 3 isoeffect curves take into account that the combined effect of the two agents could be completely independent (heteroadditive), dependent (isoadditive) or intermediate. The Mode I, Mode IIa and Mode IIb curves were produced on the isobologram graphs as follows (Kano et al, 1992).

Mode I Curve: This curve is formed on the assumption that the effects of cisplatin and HSV1716 are completely independent of each other. When a dose of HSV1716 is chosen there remains an increment in effect to be produced by cisplatin to obtain 50% cell growth inhibition. If the 2 agents were to act independently, the

addition is performed by taking the increment in doses, starting from zero, that give log survivals which add up to IC50.

Figures 24 and 25 are the dose response curves for UM-SCC-14C with HSV1716 and cisplatin in isolation. The gridlines on the graphs have been excluded. A dose of HSV1716 (V1) was chosen and the fraction affected (inhibited) was F. To obtain 50% cell growth inhibition, an increment in effect, X, remained to be produced by cisplatin (Figure 24). Therefore F+X=50. The addition was performed by taking the increment in doses, starting from zero, which produced log survivals that added up to IC50 (heteroaddition) (Figure 25). X is marked on Figure 24 and the dose of cisplatin required is marked as C1. Therefore plotting different doses of HSV1716, V1, with the corresponding dose of cisplatin, C1, produces the Mode I curve. For convenience the IC50 for each agent in isolation is plotted as 1.0 on the isobologram, therefore no units are required.

Mode IIa curve: This curve is formed on the assumption that there is some interaction between the effects of cisplatin and HSV1716. When a dose of HSV1716, V1, was chosen, an increment in effect, X, remained to be produced by cisplatin. The addition was performed by taking the increment in doses, starting not from zero but from the point on the dose response curve of cisplatin where the effect of HSV1716 had ended (F), that produced log survivals that added up to IC50 (iso-addition). The dose of cisplatin required is marked as C2 (Figure 25). Therefore plotting different doses of HSV1716, V1, with the corresponding dose of cisplatin, C2, produces the Mode IIa curve.

Mode IIb curve: This curve is formed on the assumption that there is some interaction between the effects of cisplatin and HSV1716. When a dose of cisplatin, C1, was chosen, an increment in effect, F, remained to be produced by HSV1716.

The addition was performed by taking the increment in doses, starting not from zero but from the point on the dose response curve of HSV1716 where the effect of cisplatin had ended (X), that produced log survivals that added up to IC50 (iso-addition). The dose of HSV1716 required is marked as V2 (Figure 24). Therefore plotting different doses of cisplatin, C1, with the corresponding dose of HSV1716, V2, produces the Mode IIb curve.

In summary the Combination doses V1 and C1 (Mode I), V1 and C2 (Mode IIa), and V2 and C1 (Mode IIb) will each produce 50% cell inhibition.

Representative isobolograms produced from the dose response curves of UM-SCC-14C, UM-SCC-22A and UM-SCC-22B with HSV1716 and cisplatin are shown in Figures 26, 27 and 28. The envelope of additivity, formed by the outer margins of the three isoeffect curves, is marked on each graph. Combination IC50 data from Table 13 have been plotted. All combination plots fall within the envelopes of additivity. It is possible to conclude there is additivity when HSV1716 is combined with cisplatin in all three HNSCC cell lines. Ergo the effect produced is the sum of the effect of either agent alone.

Figure 24: UM-SCC-14C DOSE RESPONSE CURVE WITH HSV1716

V1 is the chosen dose of HSV1716 used to produce the Mode I curve and Mode IIa curve. V2 is the dose of HSV1716 used to produce the Mode IIb curve.

F is the level of cell inhibition resulting from V1. X is the level of cell inhibition to increase F to 50% (F+X=50).

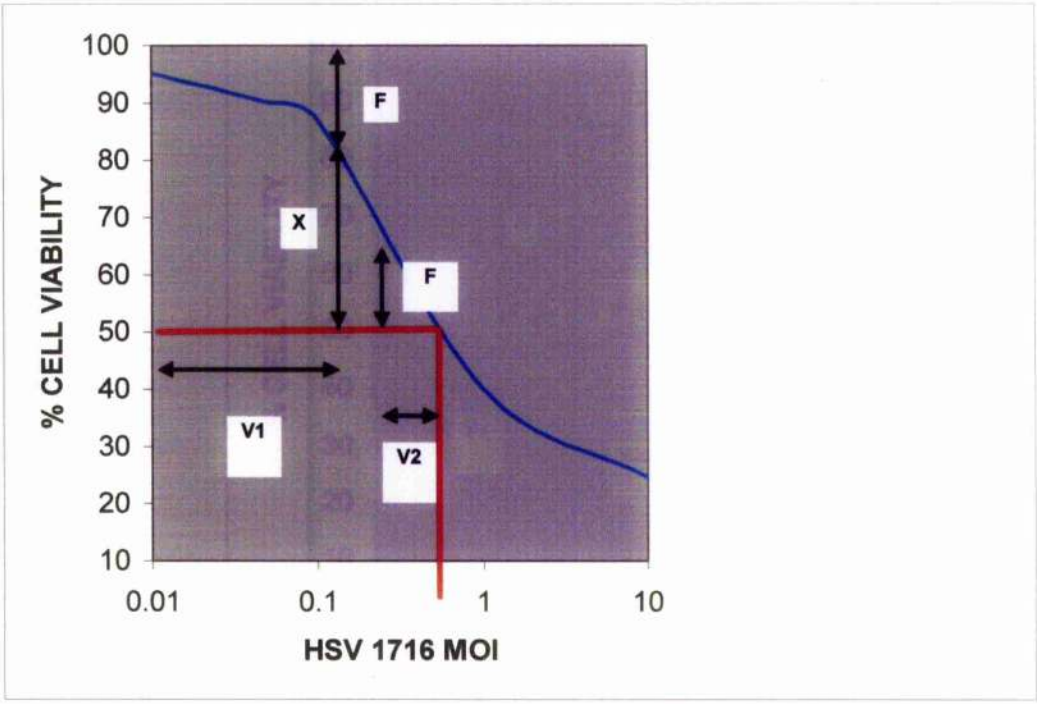


Figure 25: UM-SCC-14C DOSE RESPONSE CURVE WITH CISPLATIN

C1 is the dose of cisplatin used to produce the Mode I curve. C2 is the dose of cisplatin used to produce the Mode IIa curve.

X is the level of cell inhibition resulting from C1. F is the level of cell inhibition to increase X to 50% (F+X=50).

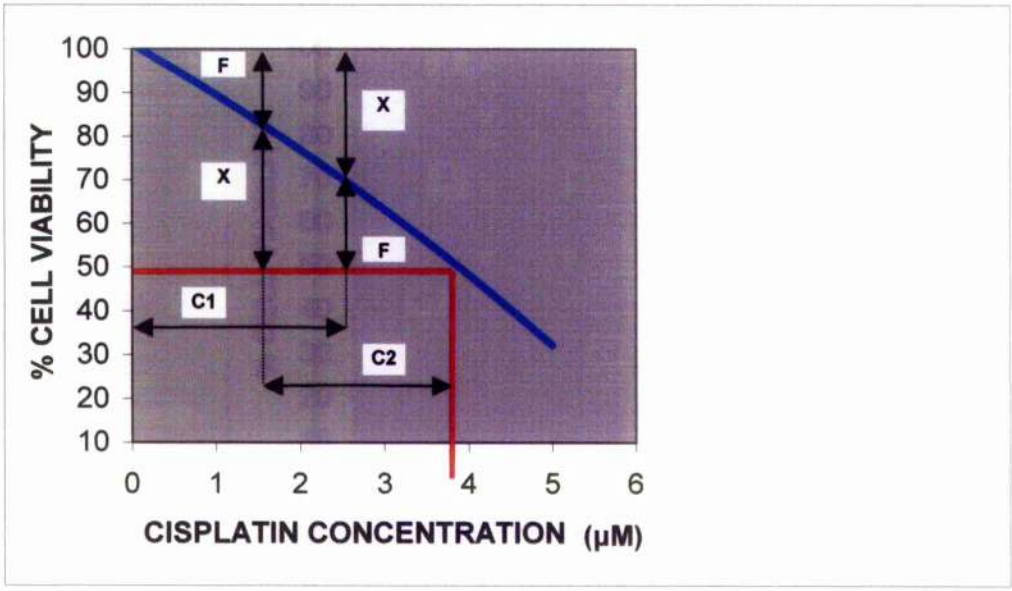


Figure 26: IC50 ISOBOLOGRAM: UM-SCC-14C WITH HSV1716 AND CISPLATIN

The outermost boundaries of the 3 isoeffect curves form an envelope of additivity. IC50 combination data are plotted and fall within the envelope of additivity. For convenience the IC50 for each agent in isolation is plotted as 1.0 on the isobologram, therefore no units are required.

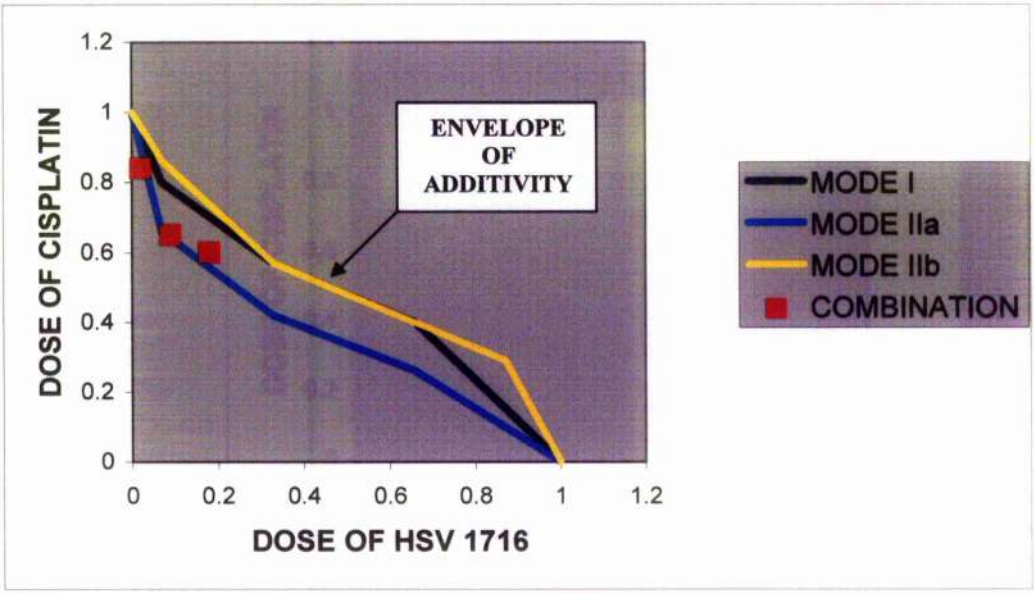


Figure 27: IC50 ISOBOLOGRAM: UM-SCC-22A WITH HSV1716 AND CISPLATIN

The outermost boundaries of the 3 isoeffect curves form an envelope of additivity. IC50 combination data are plotted and fall within the envelope of additivity. For convenience the IC50 for each agent in isolation is plotted as 1.0 on the isobologram, therefore no units are required.

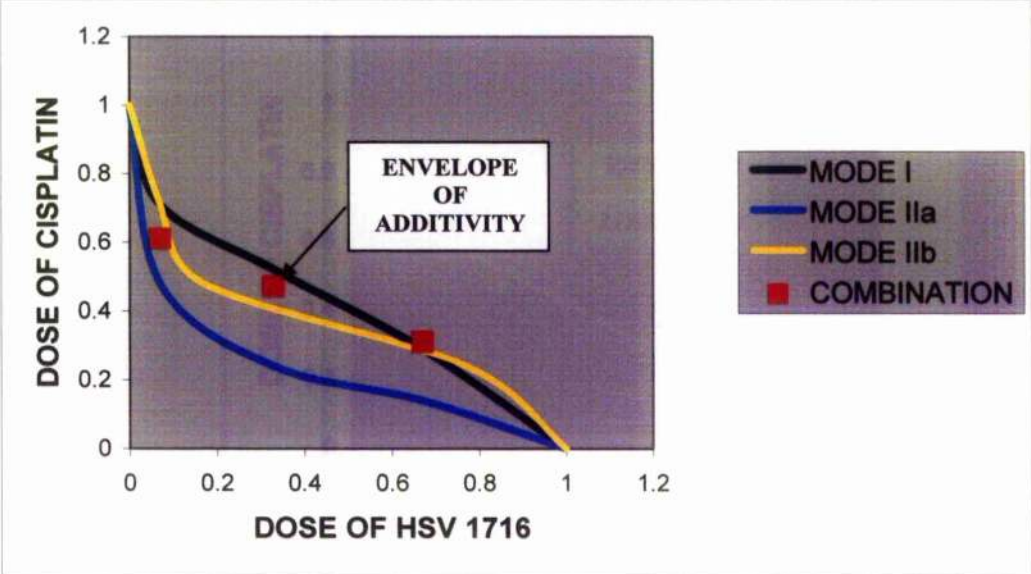
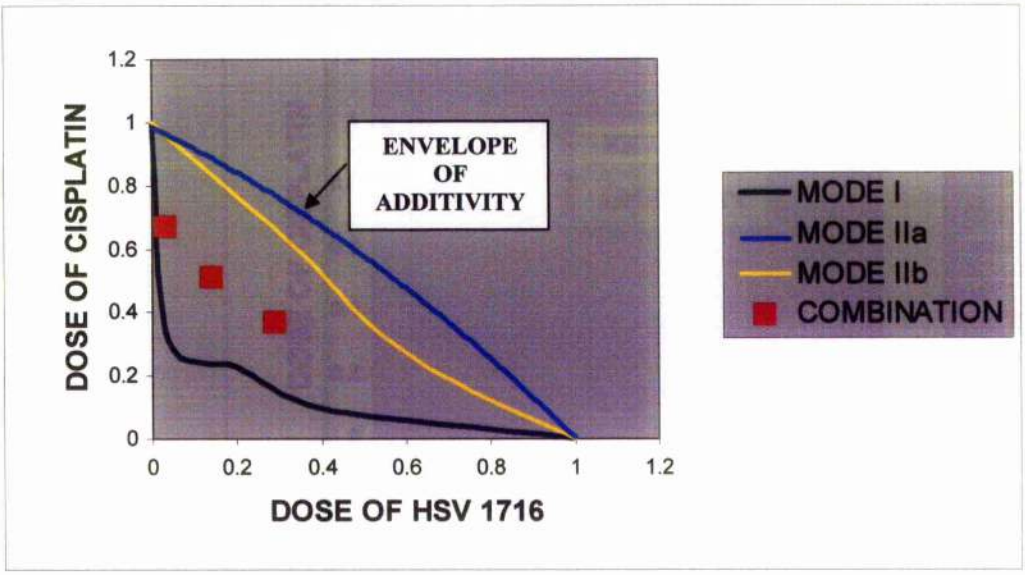


Figure 28: IC50 ISOBOLOGRAM: UM-SCC-22B WITH HSV1716 AND CISPLATIN

The outermost boundaries of the 3 isoeffect curves form an envelope of additivity. IC50 combination data are plotted and fall within the envelope of additivity. For convenience the IC50 for each agent in isolation is plotted as 1.0 on the isobologram, therefore no units are required.



In summary, we have performed the first studies of the cytotoxic effects of HSV1716 against HNSCC. Three HNSCC cell lines were used. The growth kinetics of HSV1716 in HNSCC cell lines were studied, and each cell line was found to be permissive for replication of HSV1716. HSV1716 was found to be cytotoxic to HNSCC, and dose response curves were produced for each cell line. IC50 values were calculated for each cell line. Cisplatin was found not to be toxic to HSV1716. Cisplatin cytotoxicity studies produced IC50 values showing trends and error margins comparable to existing published results. Cisplatin was not found to be toxic to HSV1716, making them suitable agents for combination cytotoxicity studies. The cytotoxic effects of HSV1716 and cisplatin do not follow first order kinetics and so isobologram analyses of the data from combination studies were performed. Additivity was observed when HSV1716 was combined with cisplatin in all three HNSCC cell lines. Having shown HSV1716 alone and combined with cisplatin to be efficacious in HNSCC, we were able to proceed with a phase I toxicity clinical trial.

4.2 PHASE I STUDY OF PREOPERATIVE INTRATUMOURAL INJECTION OF HSV1716 IN ORAL SQUAMOUS CELL CARCINOMA

4.2.1 PATIENT CHARACTERISTICS

A full target population of 20 patients was recruited. The baseline characteristics of the patients treated are shown in Table 14. 15 patients were male and 5 were female. The mean age was 61 years, and all patients had a Karnofsky performance score equal to or above 70%. All patients had a positive history of heavy smoking. 2 patients had a previous history of HNSCC. One of these presented with a metachronous primary malignancy and the other with recurrent disease. 15 patients presented with advanced stage III or IV disease. 18 patients were HSV-1 seropositive at presentation, and 2 were seronegative. Table 15 show the treatment programme for each patient. The screening immunology blood tests from each patient are shown in Table 16, along with the TNM stage of each tumour. Patients B5, C3 and D4 were all notably immunosuppressed. Each of these patients presented with advanced stage III and IV disease, with pT4N0, pT3N0 and pT3N2 tumours respectively.

4.2.2 TOXICITY AND SAFETY

Having met the eligibility criteria, all patients were allotted to one of four groups. Following fully informed consent they received an intratumoural and normal buccal mucosal injections of HSV1716, according to the trial protocol (Table 8). All 20 patients were assessed for signs of toxicity using the National Cancer Institute Toxicity Criteria, Version 2.

Preoperative intratumoural injection of HSV1716 was tolerated well in all 20 patients. All tumours were accessible for viral injection on the ward under local anaesthetic spray (lignocaine). Some common sites for oral squamous cell carcinoma are shown in Figures 29 a, b, c. The most difficult site to access was the

AGE (YEARS)	MEDIAN	61
	RANGE	44-75
SEX	MALE	15
	FEMALE	5
KARNOFSKY PERFORMANCE STATUS	90%	13
	80%	6
	70%	1
HISTORY OF SMOKING	POSITIVE	20
	NEGATIVE	0
HISTORY OF PREVOIUS HNSCC	POSITIVE	2
	NEGATIVE	18
TUMOUR STAGING	pT1	2
	pT2	10
	pT3	3
	pT4	5
NECK STAGING	pN0	7
	pN1	4
	pN2	8
	pN3	1
PRE-INJECTION IMMUNE STATUS	HSV SEROPOSITIVE	18
	HSV SERONEGATIVE	2

Table 14: Baseline patient characteristics

Patient	Age (years)	pTNM	HSV1716 injection to tumour resection time	Surgical treatment			Oncological treatment
				Tumour	Neck	Reconstruction	
A1	75	T4N2	72 hours	Resection	MRND	Forearm radial free flap	Radiotherapy
A2	72	T2N0	72 hours	Resection	MRND	Forearm radial free flap	Radiotherapy
A3	55	T1N0	72 hours	Resection	-	Mucosal transposition flap	-
A4	66	T2N0	72 hours	Resection	MRND	Forearm radial free flap	Radiotherapy
A5	46	T2N2	72 hours	Resection	MRND	Deep circumflex iliac free flap	Chemoradiotherapy
B1	61	T2N0	72 hours	Resection	MRND	Forearm radial free flap	Radiotherapy
B2	57	T4N2	72 hours	Resection	MRND	Anterolateral thigh flap	Radiotherapy
B3	59	T4N3	72 hours	Resection	Bilateral ND	Forearm radial free flap	Chemoradiotherapy
B4	74	T3N2	72 hours	Resection	MRND	Forearm radial free flap	Radiotherapy
B5	68	T4N0	72 hours	Resection	-	Deep circumflex iliac free flap	-
C1	62	T2N1	24 hours	Resection	MRND	Forearm radial free flap	-
C2	57	T2N0	24 hours	Resection	MRND	Forearm radial free flap	Radiotherapy
C3	74	T3N0	24 hours	Resection	MRND	Forearm radial free flap	Radiotherapy
C4	75	T2N1	24 hours	Resection	MRND	Forearm radial free flap	Radiotherapy
C5	44	T1N0	24 hours	Resection	-	Split skin graft	-
D1	56	T2N2	14 days	Resection	MRND	Forearm radial free flap	-
D2	53	T2N2	14 days	Resection	MRND	Forearm radial free flap	Radiotherapy
D3	59	T2N2	14 days	Resection	MRND	Anterolateral thigh free flap	Radiotherapy
D4	47	T3N2	14 days	Resection	MRND	Forearm radial free flap	Chemoradiotherapy
D5	68	T2N0	14 days	Resection	MRND	Forearm radial free flap	Radiotherapy

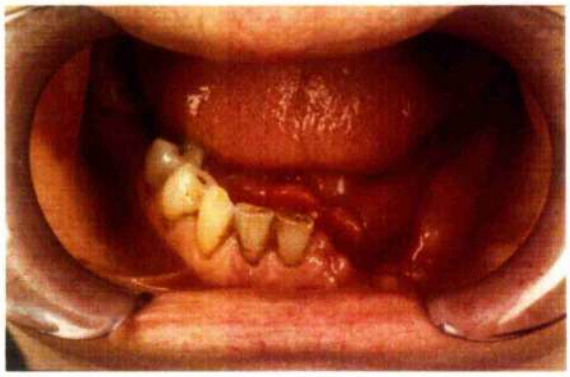
Table 15: Patient treatment programme

(MRND: Modified radical neck dissection)

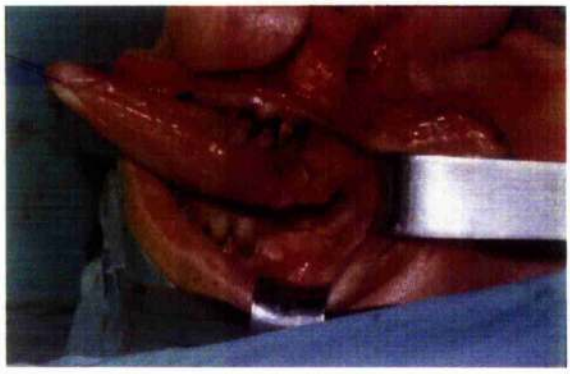
	Complement		Immunoglobulins						
Patient	C3 *(NR 0.88-1.82g/l)*	C4 *(NR 0.19-0.45g/l)*	IgG *(NR 5.3-16.5g/l)*	IgA *(NR 0.8-4g/l)*	IgM *(NR 0.5-2g/l)*	Absolute number of lymphocytes /cmm	**Number and proportion of major (T, B, NK) lymphoid cell populations**	**Number and proportion of major (CD4 T-helper, CD8 T-cytotoxic) lymphoid cell populations**	**pTNM**
A1	1.78	0.25	12.8	3.44	0.58	2222	Normal	Normal	T4N2
A2	1.43	0.28	9.53	1.9	0.75	2888	Normal	Normal	T2N0
A3	0.93	**0.15**	11.1	1.55	1.09	2287	Normal	Normal	T1N0
A4	0.96	0.23	9.51	1.91	0.73	2330	Normal	Normal	T2N0
A5	1.75	0.21	9.48	2.29	1.05	1766	Normal	Normal	T2N2
B1	1.38	**0.14**	7.8	4.01	0.95	2833	Normal	Normal	T2N0
B2	0.96	0.28	14.2	2.3	1.2	1817	Normal	Normal	T4N2
B3	1.75	0.24	8.82	2.41	0.95	1504	Normal	Normal	T4N3
B4	1.38	0.28	6.32	2.12	0.77	3218	Normal	Normal	T3N2
B5	1.59	0.33	10.8	1.49	1.55	471	**Lymphopaenic with low T, B, NK levels**	Normal	T4N0
C1	1.61	0.23	12.1	1.7	0.8	1636	Normal	Normal	T2N1
C2	1.01	0.28	**17.2**	3.83	0.77	1986	Normal	Normal	T2N0
C3	0.99	**0.18**	10.6	2.5	1.55	1262	**Normal T cell levels but low B and NK levels**	Normal	T3N0
C4	1.01	0.26	**20.2**	7.4	0.8	1795	Normal	Normal	T2N1
C5	1.36	0.35	12.4	**6.95**	0.57	2239	Normal	Normal	T1N0
D1	**1.87**	0.17	10.6	1.84	**2.81**	1253	Normal	Normal	T2N2
D2	1.25	0.23	11.4	3.65	0.37	2056	Normal	Normal	T2N2
D3	1.01	0.19	11.5	2.86	0.3	1603	Normal	Normal	T2N2
D4	0.83	0.23	16.4	3.21	1.54	2215	**Low T cell levels but normal B and NK levels**	Normal	T3N2
D5	1.13	0.19	14.7	2.41	0.51	1463	Normal	Normal	T2N0

Table 16: Immunology blood tests

a. Floor of mouth



b. Lateral border of tongue



c. Buccal mucosa

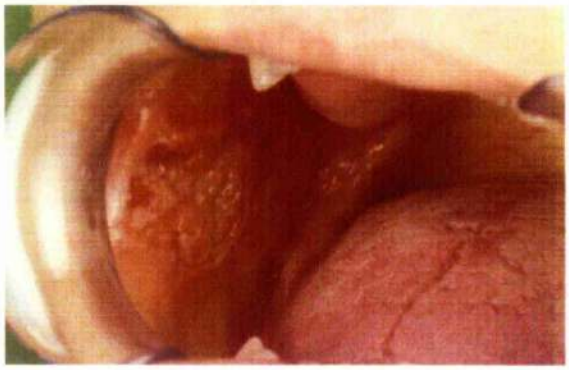


Figure 29 a, b, c: Examples of common sites for oral squamous cell carcinoma. All sites were easily accessible for preoperative intratumoural injection.

posterolateral tongue region, extending into the retromolar trigone. Some of these patients had trismus, due to tumour invasion and fixation, which made access more difficult. With the aids of a good light source and adequate tongue retraction, no significant problems were encountered. The lignocaine spray gave adequate local anaesthesia. 14 patients experienced discomfort during viral injection, but this settled in less than 5 minutes, and was no worse than the discomfort of venesection. The other 6 patients did not complain of any discomfort at all.

All patients remained stable during the 24 hour period of observation post injection. There was no change in the baseline observations for all patients. In particular there were no pyrexias, tachycardias or fluctuations in blood pressure. No patients developed any symptoms or signs of toxicity. All patients were examined 24 hours post injection. There was no notable change in the tumour or tattooed normal buccal mucosa at this time point in all patients. In particular there were no signs of change in the appearance of the tumour and no new inflammation or ulceration.

All patients progressed to tumour resection according to the trial protocol. 18 patients required a free flap reconstruction following tumour resection. 2 patients had mucosal transposition flaps. Figures 30-33 show the progression of a patient with a recurrent T4 fungating oral SCC, from presentation to tumour resection and mandibular reconstruction. 11 patients received postoperative radiotherapy, and this was declined in 1 patient. 3 patients received postoperative adjuvant chemoradiotherapy. No patients developed any symptoms or signs of potential HSV1716 related toxicity in the interval between initial injection and resection, or during the post resection period. All patients were reviewed regularly during the first

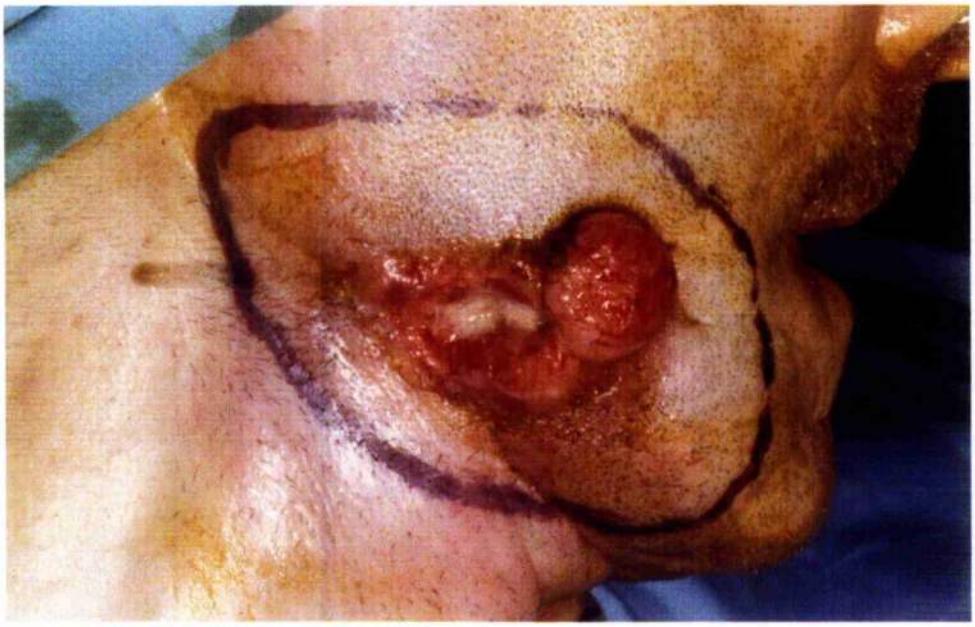


Figure 30: Fungating T4 oral cavity squamous cell carcinoma

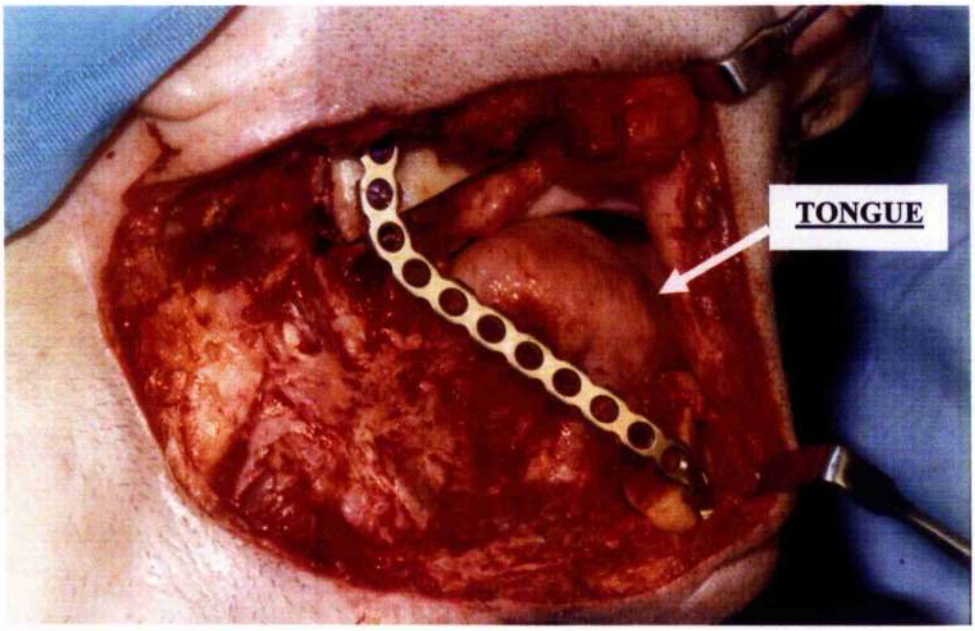


Figure 31: Titanium plate bridging the defect following tumour resection and segmental mandibulectomy

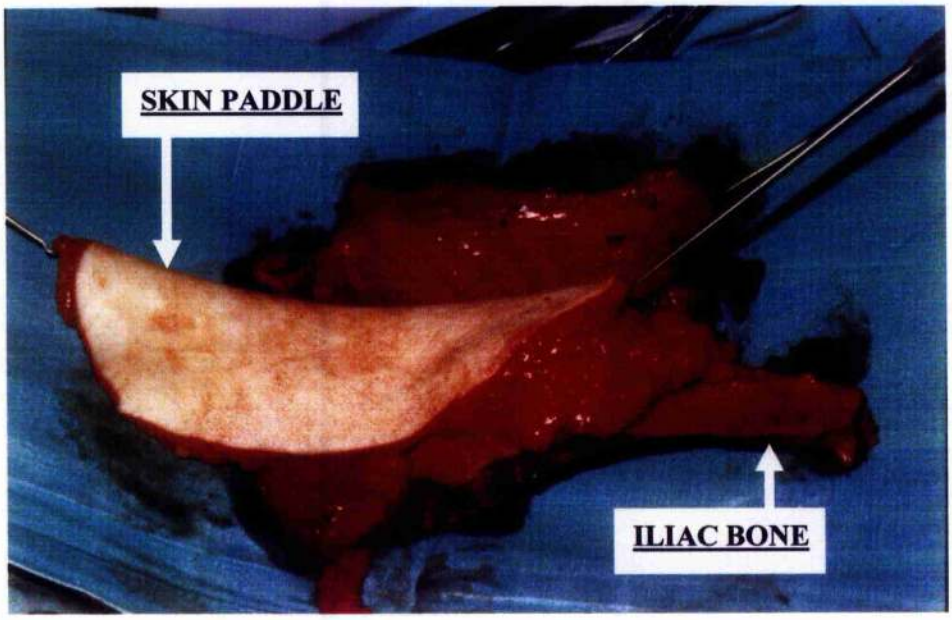


Figure 32: Osseo-myo-cutaneous DCIA flap used for mandibular reconstruction

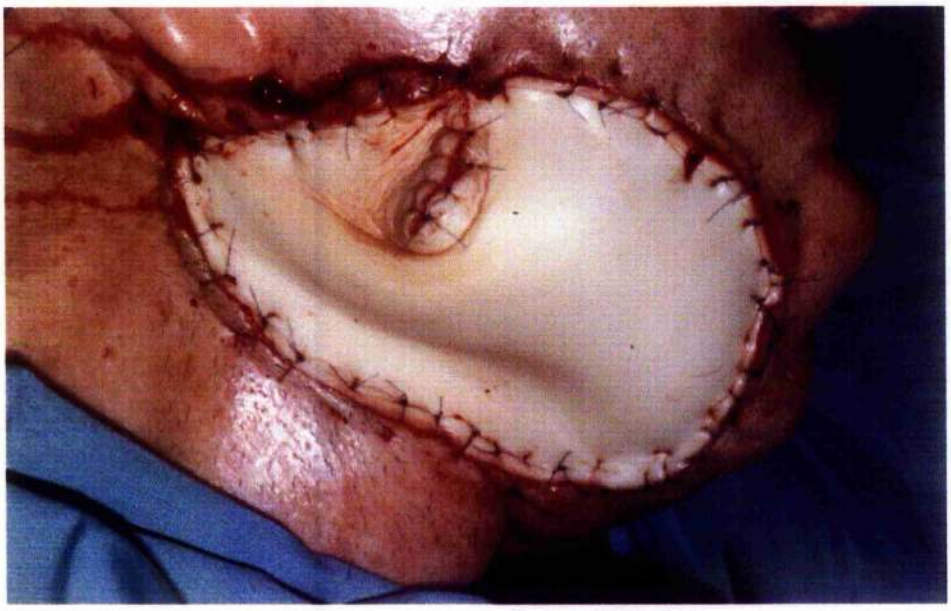


Figure 33: Post reconstruction and closure

8 weeks, and all patients with HNSCC are routinely followed up regularly for 5 years following the completion of treatment.

3 patients are known to have died following treatment. 1 patient died due to comorbitiy with cardiorespiratory disease. 1 patient died due to disease progression and another due to the development and progression of a second primary pulmonary malignancy.

4.2.3 HUMORAL IMMUNE RESPONSE

All patients had blood serum samples collected prior to virus injection, and at intervals up to 6 weeks post injection. Using the ELISA spectrophotometer, with control samples, 18 of the 20 patients treated with HSV1716 had pre-existing neutralising antibodies to HSV-1. None of these had any changes in their IgM or IgG levels post-injection. The 2 patients who were seronegative for HSV-1 pre-injection both seroconverted at 4 weeks and 5 weeks post-injection respectively, indicating an immune response.

4.2.4 BLOOD AND TISSUE ANALYSES FOR HSV1716

A) PCR FOR HSV DNA

DNA was isolated from all blood plasma samples collected and PCR analysis was carried out using primers, HS13 and HS14, specific for the UL42 locus of HSV-1 (Puchhammer-Stockl et al 1990). 7 blood samples showed evidence of HSV DNA as shown in Table 17. The blood samples were taken between 24 hours and 3 weeks post HSV1716 injection. All the samples were from patients who had received the lower dose of 10^5 pfu of HSV1716.

A typical positive PCR reaction for HSV is shown in Figure 34. Samples C, D, E and F show a positive 278 base pair band for HSV.

Resected tumour was also analysed for HSV DNA, which was detected at the injection site in 2 patients. Both these patients were injected with the higher dose of $5x10^5$ pfu of HSV1716, one at 24 hours pre-resection and the other at 72 hours pre-resection. In the latter patient, HSV DNA was detected at a distal site, furthest from the injection site. The tumour is shown in Figure 29 c. Both patients also had HSV DNA detected in the normal buccal mucosa. One other patient had HSV DNA detected in the normal buccal mucosa specimen, having been injected with 10^5 pfu of HSV1716. No tissue samples were positive for HSV DNA in the group injected 2 weeks pre-resection.

PATIENT	SAMPLE
A1	24 HOURS POST INJECTION
A2	3 WEEKS POST INJECTION
A3	1 WEEK POST INJECTION
A4	1 WEEKS POST INJECTION
A4	3 WEEKS POST INJECTION
A5	24 HOURS POST INJECTION
A5	1 WEEK POST INJECTION

Table 17. Positive blood samples for HSV DNA, analysed by PCR.

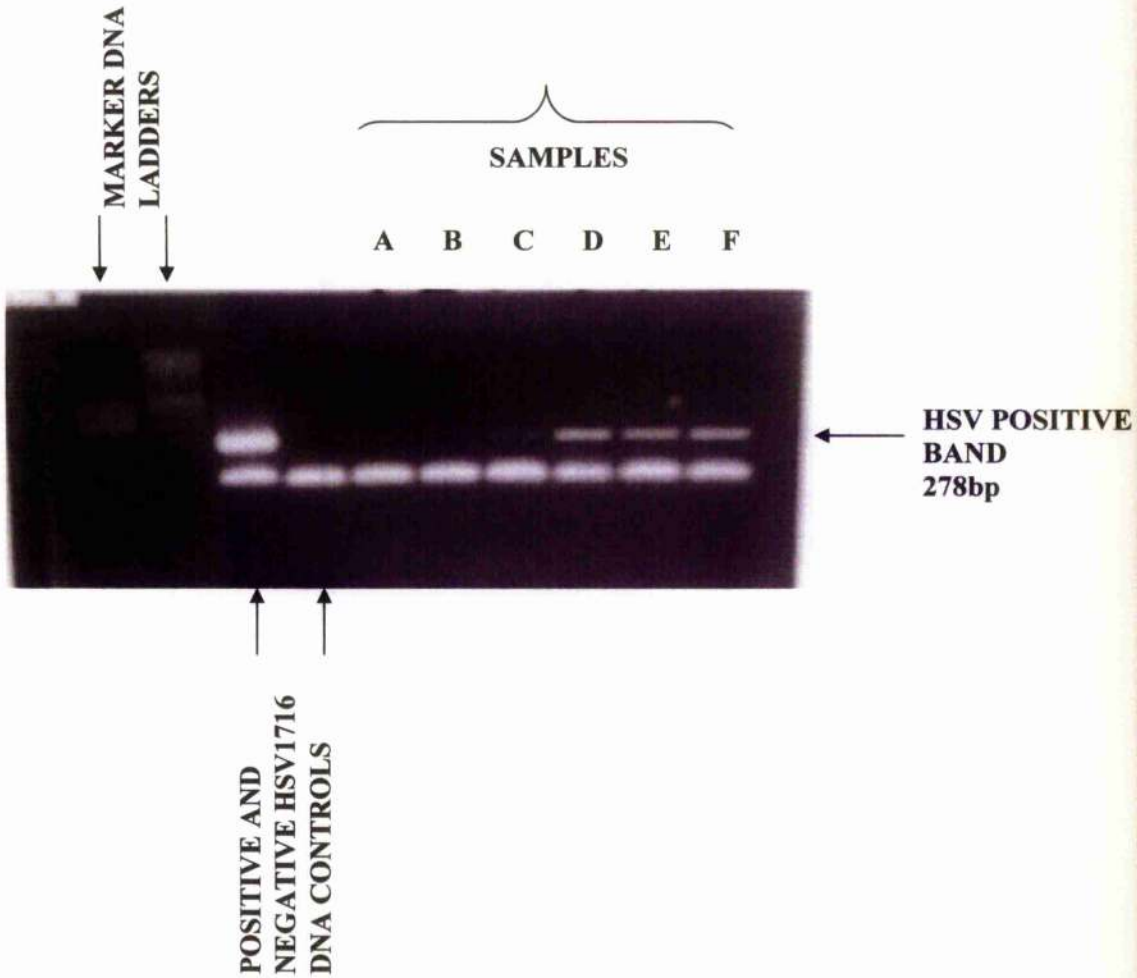


Figure 34. HSV analysis of blood DNA by PCR

Samples:	**A**	***Patient A1 3 weeks post injection***
	B	***Patient A2 1 week post injection***
	C	***Patient A3 1 week post injection***
	D	***Patient A4 1 week post injection***
	E	***Patient A4 3 week post injection***
	F	***Patient A5 1 week post injection***

B) H&E EXAMINATION AND IMMUNOHISTOCHEMISTRY FOR HSV ANTIGEN

Tumour specimens were processed in the pathology department at Glasgow Royal infirmary. The specimens were either fixed in formalin immediately following resection, or larger specimens were pinned out, labelled and sent fresh to enable better gross examination by the pathologist. The specimens were then processed and infiltrated with paraffin, as an embedding agent. Planes for sectioning were then chosen to produce slides for H&E examination, immunohistochemistry and in-situ hybridisation. 5μm sections were cut using a microtome. Figure 35 demonstrates perpendicular planes through a tumour and injection site. In larger tumours, planes

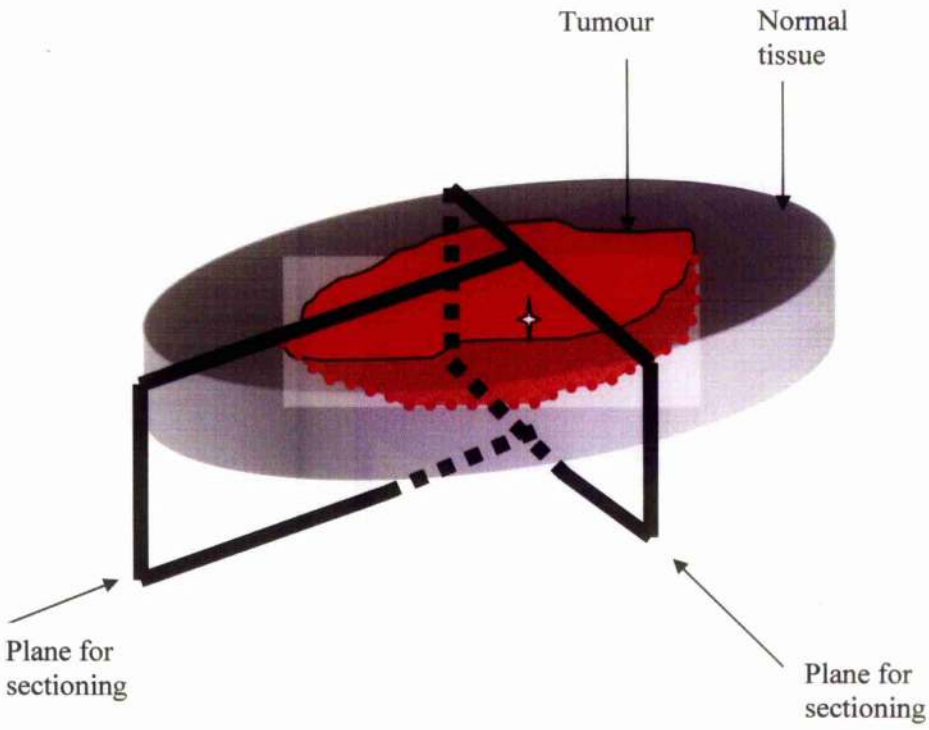


Figure 35: Schematic diagram demonstrating perpendicular planes for sectioning through a tumour and injection site, marked ✦

were also chosen through sections of tumour distal to the injection site. All tissue processing was performed by experienced technicians to the high standards required for histopathology reporting. All planes for sectioning of tumour and normal buccal mucosa were chosen by Mr. Alastair Mace and Professor MacDonald, Consultant Head and Neck Pathologist, who both analysed the final H&E, immunohistochemistry slides and *in-situ* hybridisation slides.

H&E slides of all tumour sections were analysed and no unexpected inflammation or necrosis was noted in any sections. Histology in Figures 36a and 36b confirms SCC arising from severely dysplastic surface epithelium. The tumour is moderately well differentiated with a cohesive invasive front. There is no perineural or vascular invasion.

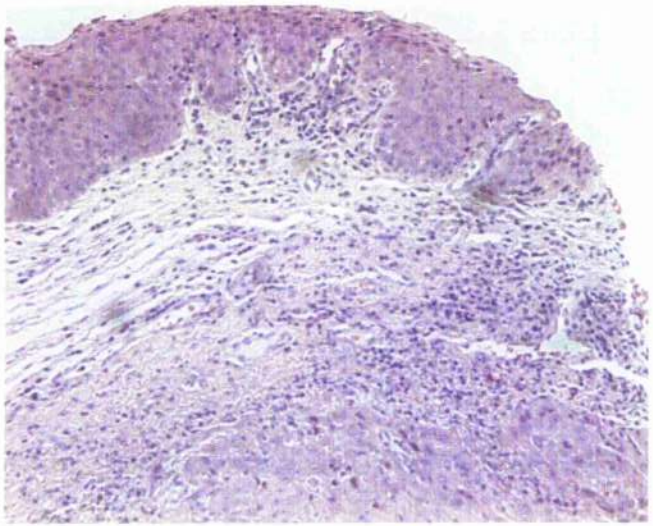
A consistent finding in the injected normal buccal mucosa sections was inflammatory lymphocyte infiltrations, and there was no evidence of necrosis. Figure 37 shows an H&E section from normal buccal mucosa injected with HSV1716. Histology confirms lightly keratinised epithelium with no dysplasia. Mild non specific inflammatory changes are present in the lamina propria.

Tumour tissue and normal buccal mucosa sections were analysed by immunohistochemistry using a polyclonal antibody to HSV-1 (DAKO). All immunohistochemistry was performed in the West of Scotland Regional Referral Unit at Glasgow Royal Infirmary. HSV specific antigen was not detected by the antibody in all tumour sections and normal buccal mucosa sections (Figure 38a). The positive and negative control slides stained appropriately each time (Figure 38b).

C) *IN SITU* HYBRIDISATION FOR HSV ANTIGEN

Tumour tissue and normal buccal mucosa specimens were analysed by *in situ* hybridisation using a digoxigenin labelled HSV DNA probe. All 5μm sections of

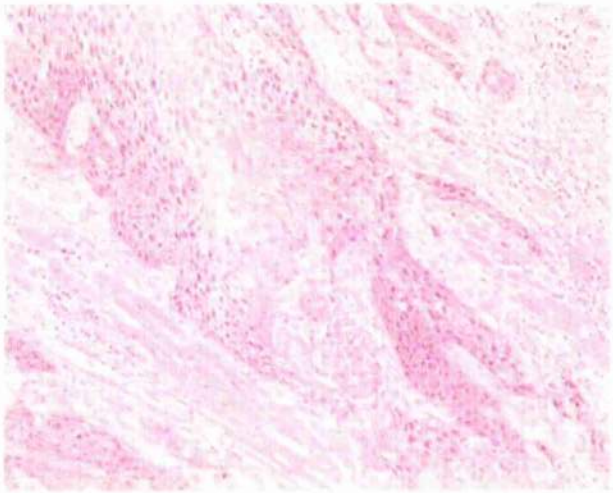
a.



Figures 36 a and b

H&E. Squamous cell carcinoma. Histology confirms SCC arising from severely dysplastic surface epithelium. The tumour is moderately well differentiated with a cohesive invasive front. There is no perineural or vascular invasion.

b.



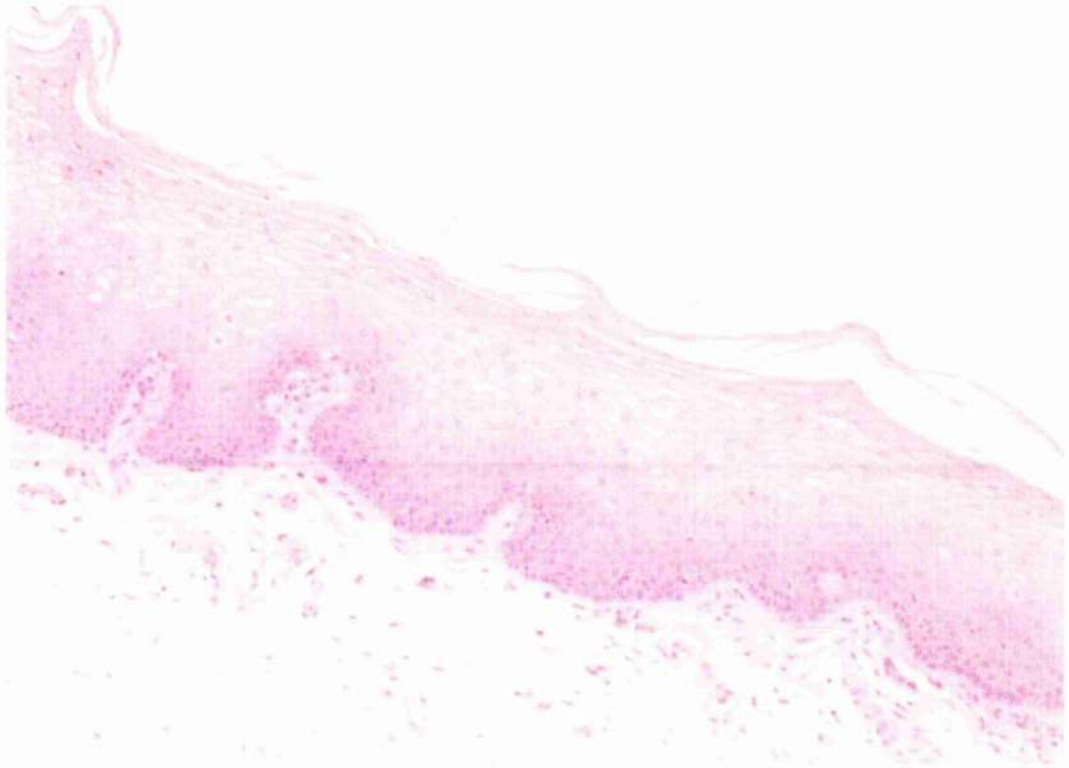


Figure 37: H&E Normal buccal mucosa injected with HSV1716

Histology confirms lightly keratinised epithelium with no dysplasia. Mild non specific inflammatory changes with lymphocyte infiltration into the lamina propria.

Figure 38a

HSV immunohistochemistry. SCC

(A polyclonal antibody to HSV-1 (DAKO) was used. There is no positive staining

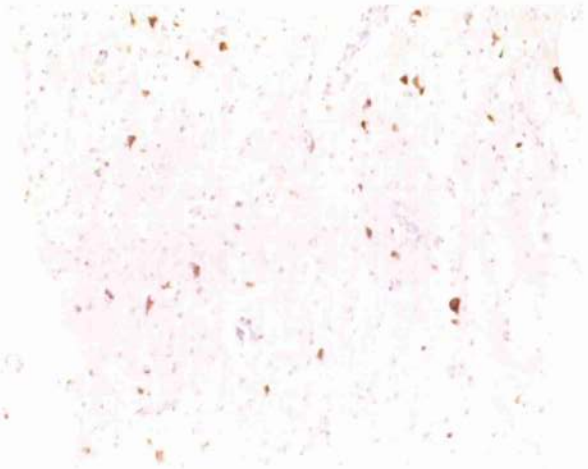


Figure 37b

HSV immunohistochemistry positive control.

Dark positive staining neurones in section of encephalitic brain.

buccal mucosa and tumour were analysed at the injection sites. Positive staining of HSV DNA was not detected in tumour tissue or normal buccal mucosa (Figure 39a). The positive and negative control slides stained appropriately each time (Figure 39b).

D) DETECTION OF INFECTIOUS HSV1716

Tissue samples of normal buccal mucosa and biopsies from the injection sites and distal sites in larger tumours were homogenised and processed to allow detection of infectious HSV1716. Blood sera from every blood sample taken from all patient samples were also processed to allow detection of infectious HSV1716. No infectious virus was recovered from all blood samples and tissue biopsies.

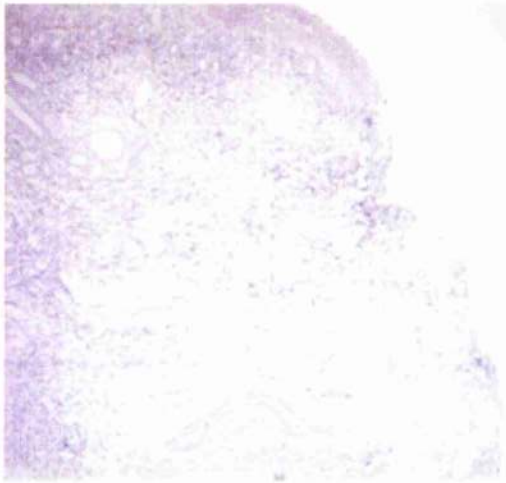


Figure 39a

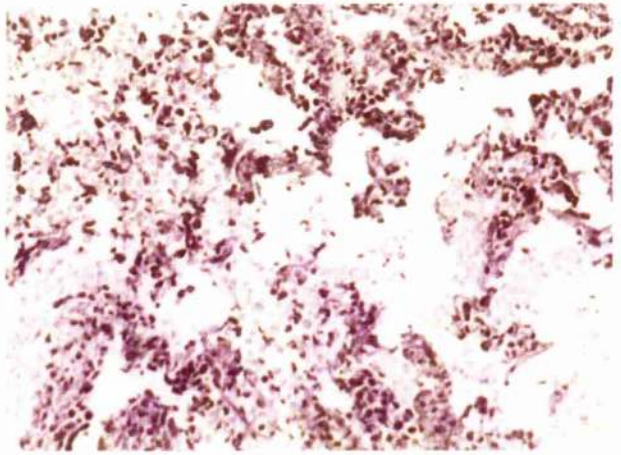
HSV *in situ* hybridisation SCC.

A digoxigenin labelled HSV DNA probe was used. Ther e is no positive staining.

Figure 39b

HSV *in situ* hybridisation positive control.

Dark positive staining is present



CHAPTER 5

DISCUSSION

5.1 RATIONALE

HSV1716 has been characterised as a selectively replication competent virus and a potential novel cancer therapy. It has been shown to induce tumour regression in models of a number of tumour types. Improved survival was seen following intratumoural injection of medulloblastoma tumours in a murine model (Lasner et al, 1996). Significant tumour regression and improved survival was seen following HSV1716 intratumoural injection of melanoma tumour deposits grown in the brains of immunocompetent C57B mice (Randazzo et al, 1995). Similar findings were seen when intracerebral deposits of human embryonal carcinoma cells were injected with HSV1716 (Kesari et al, 1995). Effective oncolysis of malignant mesothelioma, with tumour regression and improved survival, was seen when intraperitoneal deposits were injected with HSV1716 (Kucharczuk et al, 1997). A murine model involving subcutaneous human melanoma was also successful in showing selective replication and efficient oncolysis (Randazzo et al, 1997). Immunohistochemistry confirmed the viral replication to be restricted to the tumour cells.

In a clinical setting, a safety profile was initially established in a phase I trial involving the intratumoural injection of nine immunocompromised patients with glioblastoma, in doses up to 10^5 pfu (Rampling et al, 2000). There was no evidence of toxicity. Immunohistochemical analysis showed no significant immunoreactivity of tumour cells or adjacent brain tissue for HSV-1 using monoclonal antibodies. There was no evidence of HSV1716 or wild type genomes by PCR. Tissue cultures were negative. This trial was followed by a proof of principle study (Papanastassiou et al, 2002). Twelve patients with glioma tumours had their intratumoural injections four to

nine days post inoculation with HSV1716 at a dose of 10^5 pfu. Again there was no toxicity and in 2 patients infectious HSV1716 was recovered from the injection site at titres in excess of the input dose. The same two patients were seronegative for HSV-1 pre-injection. They both seroconverted and initial changes in specific IgG were detected at 19-21 days post-injection. It was only in these patients that immunohistochemistry analysis of tumour tissue for HSV antigen was positive from two patients. HSV DNA was detected by PCR at the sites of inoculation in 10 patients and at distal tumour sites in four. A tumour biopsy taken at post mortem from one of the patients, 251 days post-injection, was PCR positive for HSV DNA. One further phase I study was conducted involving patients with high-grade glioma (Harrow et al, 2004). Twelve patients received injections of HSV1716, at a dose of 10^5 pfu into the brain adjacent to excised tumour. There was no toxicity and three patients notably survived significantly longer than expected from a disease with a median survival of approximately nine months.

A phase I trial involving melanoma patients had also been conducted (MacKie et al, 2001). Five patients received intratumoural injections of HSV1716 into subcutaneous nodules of metastatic melanoma. There was no toxicity. In one patient, flattening of previously palpable tumour nodules was seen 21 days after two direct injections of HSV1716. In all three patients who received two or more injections there was microscopic evidence of tumour necrosis. Immunohistochemistry analysis showed evidence of viral replication within the limits of the tumour mass. There was no antigen staining in the adjacent normal connective tissue and the basal layer of the

epidermis. This latter result suggested that the normal melanocytes were antigen-free. There was no evidence of infectious virus in any of the samples.

The objective of this thesis was to demonstrate potential and safety of HSV1716 as a head and neck cancer therapy. HSV1716 has not been previously studied in HNSCC. We aimed to determine whether HSV1716 would replicate in and kill HNSCC cell lines. Also to determine if the cytotoxic effects *in vitro* were enhanced when combined with the conventional chemotherapeutic agent cisplatin. In a clinical setting we aimed to establish a safety profile for HSV1716 in HNSCC.

In HNSCC, there is a great need for novel therapies, in particular to treat locoregional recurrent disease. Current therapies for this disease, including re-irradiation and chemotherapy, have poor response rates and short progression free intervals. The disease and these treatments engender significant morbidity, including dysarthria, dysphagia and pain. A locoregional therapy for this disease, such as intratumoural injection of HSV1716, would be an attractive treatment option. Combining therapies has the potential for enhanced efficacy, and reduced dose requirements and adverse reactions of conventional chemotherapy. As the effects of HSV1716 on HNSCC have not been studied before, we needed to establish if these cells were permissive for replication of the virus. We were then able to determine if HSV1716 was cytotoxic to HNSCC, and whether any cytotoxicity is enhanced when combined with the conventional chemotherapy agent cisplatin *in vitro*. For this therapy to be useful in head and neck cancer it was necessary to investigate whether direct intratumoural injection of HSV1716 is tolerated well and is safe. We conducted a Phase I study involving 20 patients with oral squamous cell carcinoma to evaluate this.

5.2 LABORATORY STUDIES: HNSCC AND HSV1716

In vitro experiments using other replicating herpes simplex viruses, in head and neck cancer, have already been reported. G207 is a replication competent oncolytic virus and laboratory studies have demonstrated cytotoxicity against HNSCC. It is derived from HSV-1 strain F. It has multiple mutations including deletions at both loci of the ICP34.5 gene and insertion of a LacZ reporter gene into the ICP 6 gene (Mineta et al, 1995). The ICP6 gene encodes the large subunit of HSV ribonucleotide reductase, and loss of its expression decreases the ability of G207 virus to proliferate in nondividing cells. The specificity for tumour cell lysis is increased. The multiple mutations of G207 are thought to minimise the chance of reversion to wild-type virus. *In vitro* and *in vivo* studies showed G207 to infect and cause efficient lysis of HNSCC (Carew et al, 1999; Chahlavi et al, 1999). Carew et al, 1999, used human SCC cell lines and trypan blue to assess cell viability. Cell death progressively increased with increasing MOIs. Animal studies involved direct intratumoural injections into murine flank tumours as well as selective intra-arterial perfusion of oral cavity tumours. All experiments showed inhibition of tumour growth.

NV1020 is another multi-mutated HSV-1 oncolytic virus, which has shown cytotoxicity to HNSCC *in vitro* and *in vivo*. It has deletions of one copy of ICP 34.5 and the virulence-associated virion gene, UL 56. It also has a deletion in the thymidine kinase locus. As NV1020 was originally designed as a potential HSV vaccine, a fragment of HSV-2 DNA is inserted into the UL/S junction. This junction also contains an exogenous copy of the HSV-1 thymidine kinase gene under control of the strong ICP 4 promoter, in addition to a duplication of the UL5/6 sequences. *In*

vitro studies showed NV1020 to be highly cytotoxic to five human HNSCC lines (Wong et al, 2001). Tumour regression was seen following injections into flank tumours in a murine model. Biopsies from tumours identified areas of necrosis 24 hours post-injection. There have been no clinical trials involving G207 or NV1020 and head and neck cancer patients.

Using HSV1716 we found all 3 cell lines were permissive to infection with variable sensitivity and variable cytotoxic effects. It therefore seems likely that this virus may be efficacious by intratumoural injection, though *in vivo* experiments are required to validate this. The use of oncolytic viruses in isolation may not necessarily produce an efficacious effect comparable to, or better than currently available therapies. Combining agents with different mechanisms of cytotoxicity is an attractive approach and gives a number of potential advantages. It gives potential for using lower doses, maximising the therapeutic effect and minimising side effects. Combining therapies also gives potential for enhanced effects, through different mechanisms of cytotoxicity. These interactions can be antagonistic, additive or synergistic.

This approach has already been reported by Toyoizumi et al, 1999. They combined HSV1716 with cytotoxic agents but in a different tumour type. In-vitro studies in non-small cell lung cancer showed, with isobologram analysis, synergistic effects between HSV1716 and mitomycin C (MMC). The synergistic dose of MMC neither augmented nor inhibited viral replication *in vitro*. The mechanisms of action involved in this synergy are unclear. The combination of MMC with HSV1716 yielded an additive effect in a murine xenograft model. It was reasoned that the difference between the *in vitro* and *in vivo* results, was related to the duration of MMC cell

contact. Additive effects were found *in vitro* combining HSV1716 with *cis*-platinum II, methotrexate or doxorubicin.

In vivo cytotoxicity studies involving oncolytic HSV-1 mutant G207 combined with cisplatin showed additive tumouricidal effects in one of two established human HNSCC murine flank tumours (Chahlavi et al, 1999). No increased toxicity was noticed. There was no enhanced kill in the other tumour model, and it was noted that the cell type involved was less sensitive to cisplatin. The G207 and cisplatin appeared to be acting independently. G207 and HSV1716 undergo extensive DNA replication and transcription as they replicate in and kill tumour cells. These processes could be blocked by cisplatin, so it was necessary to determine whether cisplatin was toxic to the virus. Cisplatin blocks DNA replication by producing damaging intrastrand DNA cross-links (Pinto et al, 1985; Heiger-Bernays WJ et al, 1990). Cisplatin was not found to be toxic to G207, corresponding with our results for HSV1716. We did not use any cell lines which are poorly sensitive to cisplatin. This may have given us information regarding any dependency between HSV1716 and cisplatin.

Heise et al, 1997, investigated the cytotoxic effects of the mutated adenovirus ONYX-015, combined with cisplatin and 5-fluorouracil (5FU) in a HNSCC tumour xenograft. Cisplatin and 5FU were found to be more effective than chemotherapy or virus treatment alone. The median survival was significantly increased with combination therapy, compared with chemotherapy alone.

Khuri et al, 2000, combined ONYX-015 with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. In this study, enhanced efficacy was found with the combination of agents compared to responses reported in the literature with

cisplatin and 5-fluorouracil alone. Replicating herpes simplex viruses may have potential advantages over adenoviruses due to their more potent replication capacity producing a greater cytopathic effect. We therefore wanted to evaluate the effect of combining HSV1716 with cisplatin in the 3 head and neck cell lines studied. These 3 cell lines have already been studied for cisplatin sensitivity as reported by Welters et al, 1997. In our study we were able to produce similar results demonstrating that cell line 22A was the most sensitive and 14C the least sensitive. When we evaluated the effect of combining HSV1716 with cisplatin we found additive cytotoxicity using isobologram analysis. Further *in vivo* studies are warranted to evaluate the effects of these combined agents in nude mouse HNSCC xenografts. If this is observed then the potential exists for utilising these agents in patients with locally advanced or locoregional recurrent head and neck cancer in a phase I trial.

We have shown that HNSCC is a potential target for the selectively replicating herpes simplex virus HSV1716. More importantly we have shown that combining this agent with cisplatin has additive cytotoxicity in this cell type. This combination of agents may therefore be potentially useful as a novel therapy in patients with locally advanced or locoregional recurrent head and neck cancer. Since HSV1716 has already been used in clinical trials in glioma and melanoma with no serious toxicity and with evidence of efficacy, our *in vitro* experiments in HNSCC enabled us to conduct a phase I trial with this agent in HNSCC by intratumoral injection. We aimed to determine if HSV1716 could be injected easily, comfortably and safely in patients with oral squamous cell carcinoma

5.3 PHASE I STUDY OF PREOPERATIVE INTRATUMOURAL INJECTION OF HSV1716 IN ORAL SQUAMOUS CELL CARCINOMA

HSV1716 has not been previously investigated in HNSCC. The primary objective of this Phase I study was to determine the safety of a single intratumoural injection of HSV1716 in patients with oral squamous cell carcinoma. The results obtained showed the therapy to be completely safe with no toxicity. The maximum dose injected was $5x10^5$ pfu. The procedure was technically uncomplicated and well tolerated under local anaesthetic. The lack of toxicity is encouraging and suggests higher doses could be used in future trials.

HSV1716 injection did not cause any clinically apparent tissue reaction. There were no signs of increased inflammation or ulceration of the injected tumour or buccal mucosa. H&E slides of sections of tissue at the tumour injection site did not identify any unexpected inflammation or necrosis. H&E slides of sections of tissue of injected normal buccal mucosa revealed lymphocyte infiltrations and no necrosis. HSV DNA was detected by PCR in injected normal mucosa in 3 patients up to 72 hours post-injection. There was no infectious virus recovered from any normal mucosa samples and no positivity in immunohistochemistry or *in situ* hybridisation analysis. Overall HSV1716 injection of normal mucosa appears to have consistently caused an inflammatory reaction, but no necrosis and we have found no evidence of viral replication. HSV1716 inoculation of normal tissue has not been investigated before. Nemunaitis et al, 2000, reported results following the inoculation of normal mucosa with the selectively replicating adenovirus ONYX-015. They identified the presence and/or replication of ONYX-015 in tumour tissue on days 5-14 post injection, but not

in adjacent normal mucosa. However caution is needed in interpreting these results as their injection protocol involved only direct intratumoural injections, up to the periphery of the tumours. There were no separate injections of normal mucosa, distant from the tumour, and no separate analyses of the normal mucosa.

The use of intratumoural injection of oncolytic viruses in HNSCC has already been reported using ONYX-015 (Ganly et al, 2000; Nemunaitis et al, 2000; Khuri et al, 2000). Replicating herpes simplex viruses may have potential advantages over adenoviruses due to their inherent greater replication capacity producing a more potent cytopathic effect. In the ONYX-015 studies far higher doses of virus were used, up to 10^{11} pfu , with no significant toxicity reported. A different injection protocol was used in these studies. At each injection the ONYX-015 was distributed evenly along at least 8 needle tracts radiating from a central penetration site. Also the virus was diluted to a volume estimated to be 30% of the tumour volume. Our approach with HSV1716 was to concentrate the virus in a small defined area of the tumour, and the virus was injected in a concentrated small volume. Biopsies were then taken at the time of tumour resection from the virus injection site and from distal sites, far away from the injection site. HSV DNA was detected by PCR in only one biopsy taken from a site distant from the injection site. There was no infectious HSV1716 recovered from the specimens and no positive identification of HSV in the immunohistochemistry or *in situ* hybridisation analyses. Overall the lack of evidence of HSV1716 in the tumours at resection, and the lack of evidence of antitumour effect is disappointing. Using a higher dose of HSV1716 and distributing the virus evenly throughout the tumours in a larger volume may be more beneficial. Ganly et al, 2000,

noted increased pain associated with higher injection volumes, but this could be aided by the use of local anaesthetic injections in addition to a spray. It is possible that there is significant leakage of virus from the needle penetration site, and using a higher dose of virus with a wider distribution would aid this. Further, it has been shown in an animal study in nude mice that the distribution of an oncolytic virus is more widespread with a multi-injection protocol. (Heise et al, 2000). Two clinical trials with ONYX-015 have used a multi-injection protocol in HNSCC. Both studies demonstrated selective intratumoural replication and necrosis in patients.

Biological activity in HNSCC following HSV1716 injection in humans is lacking. HSV DNA was only identified at the injection sites in 2 patients, 1 at 24 hours and 1 at 72 hours post-injection. There was only one potential sign of intratumoural HSV1716 replication, from HSV DNA identified in a tumour biopsy distant from the injection site. No conclusions can be drawn from this single result. Possibilities of a contaminated sample and the detection of wild type virus have to be considered.

Further studies need to investigate possible immune factors which could affect viral spread in HNSCC in humans. Evidence suggests the presence of humoral immunity is not a crucial factor. Two out of 20 patients injected with HSV1716 were seronegative for HSV antibodies prior to injection. Both seroconverted within 5 weeks of their injection, indicating an immune response. The seropositive patients did not have any changes in their HSV neutralising antibodies post injection. Ganly et al, 2000, noted all but one of the 22 patients treated to develop increased antibody levels, following a single injection protocol. The clinical studies with ONYX-015 have found no correlation between the adenovirus neutralising antibody titres before and after

treatment, and the measurable tumour response. Patients who received a multi-injection protocol were noted to have tumour responses despite as many as 5 cycles of prior viral therapy and very high antibody titres (Khuri et al, 2000; Nemunaitis et al, 2000). Following HSV1716 injection in oral SCC, the patient who had HSV DNA detected by PCR from a distal biopsy site was seropositive. This data corresponds to results from a clinical study involving the intratumoural injection of HSV1716 in patients with glioblastoma multiforme (Papanastassiou et al, 2002). HSV DNA was identified by PCR analysis of biopsies from distal tumour sites in 4 seropositive patients. Such a result cannot be extrapolated too far as the brain has a distinct immune system. However further evidence that HSV antibodies do not stop HSV1716 from replicating and killing tumour cells was noted in a clinical trial injecting HSV1716 into cutaneous melanoma nodules. 2 Injections into the same melanoma nodule in a seropositive patient resulted in tumour flattening (Mackie et al, 2001). This data supports the theory that antibody penetration into solid tumours is markedly reduced (Jain et al, 1994). Seropositivity and rising antibody levels seem to have no effect on repeat intratumoural administration, and would only become important in repeat systemic viral injection.

Further studies need to look at the roles of cell mediated immunity and local immunity following the intratumoural injection of HSV1716 in HNSCC. Cell mediated immunity (CMI) is mediated by cytotoxic T lymphocytes. It involves the activation of macrophages, the production of antigen-specific cytotoxic T-lymphocytes and the release of cytokines in response to an antigen. CMI is stimulated by HSV antigens produced in the host cell and presented along with major

histocompatibility complexes on the cell surface. This can cause early elimination of virus. McKie et al, 1998, studied the immune response induced following direct intracerebral inoculation of HSV1716 in immune naive mice. They identified a time-dependent immune response, consisting of a mild infiltration by macrophages and T-lymphocytes. This occurred mainly at the site of inoculation with a maximal response at approximately 7 days post-inoculation. The central nervous system is however an immunologically privileged site. It is able to exclude immune components of the immune system by the blood-brain barrier. Neural astrocytes and microglial cells play a major role in the generation of the intracerebral immune response (Fontana et al, 1987; Hart et al, 1995). These are important differences when considering the fate of HSV1716 injection into solid tumours such as HNSCC.

Investigations are needed into the interactions between HSV1716 replication in HNSCC cells and the immune system. A quantitative and qualitative analysis of helper T lymphocyte (CD4), cytotoxic T lymphocyte (CD8) and macrophage (CD68) infiltration following viral injection is indicated. A qualitative analysis is required as it is documented that T lymphocyte function is impaired in HNSCC (Pandit et al, 2000; Bailet et al, 1997). Incorporated into this should be a study of the role of cytokines and their impact on HSV1716 replication. Tumours secreting the cytokine interferon may neutralise viral spread. It is documented that T-cell derived gamma interferon (IFN-γ) can directly inhibit *in vivo* the replication of HSV-1, and may have a synergistic role with IFN-α and IFN-β (Sainz et al, 2002). Head and neck cancer cells secrete cytokines and it has been shown that cytokine levels and activities are increased by cisplatin in patients with HNSCC (Okamoto et al, 2000). This increase

in antitumour immunity could therefore have a negative effect on HSV1716 replication when contemplating combining HSV1716 and chemotherapy agents in humans. This needs further investigation.

5.4 FURTHER DEVLOPMENTS

In summary we have shown *in vitro* that the selectively replication competent oncolytic virus, HSV1716, is able to replicate in and kill HNSCC cell lines. Cytotoxicity studies have shown, following isobologram analysis, that there is additive enhanced cell kill when HSV1716 is combined with the conventional chemotherapy agent cisplatin. We do not know the relationship between the mechanisms of action of these 2 agents and this needs further investigation. They could act completely independently of each other. We have shown that cisplatin is not toxic to HSV1716. Further *in vivo* studies are warranted to evaluate the effects of these combined agents in nude mouse HNSCC xenografts. If this is observed then the potential exists for utilising these agents in patients with locally advanced or locoregional recurrent head and neck cancer in a phase I trial. Potentially higher doses of HSV1716 could be used, with an aim of injecting higher volumes and distributing the virus evenly throughout the tumour.

Following completion of a Phase I toxicity study we have shown that HSV1716 can be injected into patients with oral SCC readily, comfortably and safely. There was no toxicity in any of the 20 enrolled patients. Current therapies for recurrent head and neck cancer, such as tumour debulking, further irradiation and chemotherapy have all produced poor responses of limited duration, and significant morbidity. HSV1716 has the potential to complement and improve conventional therapies.

Overall these findings are ideal attributes for a replication selective oncolytic virus. However we found little evidence of biological activity in the tumour specimens analysed in the clinical trial. ONYX-015 also demonstrated less cell killing potential in a clinical setting than was expected from preclinical studies (Bischoff et al, 1996). It is known that although viruses rapidly spread in cell culture monolayers, viral spread within a solid tumour mass is often limited (Heise et al, 1999). The tumour matrix may present potential physical limitations to viral spread, including fibrosis, intermixed normal cells and necrotic regions. Less than 20% of the heterogeneous cells that make up oral tumours are in the S-phase of replication. This is reported to be an important factor limiting the replication of oncolytic HSV-1 (Shillitoe et al, 2005). It will be important to further evaluate approaches that improve viral spread within a tumour, as it is one of the most important factors for antitumour efficacy following intratumoural injection. Experiments could be performed to confirm the PCNA expression in tumour cells from biopsies, and to quantify the percentage of cells in different stages of the cell cycle. If a small percentage of cells are found to be in the S-phase of replication, this will have a limiting factor on the replicative capability of HSV1716.

Better understanding of the binding and subsequent replication of the virus in the tumour cells could identify mechanisms to increase the efficacy *in vivo*. A number of studies have reported that primary cancer cells from humans express only low levels of the primary adenovirus receptor, and are therefore poorly infected by adenoviruses (Dmitriev et al, 1998; Miller et al, 1998; Li et al, 1999). An *in vivo* study with replicating adenovirus found that the absence of the primary cellular receptor on

tumour cells restricts the oncolytic potency of the virus (Douglas et al, 2001). This suggests the efficacy of replicating adenoviruses could be improved by altering the virus to allow infection via a different pathway. Shinoura et al, 1999, reported the potency of a replicating adenovirus in glioma cell lines, *in vitro* and *in vivo*, could be improved by the addition of 20 lysine residues to the COOH terminal of the fibre protein. This allowed the virus to bind to cellular heparin sulphate receptors.

The binding of HSV to cells and subsequent replication is different to adenovirus. Cell surface carbohydrate molecules frequently serve as initial receptors for viruses. The initial virus-cell contact is followed by multiple interactions between numerous copies of the viral attachment proteins and receptor molecules. The heparin sulphate proteoglycan is the molecule first identified to serve as an initial receptor for HSV-1 (WuDunn and Spear, 1989). Heparin sulphate proteoglycans are found on the surface of cells susceptible to HSV infection (Lycke et al, 1991; Gerber et al, 1995). Removal of heparin from the cell surface has been shown to render the cells at least partially resistant to HSV infection by reducing virus attachment to the cell surface (Shieh at al, 1992). Syndecan is a family of four transmembrane heparin sulphate proteoglycans. Syndecan-1 expression has been shown to be reduced during malignant transformation of various epithelia. This loss correlates with the histological differentiation grade of squamous cell carcinoma. In HNSCC, positive syndecan-1 expression correlates with a better clinical prognosis (Inki and Jalkanen, 1996). This indicates replication of HSV will be poorer in aggressive HNSCC tumours. Investigations are therefore needed into the binding of HSV1716 with HNSCC cells, and the subsequent replication in the solid tumour matrix.

For HSV1716 to have a therapeutic role in HNSCC we need to investigate the immune response to HSV1716 inoculation, and its impact on viral replication and spread. Neutralising antibodies do not appear to block replication following intratumoural injection (Nemunaitis et al, 2000; Ganly et al, 2000). However IgM and complement have been shown to impede intravenous efficacy of oncolytic HSV-1 (Ikeda et al, 1999; Ikeda et al, 2000). Intravenous delivery of HSV-1 oncolytic viruses, NV1020 and G207, was found to be less effective than regional hepatic arterial delivery in treating hepatic nodules in mice previously exposed to the virus. Viral inactivation by the host appears to significantly limit systemic herpes oncolytic efficacy and the role of HSV1716 as a systemic therapy remains a very distant prospect. Tumour targeting with cell-specific delivery of virus will improve the systemic treatment of distant metastatic disease. There is some inherent targeting as the capillaries present in tumours are leakier than in normal tissue and this allows viral particles to selectively concentrate in the tumour. Active tumour targeting has been shown *in vivo* by attaching ligands, such as basic fibroblast growth factor or epidermal growth factor to an adenovirus capsid (Gu et al,, 1999; Curiel, 1999). Jakubczak et al, 2001, demonstrated increased targeting of adenovirus type 5 by modifying the protein coat, allowing increased infectibility in coxsackievirus-adenovirus receptor (CAR) deficient tumours.

Cell-mediated immunity and local immunity will limit efficacy of oncolytic viruses and could be a significant reason for the lack of evidence of biological activity in our study. It is notable however that the immune response is not all inhibitory. Viral replication within tumours can lead to the induction of cytokines with antitumoural

properties. This concept can be harnessed to produce a therapeutic effect. *In vivo* studies have demonstrated inhibition of tumour growth by stimulating localised inflammatory or immune responses by transducing tumour cells with cytokines and growth factor genes such as inteleukin IL-1 (Douvedani et al, 1992), IL-2 (Fearon et al, 1990), IL-4 (Golumbek et al 1991; Tepper et al, 1994), interferon (IFN-γ) (Porgador et al, 1992; Restifo et al, 1993) and tumour necrosis factor (Blankenstein et al, 1991; Asher et al, 1991). IL-12 is a cytokine secreted by antigen presenting cells such as dendritic cells, monocytes, macrophages and B-lymphocytes. It is an important mediator in cell mediated immunity by promoting helper T-lymphocyte development. It has been shown to increase antitumour effects in a murine HNSCC model (Myers et al, 1998). Wong et al, 2004, studied the effects of intravenous herpes oncolytic viruses NV1023 and NV1042 in a pulmonary metastatic HNSCC murine model. These viruses are identical except that the murine IL-12 gene has been inserted into the deleted joint sequence. Both have equivalent oncolytic efficacy in lysing SCC *in vitro*, but NV1042 administered intratumourally exhibited significantly greater therapeutic effects in mice bearing SCC tumours. IL-12 expression by NV1042 generated an antitumoural immune response that enhanced oncolytic efficacy (Wong et al. 2001). Animals with disseminated SCC that were treated with systemic NV1042 survived significantly longer compared with NV1023 treated animals. It is notable that during animal CD4 and CD8 depletion studies the survival advantage of NV1042 was almost abolished. This indicates IL-12 benefit was mediated through T-lymphocyte activity. Understanding the limiting impact of the immune response on viral replication, and the potential enhanced antitumoural

effects, will be crucial in the future investigation of the potential therapeutic role of HSV1716 intratumoural injection in locally advanced or locoregional recurrent head and neck cancer.

Another approach to try and increase the antitumour activity of HSV1716 in HNSCC involves altering the virus to express cytotoxicity enhancing proteins. Nakamori et al, 2003, increased the fusogenic capability of HSV-1 by screening for a syncitial HSV after random mutagenesis and insertion of a hyperfusogenic glycoprotein from gibbon ape leukaemia virus into the viral genome. The modified virus kills tumour cells efficiently through both replication and cell membrane fusion. This dramatically increased the cytotoxicity of the virus following injection into the abdominal cavity of mice bearing human ovarian cancer. Fu et al, 2002, constructed an oncolytic virus with a syncitial phenotype, which was derived from the oncolytic HSV-1 virus G207. There was a dramatic antitumour activity *in vivo* against pulmonary metastatic breast cancer, and notably greater than G207. Increasing the cell membrane fusion of HSV1716 in HNSCC could be explored. Any alterations to the virus, for example changes to its phenotypic characteristics, must not be detrimental to its replicative viability and its fundamental clinical safety.

Combining oncolytic viruses with other treatments has the potential of achieving greater antitumour effects than either of these therapies alone. We have shown enhanced cytotoxicity *in vitro* when HSV1716 is combined with cisplatin in HNSCC. Further studies could be done to investigate the interaction between HSV1716 and radiotherapy, another mode of treatment in HNSCC. A similar *in vitro* MTS assay methodology could be adopted. Enhanced cytotoxicity of glioma cells was found

when HSV1716 was combined with radiotherapy (Harrow SJ, personal communication). ONYX-015 when combined with radiotherapy enhanced tumour control greater than that of either monotherapy in a human colorectal carcinoma murine model (Rogulski et al, 2000). Enhanced therapeutic effects were also observed when treatment with the HSV-1 mutant NV1020 was combined with ionising radiation both in human U-87 malignant glioma and Hep3B hepatoma murine models (Advani et al, 1998; Bradley et al, 1999; Chung et al, 2002) Overall radiation does not appear to impair, and potentially enhances viral replication of HSV1716, ONYX-015 or NV1020. This indicates that, unlike human genomes, viral genomes suffer relatively little damage by ionising radiation. Multimodal cancer management with therapies which have different safety profiles has the advantage of potential enhanced efficacy without increased toxicity. It may be possible to lower treatment doses, thereby decreasing possible toxicity, and still achieve greater efficacy than with single therapies. Also there should be no overlapping resistance between HSV1716 and cisplatin or radiotherapy.

In conclusion, the preclinical studies involving HSV1716 in HNSCC cells alone and in combination with cisplatin are encouraging. There is *in vitro* evidence of enhanced cytotoxicity when HSV1716 is combined with the conventional chemotherapy agent cisplatin. Intratumoural injection of HSV1716 into human oral squamous cell carcinoma can be performed readily, comfortably and safely. As has been seen with other oncolytic viruses, the effective application is not as straightforward as laboratory studies might have indicated. The principle problem areas involve optimising the delivery and distribution of HSV1716 into a dense heterogeneous SCC

tumour cell matrix. Increasing our knowledge of the interactions between HSV1716, the HNSCC tumour cell and the immune system will help to optimise antitumour efficacy. This will maximise its ability to disseminate throughout a tumour mass and endure efficacy, despite encountering the immune response. Overcoming these hurdles will enable HSV1716 to be used as a multimodal head and neck cancer treatment, with the golden aim of destroying tumours and improving the survival of cancer patients.

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