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New treatment modalities in superficial bladder cancer

PhD thesis

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List of Abbreviations

AAV	adeno-associated virus
ACV	acyclovir
ALVAC	canarypox virus
ATCC	American Tissue Culture Collection
BAC	bacterial artificial chromosome
BCG	Bacille Calmette-Guerin
BCL-2	B-cell lymphoma 2
CAR	coxsackie adenovirus receptor
CCD camera	charge-coupled device camera
CD	cytosine deaminase
CI	combination index
CIS	carcinoma in situ
CMV	cytomegalovirus
Cox-2	cyclooxygenase 2
Cx26	connexin 26
dCK	deoxycytidine kinase
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPD	dihydropyrimidine dehydrogenase
dTMP	deoxy thymidine monophosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxy uridine monophosphate
EAU	European Association of Urology
EBV	Epstein Barr virus
ECACC	European Collection of Cell Cultures
ED50	median effect dose 50
EMDA	electromotive drug administration
EORTC	European Organisation for Research and Treatment of Cancer
ERKs	extracellular signal-regulated kinases
Fa-CI	fraction affected-combination index
FCS	foetal calf serum
FGM	full growth media
FMG	fusogenic membrane glycoproteins
GADD34	growth arrest and DNA damage 34 protein
GAG	glycosaminoglycan
GALV	gibbon ape leukemia virus
GCV	ganciclovir
GM-CSF	granulocyte macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
HBSS	Hanks Balanced Salt Solution(
hCNT	human concentrative nucleoside transporters
hENT	human equilibrative nucleoside transporters
HER-2	human epidermal growth factor receptor 2

HIV	human immundefficiency virus
HRP	horseradish peroxidase
HSV	herpes simplex virus
HSV-TK	herpes simplex virus tymidine kinase
HVEM	herpesvirus entry mediator
IAA	indole-3-acetic acid
ICP34.5	infected cell protein 34.5
IFN-γ	interferon gamma
IL1	interleukin 1
ISUP	International Society of Urological Pathology
LD_{50}	lethal dose 50
MAb	monoclonal antibody
MAP	mitogen-activated protein kinases
MEK	MAP/ERK kinase
MHC	major histocompatibility complex
MMC	mitomycin C
MOI	multiplicity of interest
MTS assay	mithocondrial tetrazolium salt assay
MuLV	murine leukemia virus
NAT2	N-acetyltransferase 2
NK cell	natural killer cell
NMIBC	non muscle invasive bladder cancer
NSCLC	non-small cell lung cancer
NYVAC	attenuated vaccinia virus(
PDT	photodynamic therapy
pfu	plaque forming unit
PIG-U	phosphatidylinositol glycan class U
PKR	protein kinase R
PNP	purine nucleoside phosphorylase
ProTα	prothymosin alpha
PUNLMP	papillary urothelial neoplasms of low malingnant potential
QRT-PCR	quantitative reverse transcription polymerase chain reaction
RB	retinoblastoma
RCR	replication competent retroviral vector
RNA	ribonucleic acid
RR	ribonucleotide reductase
RSV promoter	Rous sarcoma virus promoter
RT	room temperature
SUP	suppressor mutant virus
TCC	transitional cell cancer
TK	thymidine kinase
TNFα	tumor necrosis factor alfa
TNM	tumour - lymph nodes - metastasis system
TS	thymidylate synthase
TURBT	transurethral resection of bladder tumour
UDK	uridine kinase
UDP	uridine phosphorylase

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UK	United Kingdom
UMP	uridine monophosphate
UPRT	uracil phosphoribosyltransferase
VSV	vesicular stomatitis virus
VZV	varicella-zoster virus
WHO	World Health Organisation
2’d-5F- uridine	2’ deoxy 5-fluoro uridine
5-FC	5-fluorocytosine
5-FdUMP	5-fluoro deoxyuridine monophosphate
5-FU	5-fluorouracil
5-FUTP	5-fluorouridine triphosphate

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1 Introduction

1.1 Bladder cancer

1.1.1 Incidence

In Hungary approximately 2,600 new cases of bladder cancer are diagnosed every year making it the fifth commonest cancer in men and the eleventh commonest cancer in women. Bladder cancer is a heterogeneous disease with a variable natural history. At one end of the spectrum, low grade Ta tumors have a low progression rate and require initial endoscopic treatment and surveillance, but rarely present a threat to the patient. At the other extreme, high grade tumors have a high malignant potential associated with significant progression and cancer death rates. The incidence of the disease increases by age and it occurs most commonly in people between 50 and 70 years of age. The average age at diagnosis is 65 years. Bladder cancer is 3 to 4 times more common among males than females. On the other hand, it has been suggested that the stage-adjusted survival of bladder cancer among women is worse than among men (Mungan et al. 2000).

The incidence is higher in Caucasians than in African Americans and these differences may be partly due to genes, as studies have shown that certain genetic polymorphisms linked to an increased risk of bladder cancer (for example, the N-acetyltransferase 2 (NAT2) slow acetylator polymorphism) are much more prevalent in Caucasian than non-white populations (Garcia-Closas et al. 2005). There is a positive social class gradient for bladder cancer in both sexes. Surveys of cancer incidence and mortality suggest that parous women have a lower risk of bladder cancer than nulliparous women, probably due to hormonal changes related to pregnancy, and that the risk may decrease with increasing parity (Cantor et al. 1992; Green et al. 1988; Miller et al. 1980; Plesko et al. 1985)

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1.1.2 Risk factors

Cigarette smoking is the principal preventable risk factor for bladder cancer in both men and women. In Europe it is estimated that up to half the bladder cancer cases in men and a third in women are caused by cigarette smoking. Current smokers have around three times the risk of developing bladder cancer compared to non-smokers while ex-smokers have double the risk of non-smokers. Risk is positively associated with both increasing dose and duration of smoking (Brennan et al. 2000; Brennan et al. 2001; NICE 2002). The causative agents amongst others are thought to be alpha- and beta- naphtylamine, which are secreted into the urine of smokers.

Bladder cancer was one of the first cancers shown to be industrially associated. In 1895 Rehn reported cases of bladder cancer in a German aniline dye factory (Rehn 1895). Altogether it is estimated that between 5 and 10% of male bladder cancer cases in Europe are caused by occupational exposure (Kogevinas et al. 2003). This proportion may be higher in countries with less-regulated industrial processes. Workers in chemical, dye, rubber, petroleum, leather and printing industries are at increased risk. Excess risks have been frequently observed among painters, which is thought to be due to exposure to possible carcinogenic constituents of paints like benzidine, polychlorinated biphenyls, formaldehyde, and asbestos and solvents like benzene, dioxane, and methylene chloride (Steenland and Palu 1999). The latency period between exposure and tumour development may be prolonged. A moderately increased risk is also found among leather workers and shoe makers, although the responsible agent is still un-known (Marrett et al. 1986). An excess risk of bladder cancer is also observed in aluminum, iron, and steelworkers, which may be the result of exposure to aromatic amines and polycyclic aromatic hydrocarbons in coal-tar pitch volatiles (Gaertner and Theriault 2002; Romundstad et al. 2000; Theriault et al. 1984). Several studies have been performed on chlorinated drinking water and bladder cancer, and all of these reported increased risks (King and Marrett 1996; Koivusalo et al. 1998). Other studies have evaluated the association between ingestion of arsenic in drinking water and the risk of bladder cancer.

Chronic urinary tract infection is associated with the development of bladder cancer, especially invasive squamous cell carcinoma (Kantor et al. 1984). Cyclophosphamide, an alkylating agent used in the treatment of malignant neoplasms, particularly

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lymphoproliferative and myeloproliferative diseases, increases the risk of bladder cancer (mainly urothelial carcinoma) with a clear dose-response relationship (Fairchild et al. 1979; Travis et al. 1995). A chelating agent is now given with cyclophosphamide to reduce this risk. Treatment with radiotherapy to the pelvic area for cancers such as cervical cancer, prostate cancer, kidney cancer, fallopian tube cancer and testicular cancer can increase the risk of bladder cancer (Kaldor et al. 1995).

Ingestion of artificial sweeteners has been proposed to be a risk factor, but several, studies have failed to confirm any association (Elcock and Morgan 1993; Howe et al. 1977). Physical trauma to the urothelium induced by infection, instrumentation, and calculi increases the risk of malignancy (Hicks et al. 1982). Most studies show a two-six-fold increased risk of bladder cancer in first-degree relatives of bladder cancer patients, with a higher risk if the relative was diagnosed before the age of 45. (Randi et al. 2007).

Squamous cell carcinoma of the urinary bladder has been known to be associated with *Schistosoma haematobium* infection for many years. The epidemiologic association is based both on case-control studies and on the close correlation of bladder cancer incidence with the prevalence of *S. haematobium* infection within different geographic areas (Bedwani et al. 1998; Gelfand 1967; Lucas 1982).

1.1.3 Staging

The stage of a cancer describes its size and whether it has spread. The most appropriate treatment depends on the stage of the cancer. The most commonly used staging system for bladder cancer is the TNM system of the European Association of Urology (EAU), in which **T** is the size of the **t**umour, **N** is whether it has spread to the nearby lymph **n**odes, **M** is whether the cancer has spread to other parts of the body (**m**etastases) (Figure 1.1).

Tumour size (T):

Superficial bladder cancer

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CIS CIS (carcinoma in situ) is a high –grade (anaplastic) carcinoma confined to the urothelium, but with flat non-papillary configurations. CIS appears as reddened and velvety mucosa and is slightly elevated but sometimes not visible.

Ta These tumours are confined to the urothelium, have a papillary configuration of their exophytic part and do not penetrate from the urothelium into the lamina propria or detrusor muscle.

T1 tumours generate from the urothelium but penetrate the basement membrane which separates the urothelium from the deeper layers. T 1 tumours invade the lamina propria but not so deep that they reach the detrusor muscle.

Invasive bladder cancer

T2 The cancer has started to grow into the muscle of the bladder wall under the connective tissue layer.

T2a The tumour invades the superficial muscle.

T2b The tumour invades the deep muscle.

T3 The cancer invades the perivesical tissue.

T3a microscopically

T3b macroscopically

Locally advanced bladder cancer

T4 The cancer has spread outside the bladder to any of the following: the prostate, uterus, vagina, pelvic or abdominal wall.

T4a The cancer has spread to the prostate, uterus or vagina.

T4b The cancer has spread to the pelvic or abdominal wall.

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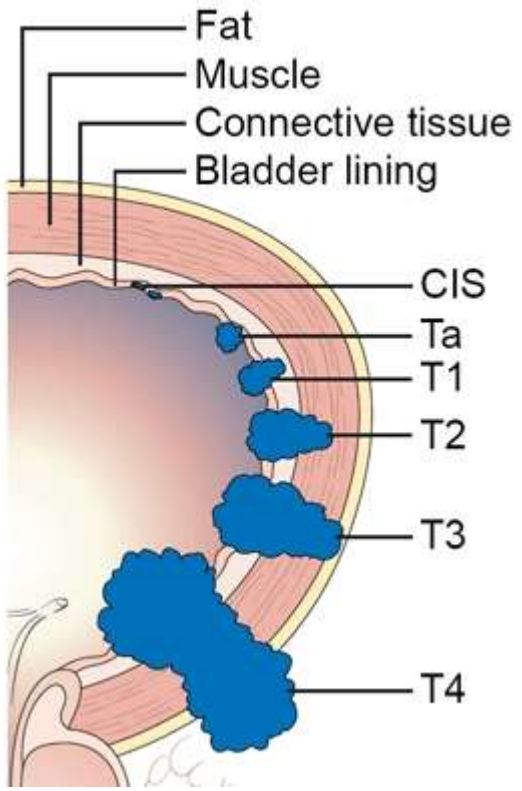

Figure 1.1 T stages of bladder cancer

Lymph nodes (N):

The N refers to whether the cancer cells have spread into the lymph nodes.

N0 There are no cancer cells in any lymph nodes.

N1 There are cancer cells in one lymph node smaller than 2cm across.

N2 There are cancer cells in one affected lymph node larger than 2cm, but smaller than 5cm, or more than one node affected, but all of them smaller than 5cm across.

N3 There are cancer cells in at least one affected lymph node larger than 5cm across.

Metastases (M):

M0 if the cancer cells have not spread.

M1 is when the cancer cells have spread to other parts of the body. If bladder cancer spreads, it's most likely to go to the bones, the lungs or the liver.

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1.1.4 Grading

The grading system is also very important in the classification of bladder cancer. The grading system describes the anaplastic degree of the cancer cells.

Grade 1 is well differentiated tumour.

Grade 2 is moderately differentiated tumour.

Grade 3 is poorly differentiated tumour.

1.1.5 Histopathology

The normal urothelium is composed of 3-7 layers of transitional cell epithelium resting on a basement membrane composed of extracellular matrix (collagen, adhesive glycoproteins, glycosaminoglycans). The epithelial cells vary in appearance. The basal cells are actively proliferating cells resting on the basal membrane; the luminal cells, perhaps the most important feature of normal bladder epithelium, are larger umbrella-like cells that are bound together by tight junctions. Beyond the basement membrane is loose connective tissue, the lamina propria, in which occasionally smooth-muscle fibres can be identified. The fibres should be distinguished from deeper, more extensive muscle elements defining the true muscularis propria. The muscle wall of the bladder is composed of muscle bundles coursing in multiple directions. As these converge near the bladder neck, 3 layers can be recognized: inner and outer longitudinally oriented layers and a middle, circularly oriented layer.
According to the WHO/ISUP system all bladder tumour cases are devided into four groups.

1.1.5.1 WHO/ISUP system

1.1.5.1.1 Papilloma

The WHO/ISUP system has very restrictive histologic features for the diagnosis of papilloma, where normal appearing urothelium lines papillary fronds. Defined as such, it is a rare benign condition typically occurring as a small, isolated growth seen

primarily in younger patients. The majority of these lesions once excised will not recur (McKenney et al. 2003).

1.1.5.1.2 Papillary Urothelial Neoplasms of Low Malingnant Potential (PUNLMP)

The category of PUNLMP was derived to describe lesions that do not have cytologic features of malignancy, yet have thickened urothelium as compared to papilloma. There is no or very little variation of nuclear features or the pattern of organization. Having a category of PUNLMP avoids labeling a patient as having cancer with its psychosocial and financial implications, although they are not diagnosed as having a benign lesion, whereby they might not be followed as closely. The current classification system allows for designation of a lesion (papillary urothelial neoplasm of low malignant potential), that biologically has a very low risk of progression, yet is not entirely benign.

1.1.5.1.3 Low and High grade papillary carcinoma

The WHO/ISUP system classifies papillary urothelial carcinoma into only 2 grades. Low grade papillary urothelial carcinoma exhibits an overall orderly appearance but has minimal variability in architecture and/or cytologic features, which are easily recognizable at scanning magnification. High grade papillary urothelial carcinomas are characterized by a disorderly appearance due to marked architectural and cytologic abnormalities, recognizable at low magnification. It is important to remember that a single papillary urothelial neoplasm may contain a spectrum of cytologic and architectural abnormalities.

Bladder cancer can be classified by the histopathological type.

1.1.5.2 Histopathological type

1.1.5.2.1 Transitional cell carcinoma

Approximately 90% of all bladder cancers are transitional cell carcinomas. These tumours most commonly appear as papillary, exophytic lesions, less commonly they may be sessile or ulcerated. Whereas the former group are usually superficial in nature, sessile growths are often invasive.

1.1.5.2.2 Nontransitional cell carcinomas

1.1.5.2.2.1 Adenocarcinoma

Adenocarcinomas account for less than 2% of all blader cancer. Primary adenocarcinomas of the bladder may be preceded by cystitis and metaplasia. Histologically adenocarcinomas are mucus secreting and may have glandular, colloid or signet-ring patterns. Whereas primary adenocarcinomas often arise along the floor of the bladder, adenocarcinomas arising from the urachus occur at the dome. Both tumour types are often localized at the time of diagnosis, but muscle invasion is usually present. Five-year survival is usually less than 40%, despite aggressive surgical management (Abenoza et al. 1987; Kramer et al. 1979; Malek et al. 1983; Wright et al. 1988).

1.1.5.2.2.2 Squamous cell carcinoma

Squamous cell carcinoma account for between 5% and 10% of all bladder cancers and is often associated with a history of chronic infection, vesical calculi, or chronic catheter use. It may also be associated with bilharzial infection owing to Schistosoma haematobium, because squamous cell carcinoma accounts for approximately 60% of all bladder cancers in Egypt, parts of Africa and Middle East, where this infection is prevalent (El-Bolkainy et al. 1981). These tumours are often nodular and invasive at the time of diagnosis. Histologically they appear as poorly differentiated neoplasms composed of polygonal cells with characteristic intercellular bridges.

1.1.5.2.2.3 Undifferentiated carcinomas

Undifferentiated bladder carcinomas, which are rare (accounting for less than 2%), have no mature epithelial elements. A small cell type has been described that histologically resembles similar lesions of the lung (Mills et al. 1987).

1.1.5.2.2.4 Mixed carcinoma

Mixed carcinomas constitute 4-6% of all bladder cancers and are composed of transitional, glandular, squamous or undifferentiated patterns. The most common type comprises transitional and squamous cell elements (Murphy 1989). Most mixed carcinomas are large and infiltrating at the time of diagnosis.

1.1.5.2.2.5 Rare epithelial and nonepithelial cancers

Rare epithelial carcinomas identified in the bladder include villous adenomas, carcinoid tumours, carcinosarcomas and melanomas. Rare nonepithelial cancers of the urinary bladder include pheochromocytomas, lymphomas, choriocarcinomas and various mesenchymal tumours (hemangioma, osteogenic sarcoma and myosarcoma) (Murphy 1989). Cancers of the prostate, cervix and rectum may involve the bladder by direct extension. The most common tumours metastatic to the bladder include (in order of incidence) melanoma, lymphoma, stomach, breast, kidney and lung (Goldstein 1967; Murphy 1989).

1.2 Non-muscle invasive (superficial) bladder cancer

1.2.1 Non-muscle invasive bladder cancer

Approximately 80% of all bladder cancer cases are non muscle invasive bladder cancer (NMIBC) defined as stage Ta-T1, grade 1-3 and carcinoma in situ (CIS). Historically these tumours were viewed as a homogeneous group, referred to as ‘superficial’ bladder cancer and treatment was based at reducing recurrences. In the 1980’s it was recognised that a subgroup of these patients with T1 G3 tumour and/or CIS had a significant rate of progression to muscle invasive disease and a poor long term survival (Cookson et al. 1997) which led to the concept of ‘high risk’ NMIBC. Further studies have now shown that these ‘superficial’ tumours in fact form a heterogenous group of tumours whose 1 year recurrence and progression rates vary from 15-61% and <1-17% respectively (Table 1.1) (Sylvester et al. 2006). As it is shown in this table low and intermediate risk patients have much less risk of recurrence and progression compared to the high risk group.

Based on the developing understanding of the pathological behaviour of NMIBC, the therapeutic options for the management of NMIBC have evolved over the last half century. Initially, treatment was transurethral resection (TURBT) alone until Jones and Swinney first described the use of intravesical chemotherapy using Thiotepa in 1961 (Jones and Swinney 1961). After TURBT the use of Bacille Calmette-Guerin as intravesical immunotherapy in NMIBC was first reported by Morales in 1976 (Morales

et al. 1976). and refined with the introduction of a maintenance regime by Lamm (Lamm et al. 2000). Radical cystectomy was advocated as a treatment for 'high risk' NMIBC (Malkowicz et al. 1990). A single immediate instillation of intravesical chemotherapy has been shown to be of benefit (in terms of recurrence and progression) immediately following transurethral resection of bladder tumour (TURBT) (Sylvester et al. 2004).

Table 1.1 EORTC risk tables Sylvester 2006

	% of total	Recurrence 1 year	Recurrence 5 year	Progression 1 year	Progression 5 year
Low risk Ta G1	50	15-24	31-46	≤1	1-6
Intermediate risk Ta-1 G1-2	35	24-38	46-62	≤1-5	1-17
High risk Ta-1 G3	15	24-61	46-78	1-17	6-45

The wide spectrum of pathological behaviour of NMIBC has led to the realisation that in some cases eg a solitary pTa TCC, the main therapeutic goal should be to prevent recurrences with minimal morbidity (Hall et al. 1994), whilst in other cases relatively high morbidity is acceptable in order to prevent progression to muscle invasive disease. Out of this has been borne the concept of NMIBC risk groups. Whilst the exact definition of each risk category varies (EAU April 2008; Hall et al. 1994), two groups are clearly defined, i.e. low and high risk NMIBC.

1.2.2 Current therapeutical options for NMIBC

Current guidelines suggest that all patients with non muscle invasive bladder cancer should receive one immediate post operative instillation of chemotherapy within 6 hours after TURBT. Further treatment depends on the patients` risk of recurrence and progression to muscle invasive disease (Sylvester et al. 2006). Low risk group patients have single, small (<3cm), histologically Ta, low grade tumour. For these patients no further treatment is recommended prior to a subsequent recurrence (EAU April 2008; Hall et al. 1994). Patients with Ta-T1, high grade tumour, or with carcinoma in situ, or with combination of these are in the high risk group. For this group a further

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transurethral resection (re-TURBT) and 1-3 years of maintenance BCG (bacillus Calmette-Guérin) intravesical immunotherapy is recommended (EAU April 2008).

1.2.3 Intermediate risk NMBC

The remaining group with intermediate risk forms approximately 35% of all NMIBC and is by definition made up of patients excluded from the other risk categories. (Patients with Ta-T1, low and intermediate grade, multifocal, >3cm tumours attend to this group). Intermediate risk group patients form a heterogeneous group of patients e.g. from solitary but recurrent G1pTa to multiple recurrent G2pTa. These patients have a significant risk of recurrence of between 24% at 1 year and 62% at 5 years, and a single instillation of intravesical chemotherapy is inadequate treatment. On the other hand, the relatively high morbidity of intravesical BCG, particularly during maintenance treatment, has meant that the use of BCG in this group has not been popular, at least in Europe. The current popular therapeutic option is weekly instillation of intravesical chemotherapy for 6 weeks (induction course) with or without maintenance therapy. However, the EORTC risk tables suggest a risk of progression as high as 17% at 5 years, which suggests that some patients within the intermediate group may have a significant risk of progression, and would therefore benefit from a more intensive treatment than is currently used even if this results in higher morbidity. Unfortunately we currently cannot identify such patients individually, and so efforts are focused on new approaches to the therapy of these patients as a group, with the ultimate aim of reducing recurrences, preventing progression with acceptable morbidity.

1.2.4 Current therapeutic options for intermediate risk NMIBC

Four national and international urological association guidelines are available for NMIBC (BAUS 2008; EAU April 2008; Hall et al. 2007; Oosterlinck et al. 2005). All four guidelines recommend that all patients with intermediate risk (IR) NMIBC should receive one immediate instillation of chemotherapy after TURBT.

1.2.4.1 Induction course of intravesical chemotherapy

Whilst all the guidelines agree that further treatment is required, no clear consensus emerges as to the optimal dose, regimen and duration of treatment. The summary of product characteristics for mitomycin-C (Kyowa Hakko UK, Slough, UK) suggests that 20–40 mg of mitomycin-C in 20–40 mL of diluent is given weekly or three times a week for a total of 20 doses (Kyowa). Many urologists seem to prefer a 6-week course of weekly intravesical chemotherapy, often called an 'induction' course, perhaps because this regimen is similar to the induction regimen for intravesical BCG.

1.2.4.2 Maintenance intravesical chemotherapy

Maintenance regimens of intravesical chemotherapy are based on the hypothesis that a prolonged course of intravesical chemotherapy will reduce the recurrence rate of bladder cancer by more than one immediate instillation or an induction course, with acceptable morbidity. It is not known whether maintenance chemotherapy is necessary if one dose of intravesical chemotherapy was given immediately after TURBT, and vice versa, i.e. that if a maintenance course of intravesical chemotherapy is given for in intermediate risk NMIBC, then an immediate instillation may not be necessary. If one immediate instillation is not given, then a prolonged course of intravesical chemotherapy of 1 year might be required to achieve the same magnitude of reduction in the recurrence rate (EAU April 2008). Given the simplicity and safety of an immediate instillation, it would seem sensible to pursue the former approach rather than the latter. Finally, there is a theoretical risk of carcinogenesis with prolonged use of any chemotherapeutic agent.

1.2.4.3 Optimizing intravesical chemotherapy

It was postulated that the variable response of patients to intravesical chemotherapy has two components; lack of sensitivity of the tumour to intravesical chemotherapy and inadequate drug delivery to the tumour (Au et al. 2001). The latter might be improved by optimizing intravesical chemotherapy. Au *et al.* (Au et al. 2001) assessed, in a randomized phase III trial, four approaches combined to optimize the efficacy of intravesical mitomycin C. Patients were randomized into either standard intervention

and received 20 mg mitomycin C in 20 mL of sterile water, or optimized intervention, and were asked to refrain from drinking for 8 h before treatment, given a total of 3.9 g of sodium bicarbonate orally, and had any residual urine drained from the bladder before receiving 40 mg mitomycin C in 20 mL of sterile water. The optimized arm showed a longer median (95% CI) time to recurrence of 29.1 (14.0–44.2) months and a greater 5-year recurrence-free fraction of 41.0 (30.9–51.1)% than the standard arm, of 11.8 (7.2–16.4) months and 24.6 (14.9–34.3)% respectively (P =0.005, log-rank test for time to recurrence). Despite the relative simplicity of the optimization schedule and the improved efficacy, optimized intravesical regimens have not been widely adopted. Another simple way to increase the time of residence in the bladder is prolonged drug infusion into the bladder. Other methods have included triblock copolymers which release drug over time and in response to temperature.

1.2.4.4 BCG: Reducing side -effects

BCG therapy remains the reference standard non-surgical treatment for high-risk NMIBC and might therefore be, theoretically at least, the optimum therapy for intermediate risk NMIBC. In practice, BCG is associated with relatively high morbidity, with up to 25% of patients failing to complete a full maintenance course of BCG. To avoid this, recent studies have assessed various means of reducing the toxicity of BCG therapy whilst maintaining its therapeutic effects. The Spanish CUETO group have examined this issue. In a series of methodical trials they showed that 27 mg of BCG Connaught strain (one-third dose) had lower toxicity than 81 mg (full dose) but was as effective as a full dose for high-risk disease; 13.5 mg BCG was not as effective as 27 mg but produced the same toxicity, so 27 mg of BCG seems to be the minimum effective dose (Fernandez-Gomez et al. 2008). A further study suggested that monthly single instillations of 120 mg BCG for 1 year was as effective as 3-week courses of maintenance BCG at 3, 6, 12, 18 and 24 months, in terms of recurrence and progression rates, and with less toxicity (Ali-El-Dein 2007). The EORTC 30962 study assessed at the one-third vs full-dose BCG over 1 or 3 years of maintenance. The study closed in 2005 and the results are eagerly awaited. Finally, a randomized double-blind placebocontrolled multicentre trial showed that prophylaxis with 200 mg ofloxacin after

each BCG instillation reduced the incidence of BCG-induced side-effects (Colombel et al. 2006).

1.2.4.5 Device-assisted therapy

Another approach to improving the delivery of intravesical chemotherapy drugs to the tumour is with device-assisted therapy. The two main methods are with microwave hyperthermia to heat the bladder wall, or electromotive drug administration (EMDA). The theoretical base of EMDA (Di Stasi et al. 2006) and thermochemotherapy (Gofrit et al. 2004; van der Heijden et al. 2004) is to increase the permeability of the bladder mucosa (with BCG induced inflammation and with hyperthermia in the bladder) before administering chemotherapy eg. Mitomycin. A very small electric current increases the permeation of charged and neutral compounds through the process of electromigration and elecro-osmosis. Microwave hyperthermia was designed to be used exclusively in the setting of high-risk NMIBC and patients in whom intravesical BCG failed. The use of EMDA-potentiated mitomycin C alone has been described but it appears to be particularly effective when used in conjunction with BCG (Di Stasi et al. 2006). In this randomized trial specifically in patients with stage pT1 disease (i.e. high-risk NMIBC) the combination of EMDA, mitomycin C and BCG when used sequentially showed a higher mean (95% CI) disease-free interval than BCG alone, at 69 (55–86) vs 21 (15–54) months, respectively. That study was also unique in that it was the first study of intravesical therapy to show a reduction in disease specific mortality and overall mortality. The authors hypothesised that the mode of action in sequential BCG/EMDA mitomycin C is that BCG-induced inflammation increases the permeability of the bladder wall and improves the delivery of the mitomycin C to the tumour site. Thus sequential BCG/EMDA/mitomycin C might have a role in intermediate risk NMIBC in the future, particularly in patients thought to be at high risk of progression.

1.3 Gene therapy

Gene therapy is the treatment of disease by the introduction of normal genes into the patient to overcome the effects of those defective genes. Cancer gene therapy is the introduction of DNA or RNA which cause cellular changes that inhibit the uncontrolled

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growth of tumour cells. Several viral and nonviral vectors have been developed to achieve the delivery of the transgene to the tumour cells. Gene transfer by viral vectors takes advantage of the natural ability of viruses to enter cells and express transgenes by the infected cells. The success of these strategies depends on the high level transfer of therapeutic genetic material to the cells of interest (ie. tumour, immune cell, endothelial cell).

This approach was first applied to cancer patients using non-replicating adenoviruses or retroviruses to deliver copies of the wild type tumour suppressor gene p53 to tumours with the aim of preventing uncontrolled cell division (Roth 1996; Spitz et al. 1996). This technology uses our understanding of cancer at the molecular level and has been exploited to develop new strategies for killing cells selectively or arresting their growth (McCormick 2001). Gene therapy for the treatment of cancer was initiated with high levels of optimism and enthusiasm. Recently however, this perception has been tempered by the realisation that efficiency of gene delivery remains the most significant barrier to success (Vile et al. 2000).

The field of molecular cancer therapy embraces a range of technologies including direct attack on tumour cells using oncolytic viruses or prodrug activating systems, and viral fusiogenic envelopes. These fields are illustrated in Figure 1.2. However the categorisation of such therapies is somewhat artificial for two reasons. Firstly, one single therapy is unlikely to cure a particular cancer since evidence from conventional therapies suggests that a combination of therapies is much more likely to work in the clinic. Secondly, a particular therapy may have multiple modes of action. For example, the prodrug activating system of HSV-TK combined with ganciclovir promotes tumour reduction by directly killing tumour cells and also stimulates an immune response against the tumour (Vile et al. 1997).

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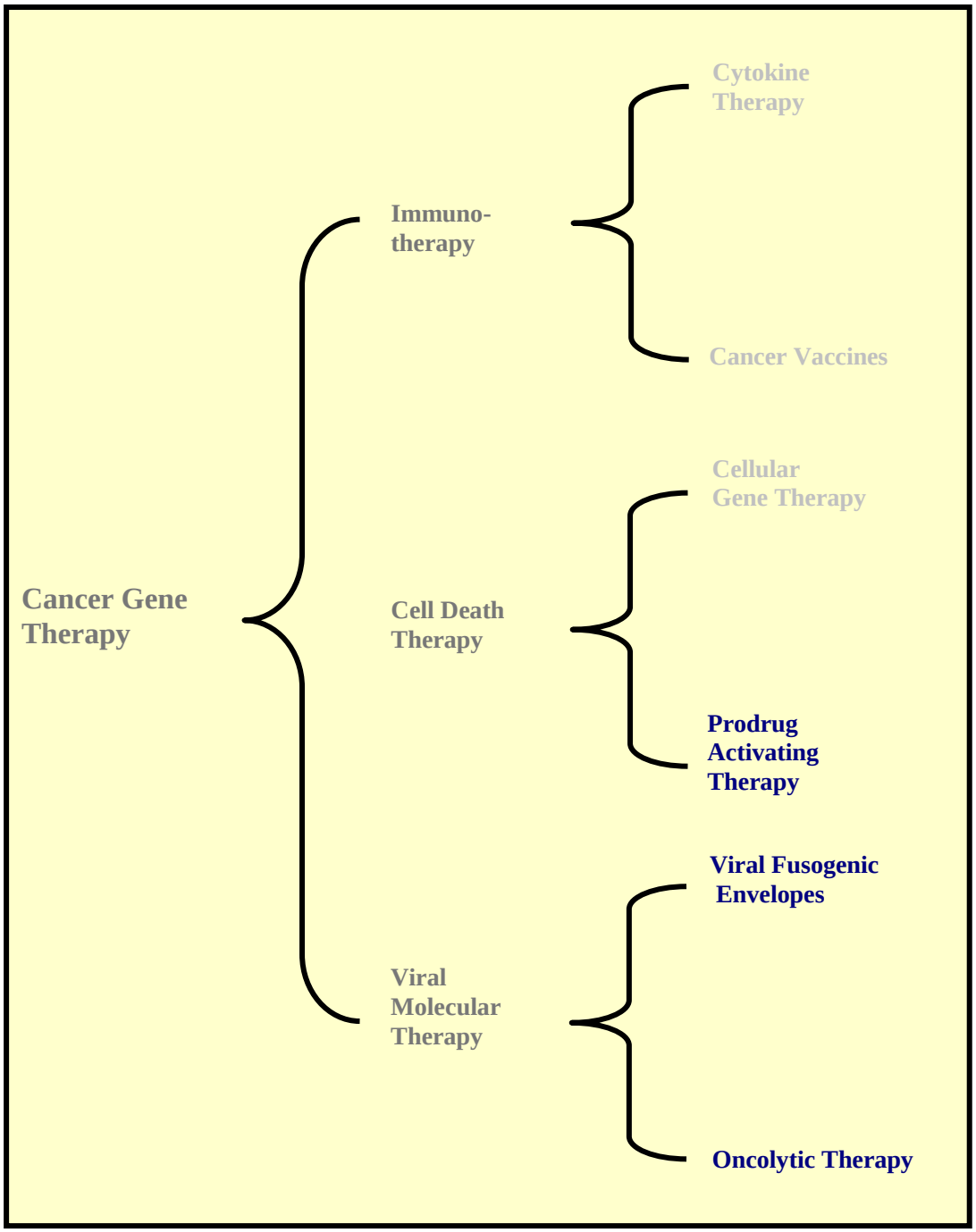

Figure 1.2 An outline of the areas of cancer gene therapy being developed. The approaches highlighted in blue are discussed in this thesis.

1.4 Gene therapy in bladder cancer

In the following part we aim to review bladder cancer gene therapy by analyzing the stages which are key to its success: route of delivery, anti-tumour approaches and vectors used.

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1.4.1 Route of delivery

1.4.1.1 Systemic administration

This route often fails in the treatment of bladder cancer due to loss of the therapeutic agent from metabolism in the liver and poor tumour penetration. There is a need for a sufficient level and an appropriate amount of time for the therapeutic agent to come into contact with the tumour cells in the bladder mucosa in order to achieve its effect which is not achieved via this route.

1.4.1.2 Intravesical administration

The bladder represents an ideal target for intravesical gene therapy in the management of bladder cancer. The vectors are in direct local tumour contact, eliminating the difficulties associated with systemic administration and ensuring a low risk of gene transfer to other organs. Easy external access is achieved through the urethra which avoids losses from the first pass metabolism in the liver and minimizes the clearance of the vector by the immune system allowing minimal systemic side effects.

The inside of the bladder consists of 6-7 layers of cells which form a permeable transitional epithelium known as the urothelium (Figure1.3). The urothelium acts as a barrier and in healthy circumstances it is almost impermeable. The main part of this barrier is the superficial cell layer of the transitional epithelium made up of umbrella cells. The umbrella cells join to each other with tight junctions and constitute a watertight barrier (Hohlbrugger 1995). The apical surface of the umbrella cells is covered by uroplakins (UPIa, UPIb, UPII, UPIII), which make up the rigid plaques. In addition the umbrella cells are covered by a hydrophilic glycosaminoglycan (GAG) layer. The GAG layer physically blocks the adhesion of foreign substances such as drugs and viruses (Lande et al. 1995; Parsons 1994). Together these layers erect a tough bladder barrier that maintains a high electrochemical gradient between the urine and the blood. The impermeability of the urothelium is frequently exploited for instilling potentially toxic agents into the bladder to achieve localized pharmalogical effect.

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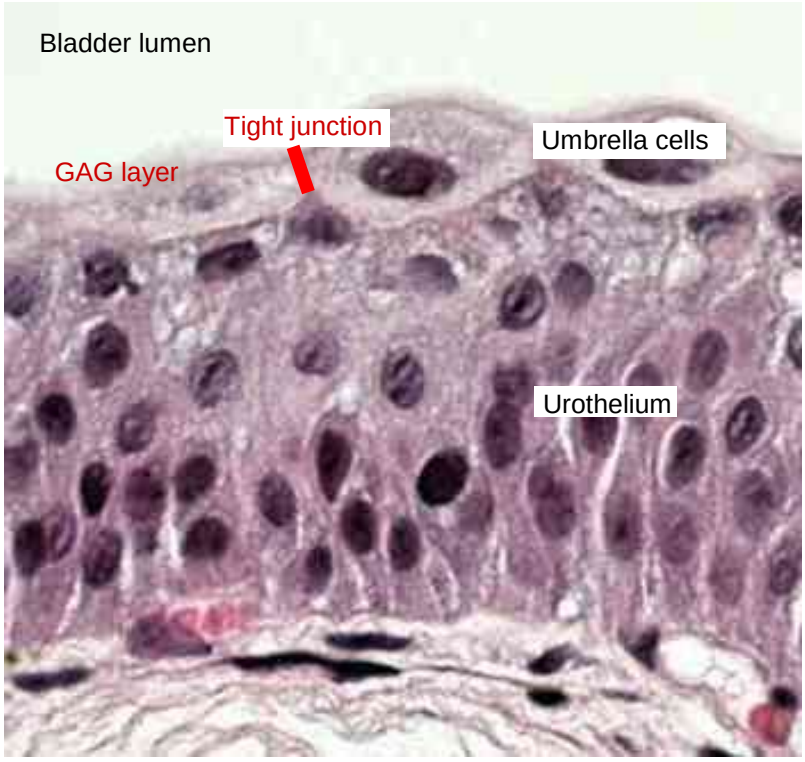

Figure 1.3 Structure of bladder transitional epithelium on a H&E staining histology sample. The localisation of tight junction and GAG layer that is not visible under normal microscope is marked in red.

Several strategies have been developed to improve the intravesical delivery of drugs in the bladder. This includes helping them cross the permeability barrier of the urothelium by both physical and chemical enhancement methods and increasing residence time in the bladder. These approaches may also improve the efficacy of some gene therapy vectors.

1.4.1.2.1 Improving permeability using physical methods.

The permeability of bladder cells can be improved with physical approaches like electromotive drug administration (EMDA), thermotherapy or iontophoresis which was further discussed in section 1.2.4.5.

1.4.1.2.2 Improving permeability using chemical approaches.

Several chemical approaches that improve the permeability of the urothelium have been investigated in the literature. Protamine sulfate interacts with the GAG layer to increase the permeability of the urothelium (Cetinel et al. 2003; Tzan et al. 1994) whilst

administration of dimethyl sulfoxide has been described to enhance the absorption of paclitaxel and pirarubicin (Chen et al. 2003; Hashimoto et al. 1992). The administration of 22% ethanol prior to the instillation of an adenoviral vector causes damage of the GAG layer and makes the treatment more effective (Engler et al. 1999). In addition a polyamide containing formulation, syn3, when used as a pre-treatment prior to intravesically administered adenovirus has been reported to enhance viral uptake (Yamashita et al. 2002). Recent studies in preclinical models with the syn3/adenoviral-based strategy have also shown an improved therapeutic effect (Benedict et al. 2004; Connor et al. 2005). A phase I study of the Big CHAP polyamide and an adenovirus coadministered intravesically led to increased transduction compared to adenovirus alone (Kuball et al. 2002). Although the use of Syn3 appears to be non toxic to the bladder tissue the use of other transducing agents has been shown to have adverse effects on the urothelium (Benedict et al. 2004).

1.4.1.2.3 Increasing residence time in the bladder

Increasing the time of residence in the bladder is an obvious strategy to increase the efficacy of a drug or therapy by increasing the duration of direct contact between drug and abnormal urothelium and ensuring constant drug concentration. This method was further discussed in section 1.2.4.3.

1.4.1.2.4 Bioadhesion

This defines the interaction between a biological surface, such as the urothelium and a polymer such as algin, chitosan, fibrinogen, gelatin, polyethhylene glycol (Ozturk et al. 2004; Tyagi et al. 2006; Ye et al. 2001) . The efficiency of drug absorption is increased after bioadhesion properties are coupled to microspheres, liposomes or nanoparticles. For example microspheres based on chitosan are strongly mucoadhesive and their instillation was able to increase the residence time and decrease the frequency of administration of acyclovir (Genta et al. 1997).

The residual urine in the bladder before instillation and the accumulated urine during instillation can cause dilution in the concentration of the administered therapeutic agent. Restricted fluid intake before and during instillation and the complete emptying of bladder is required in any kind of intravesical treatment (Au et al. 2001). Significant

differences in gene expression are achieved by varying physical parameters during intravesical instillation. Increased gene expression associated with larger volume instillation may be responsible for some reported variability of gene transfer to the bladder. Significantly more gene expression was detected in bladders instilled with a higher volume of viral vectors ($p < 0.05$). Likewise, higher instillation pressures resulted in higher transgene expression in distant organs (Siemens et al. 2001).

1.4.2 Therapeutic genes used against bladder cancer

1.4.2.1 Tumour suppressor genes

Mutations in tumour suppressor genes are prevalent in many cancers and mutation of the p53 gene is common in muscle-invasive bladder cancer (Smith et al. 2003). Reintroduction of a functional copy of the p53 gene is one route taken by scientists. Miyake et al (Miyake et al. 1998) introduced a functional copy of the p53 gene into the bladder cancer cell line (HT1376) *in vitro* and found it became resensitised to cisplatin. Further *in vivo* studies used an adenoviral vector carrying the p53 gene which was injected directly into HT1372 tumours established in nude mice. Intraperitoneal administration of cisplatin then led to massive apoptotic destruction of the tumour. Adenoviral vectors carrying the p53 gene, INGN201 and SCH58500, have been tested in phase I clinical trials for bladder cancer (Table 1.2) (Barnard 2000).

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Treatment	Clinical trial phase type	Vectors	Dose /PFU	Therapeutic gene	No of patients	Route of treatment	Clinical Response	References
CG0070-Oncolytic adenovirus	I	Oncolytic adenovirus	Dose-Escalation Trial	-	This study is ongoing	INI	This study is ongoing	Cell Genesys ClinicalTrials.gov Identifier: NCT00109655
AdCD40L	I/IIa	Adenovirus vector serotype 5, E1/E3 deletion	Dose-Escalation Trial	human CD40L gene driven by RSV promoter.	This study is ongoing	INI	This study is ongoing	Uppsala University ClinicalTrials.gov Identifier: NCT00891748
INGN 201 (Ad5CMV-p53)	I	adenovirus	Up to 7.5 $X10^{12}$ (2x)	Wild type p53	13	INI	1/13 showed evidence of tumour response. Transgene expression 7/8 patients by INI	(Pagliaro et al. 2003b)
SCH 58500 (rAd/p53) + Big CHAP polyamide	I	adenovirus	Up to 7.5 $X10^{13}$ (1x)	Wild type p53	12	INI, IT	Transgene expression 7/8 patients by INI, 0/3 patients by IT. No clinical response shown	(Kuball et al. 2002)
Dryvax vaccinia virus	I	vaccinia	Up to 10^8 (3x)	-	4	INI	3/3 high dose patients show immune response to vaccinia in tumour and normal tissue. 3/4 patients at 4-year were free of disease.	(Gomella et al. 2001)
BC-819 DNA plasmid	I/II	DNA plasmid	2-20mg (up 12X)	intracellular toxin driven by H19 gene regulatory sequences	18	INI	Eight of the 18 patients (44%) had complete marker tumor ablation or a 50% reduction of the marker lesion.	(Sidi et al. 2008)

intravesical instillation (INI), intratumoral injection (IT)

Table 1.2 Clinical trials for bladder cancer with different gene therapy vectors

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1.4.2.2 Suicide gene therapy

Prodrug activation therapy strives to deliver genes to cancer cells, which convert non-toxic prodrugs into active chemotherapeutic agents (Figure 1.4). The net gain is that a systemically administered prodrug can be converted into high local concentrations of an active anticancer drug in the tumour. To be clinically successful, both enzymes and prodrugs should meet certain requirements for this strategy (Niculescu-Duvaz et al. 1998; Xu and McLeod 2001; Yazawa et al. 2002). The prodrug activating genes should be either of non-human origin or a human protein that is absent or expressed only at low concentrations in normal tissues (Rigg and Sikora 1997; Weedon et al. 2000). To provide catalytic activity the prodrug activating genes need to be sufficiently highly expressed in tumour cells. The prodrug should be a good substrate for the activating enzyme in tumours, but not be activated by cellular enzymes in non-tumour cells. An ideal prodrug system will have a bystander effect; that is the ability to kill non-expressing neighbouring tumour cells by the export of toxic metabolites. In addition, the half-life of active drug should be long enough to induce a bystander effect, but short enough to avoid leakage of the drug into the systemic circulation. A number of suicide genes have been examined in different types of cancer including thymidine kinase, rat p450 CYP2B1, human intestinal carboxylesterase, yeast cytosine deaminase.

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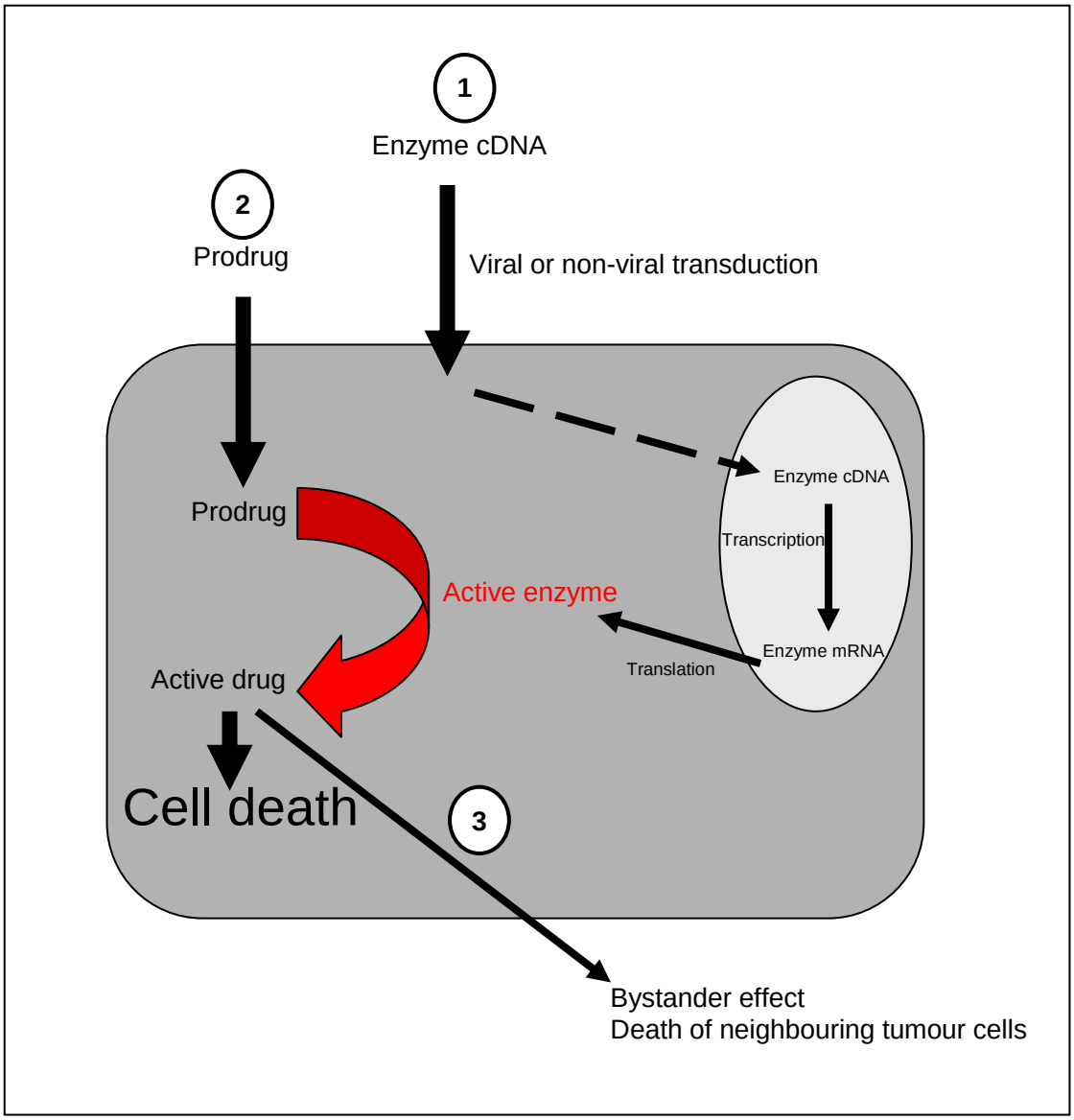

Figure 1.4 The three stages of activity of a prodrug activating system. In the first stage, a prodrug activating gene is transduced into and expressed in tumour cells. In the second stage a non-toxic prodrug is administered to the cells. The prodrug works as a substrate for the activating enzyme resulting in the production of toxic metabolites. These metabolites inhibit DNA or RNA replication resulting in cell death. The final stage is the export of these metabolites to kill the surrounding tumour tissue (bystander effect).

1.4.2.2.1 Thymidine kinase

The thymidine kinase gene system is based on the metabolism of certain purine nucleosides. Thymidine kinase catalyses the phosphorylation of the purine nucleoside analogues Ganciclovir (GCV), Acyclovir (ACV) and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracyl (FIAU) converting them to their corresponding

nucleoside monophosphates, which are catalysed to nucleoside diphosphates by mammalian nucleoside monophosphate kinases and are subsequently converted to the tri phosphate form by nucleoside diphosphate kinase (Miller and Miller 1980). The phosphorylation of these substrates by thymidine kinase into a monophosphate form (eg GCV-MP) is the rate-limiting step in the generation of a cytotoxic drug (Field 1983; Nishiyama and Rapp 1979; Oliver et al. 1985). These prodrug metabolites are able to stall DNA synthesis by inhibiting DNA polymerase and by incorporation into DNA causing chain termination (Davidson et al. 1981; Elion 1980) thus killing dividing cells. It has been shown that therapeutic concentrations of these drugs are non-toxic to cells that lack thymidine kinase, and conversely expression of thymidine kinase is non toxic to cells in the absence of these drugs (Elion 1980; Field 1983). It was tested in retrovirus, adenovirus and has been shown effective in tumour killing *in vitro* and *in vivo* (Yazawa et al. 2002). Viral-mediated transfer of the herpes simplex virus thymidine kinase gene has been demonstrated by several investigators to confer sensitivity to nucleoside analogues such as ganciclovir in brain, prostate, liver, kidney, ovary, head and neck, lung, pancreas, and bladder cancers (Nasu et al. 2000). Oncolytic HSV-1 vectors with intact thymidine kinase should also activate these prodrugs such that infected cells are killed by both the activated prodrug active and oncolysis. This may of course be counterproductive as replication of the oncolytic HSV may also be inhibited. Most studies have shown very little or no synergy between oncolytic HSV vectors and TK mediated prodrug activation, due to the inhibition of virus replication, which occurs. Suicide gene therapy using adenoviral-mediated herpes simplex virus thymidine kinase and the prodrug ganciclovir in an orthotopic murine bladder cancer model demonstrated a greater than threefold reduction in tumor growth. Long-term survival results suggested a survival benefit for the treated animals compared to the control group. (Cheon et al. 2002; Sutton et al. 1997; Sutton et al. 2000). An adenovirus-mediated thymidine kinase suicide gene therapy was tested with different promoters in the treatment of experimental bladder cancer. Cytomegalovirus-immediate-early promoter and the Rous-sarcoma-virus promoters expressing thymidine kinase were both effective (Freund et al. 2000b). A retroviral vector was also tested with thymidine kinase gene plus ganciclovir on human bladder cancer cells and on a nude

mouse model and effective transduction and reduction of tumour volume was shown (He et al. 2004).

1.4.2.2.2 E. coli/yeast cytosine deaminase enzyme

A commonly utilised enzyme prodrug combination is the *E-coli* /yeast enzyme cytosine deaminase (CD) and 5-fluorocytosine (5-FC). CD, a pyrimidine salvage enzyme, is the only known route by which cytosine is metabolised through hydrolytic deamination to uracil and ammonia. CD also deaminates the anti-fungal compound 5-fluorocytosine (5-FC) into 5-fluorouracil (5-FU), a highly toxic compound, widely used as a cancer chemotherapeutic agent (Moolten 1994). This enzyme has been found in prokaryotes and lower eukaryotes, but appears to be absent in higher eukaryotes (Austin and Huber 1993; Kilstrup et al. 1989). Consequently, mammalian cells are resistant to 5-FC but not 5-FU (Mullen et al. 1992; Pirollo et al. 2008). The cytotoxicity of 5-FU is determined by its conversion into 5-fluoro deoxyuridine monophosphate (5-FdUMP) and 5-fluorouridine triphosphate (5-FUTP) (Figure 1.5). 5-FdUMP is an irreversible inhibitor of thymidylate synthase, and depletes replicating cells of the thymidine nucleotide precursor, deoxythymidine triphosphate (dTTP), during DNA synthesis, while 5-FUTP inhibits RNA maturation and processing. The rate limiting step is the formation of an intermediary metabolite, 5-fluorouridine monophosphate (5-FUMP) (Niculescu-Duvaz et al. 1998). It has been reported that the *E. coli* uracil phosphoribosyltransferase (UPRT) can markedly potentiate the anti-tumour effects of 5-FU by converting it directly into its intermediary 5-FUMP, thereby leading to more efficient generation of its active metabolites 5-FdUMP and 5-FUTP (Tiraby et al. 1998). A construct containing a fusion between the yeast Fcy 1 and Fur 1 genes encoding cytosine deaminase and uracil phosphoribosyltransferase, respectively, is commercially available (*Invivogen)* and an identical chimera (Fcy:Fur) has been described by (Erbs et al. 2000). Fcy:Fur exhibited similar UPRTase activity to the natural yeast gene, but CDase activity was 100 fold elevated compared to the yeast CD gene alone.

The mechanism of 5-FU cytotoxicity is unclear, because it is converted to several metabolites which each have different biochemical actions (Nakamura et al. 2001). However, much interest has been placed in a metabolite 5-fluorodeoxyuridylate that

inhibits thymidylate synthase, which has been suggested to inhibit cellular DNA synthesis more than viral DNA synthesis (Nakamura et al. 2001).

The CD/5-FC prodrug activating system has been studied as a potential gene therapy strategy in a number of solid tumours. Investigators have demonstrated the efficacy of the CD gene/5-FC prodrug activating system when treating colon carcinoma (Austin and Huber 1993), pancreatic cancer (Evoy et al. 1997), breast cancer (Anderson et al. 2000; Li et al. 1997), and peritoneal carcinomatosis (Bentires-Alj et al. 2000). Adenovirus-mediated (Block et al. 2000; Ohwada et al. 1996; Topf et al. 1998) transfer of the CD gene showed enchanced local tumour control in colon carcinoma in the presence of prodrug 5-FC.

A murine leukaemia virus-based replication-competent retrovirus containing yeast cytosine deaminase gene plus 5-fluorocytosine dramatically inhibited the tumour growth in an orthotopic bladder animal model (Kikuchi et al. 2007b). An adenovirus-mediated CD gene therapy driven by human telomerase reverse transcriptase promoter was tested and found effective in combination with low-dose etoposide in bladder cancer cells (Shieh et al. 2006). A double suicide gene therapy using adenovirus mediated cytosine deaminase-thymidine kinase fusion gene with ganciclovir or 5-fluorocytosine in a murine subcutaneous bladder carcinoma model was effective by significantly inhibiting tumour growth. The treatment efficacy of combining ganciclovir and 5-fluorocytosine was superior to only ganciclovir or 5-fluorocytosine groups (Tan et al. 2006).

Expression of yeast CD from a replication competent HSV-1 oncolytic virus mediates intra-tumoral conversion of 5-FC to 5-FU, which results in enhanced tumour cell killing compared with the backbone virus alone (Nakamura et al. 2001; Simpson et al. 2006). *In vitro* studies have shown increased killing in cell lines derived from lung cancer (A549, H460), pancreatic cancer (CAPAN-1, MIA PACA-2, BXPC-3) and colon cancer (HCT 116, HT-29, SW620) without inhibiting virus replication (Nakamura et al. 2001; Simpson et al. 2006).

OncoVEX$^{GALV/CD}$, described above, express both a highly potent version of CD (Fcy::Fur) and the fusogenic glycoprotein from gibbon ape leukaemia virus (GALV). In the presence of 5-fluorocytosine (5-FC), OncoVEX$^{GALV/CD}$ showed greatly improved tumour contol *in vitro and in vivo* (Simpson et al. 2006).

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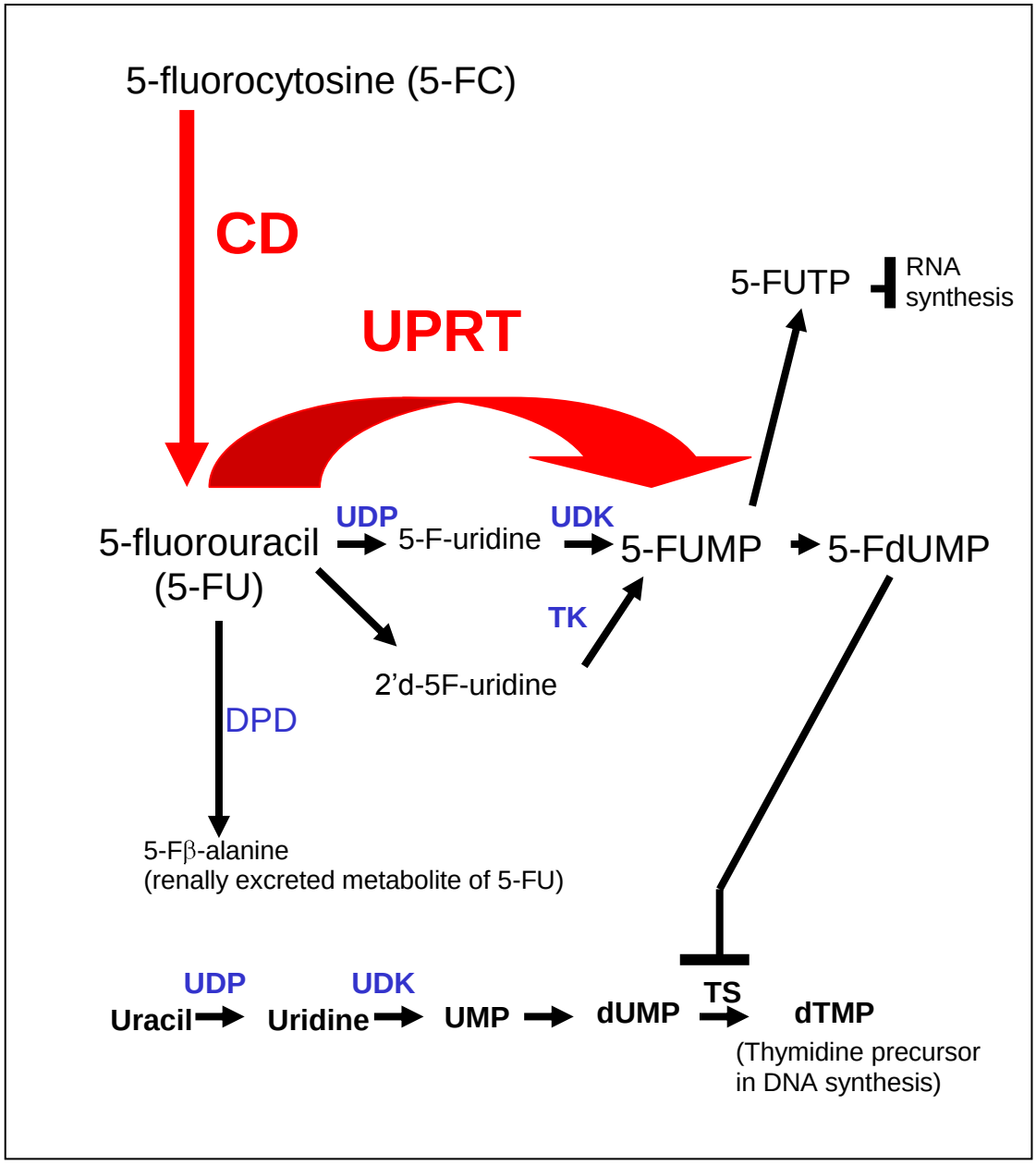

Figure 1.5 The metabolism of 5-fluoro-cytosine (5-FC) by cytosine deaminase (CD), and uracil phosphoribosyltransferase (UPRT) during DNA synthesis. CD converts 5-FC into 5-fluoro-uracil (5-FU). UPRT then converts 5-FU directly to 5-FUMP, which is then converted to its active metabolites 5-FdUMP and 5-FUTP. This bypasses the rate limiting mammalian cellular enzymes such as UDP (uridine phosphorylase), UDK (uridine kinase) and TK (thymidine kinase). This also reduces the degradation of 5-FU by the competitive enzyme, DPD (dihydropyrimidine dehydrogenase). UMP (uridine monophosphate), dUMP (deoxy uridine monophosphate), dTMP (deoxy thymidine monophosphate), 2'd-5F- uridine, (2' deoxy 5-fluoro uridine), TS (thymidylate synthase)

1.4.2.2.3 Rat p450 enzyme and cyclophosphamide

Expression of the rat p450 enzyme from hrR3 (rRp450), in the presence of cyclophosphamide, has also shown oncolysis and prodrug activation *in vitro* without inhibiting virus replication in both colon carcinoma and glioma cells (Aghi et al. 1999; Chase et al. 1998; Ichikawa et al. 2001; Pawlik et al. 2002; Tyminski et al. 2005). *In vivo* studies on liver metastases and glioma models revealed a substantial decrease in the tumour burden in all animals treated with rRp450 (+cyclophosphamide) compared to controls (Aghi et al. 1999; Chase et al. 1998; Ichikawa et al. 2001; Pawlik et al. 2002).

1.4.2.2.4 Human intestinal carboxylesterase

Recently p450 has been co-expressed from an ICP34.5- and ICP6- mutant HSV with another prodrug activating gene, intestinal carboxylesterase (Tyminski et al. 2005). This new oncolytic herpes virus (MGH2) displays increased anti-tumour efficacy against human glioma cell lines (U251, T98G) when combined with the prodrugs, cyclophosphamide and CPT-11 (Tyminski et al. 2005). *In vivo* studies using MGH2 in glioma models have indicated an additive benefit of each of the pro-drugs (Tyminski et al. 2005).

1.4.2.2.5 Sweet almond beta-glucosidase

Amygdalin as a prodrug is a naturally occurring cyanogenic glycoside, which can be cleaved by sweet almond beta-glucosidase to yield free cyanide. If amygdalin is activated specifically at the tumour site, then malignant cells are killed without the systemic toxicity usually associated with chemotherapy. Beta-glucosidase was conjugated to a tumour-associated monoclonal antibody (MAb) (HMFG1) and the conjugate has been tested in vitro. The combination of amygdalin with HMFG1-beta-glucosidase enhanced the cytotoxic effect of amygdalin by 36-fold on HT1376 bladder cancer cells (Syrigos et al. 1998).

1.4.2.2.6 Escherichia coli purine nucleoside phosphorylase

In another *in vivo* model intratumoral injection of a replication-competent retroviral vectors delivering Escherichia coli purine nucleoside phosphorylase (PNP) followed by systemically administered fludarabine phosphate, significantly inhibited the growth of pre-established KU-19-19 tumours (Kikuchi et al. 2007a).

1.4.2.2.7 Horseradish peroxidase

The plant enzyme horseradish peroxidase (HRP) and the plant hormone indole-3-acetic acid (IAA) have also been used effectively as an enzyme/prodrug combination killing T24 bladder carcinoma. The activated drug is a long-lived species able to cross cell membranes, and cell contact appears not to be required for a bystander effect to take place (Greco et al. 2000)

1.4.2.3 Fusogenic membrane glycoproteins

One of the approaches for cancer gene therapy is the transduction of tumour cells with viral fusogenic membrane glycoproteins (FMG) (Bateman et al. 2000). A number of different viruses kill their target cells by causing fusion between infected and non-infected cells, via the interaction between the viral envelope with its receptor. This fusion of uninfected cells with infected cells represents a type of bystander effect. The fusogenic portions of membrane glycoproteins have been identified for the VSV, measles virus and HIV-1 (Cathomen et al. 1998; Duus et al. 1995; Laurent-Crawford et al. 1995; Sodroski et al. 1986). Transduction of these viral glycoproteins alone into human tumour cells has been shown to induce cell to cell fusion and formation of lethal multi-nucleated syncytia (Cathomen et al. 1998; Sodroski et al. 1986). The cytotoxic activity of three envelope genes from different groups of viruses was originally evaluated (Bateman et al. 2000), these include the rhabodoviral VSV-G envelope gene, the combination of F and H genes from measles virus and a mutated version of the retroviral Gibbon Ape Leukaemia Virus (GALV *env* R-) (Bateman et al. 2000). In this early *in vitro* study all three membranes showed a high level of cell killing, with GALV *en*v R- showing the highest killing. Truncation of the GALV protein by removing 16 amino acids in the transmembrane R-peptide, which normally serves to restrict fusion of the envelope until it is cleaved during viral infection renders it constitutively highly fusogenic and therefore cytotoxic to human tumour cells (Diaz et al. 2000).

Transfection with GALV env R- or measles proteins F and H induces the formation of multi-nucleated syncytia, bound by a single cellular membrane encompassing nuclei that individually still retain clearly defined nuclear membranes (Bateman et al. 2000). Typically, these syncytia remain metabolically active and alive for anywhere from 12-120 hours post-transfection. However, by 120 hours post-transfection, >90% of cells in culture are dead (Bateman et al. 2000). Recruitment of bystander cells into the syncytium occurs by a process that appears to involve the organization of incoming nuclei along actin and microtubule bundles, such that large numbers of nuclei are localized close together. Later, adjacent nuclei can fuse together, but classic features of apoptosis, such as nuclear condensation or fragmentation and caspase activity are not detected (Bateman et al. 2002; Higuchi et al. 2000).

Early studies *in vitro* showed that transfected FMG were able to cause cell fusion in a wide range of human cell lines including: 293, Mel624 (human melanoma), HT1080 (human fibrosarcomas), Hela (cervical carcinoma) and Tel.CeB6 (human rhabdomyosarcoma) (Bateman et al. 2000). Before FMG were successfully encoded into viral vectors, they were *in vivo* tested by transfecting plasmids in tumour cell lines, followed by injection of transfected cells into the flanks of nude mice. Transfected control plasmids showed no tumour regression of Mel624 and HT1080 cells in contrast cells transfected with FMG (in this case GALV *env* R-) under the CMV promoter showed up to 100% tumour regression *in vivo* nude mice models (Bateman et al. 2000).

Early attempts to produce adenovirus virus vectors encoding GALV *env* R- failed because rapid cell fusion inhibited adenovirus replication (Diaz et al. 2000). Therefore retroviral and lenti-viral delivery was investigated both of which encountered problems in generating high titre virus stocks. Such vectors were tested against human tumour xenografts, growing in nude mice, and resulted in considerable reduction in size of tumours compared to control vectors (Diaz et al. 2000).

The authors detected, in GALV *env* R- treated tumours, several immune stimulatory molecules associated with a stress response, such as heat shock proteins (Melcher et al. 1998) the NK cell receptor (Groh et al. 1998) and murine IFN-γ. This suggests that the immune system may partly contribute to the mode of action of GALV *env* R- even in immuno-compromised animals such as nude mice.

The problems of expression were solved by encoding GALV *env* R- in HSV, initially inserted into strain G207 (Fu et al. 2003) and later a second generation virus OncoVEX^{GALV} (Simpson et al. 2006). These showed no impairment of viral replication such that virus stocks could easily be produced (Simpson et al. 2006). The expression of GALV *env* R- caused syncytia formation in tumour cells of a variety of origins including from colon, brain, pancreas, lung, liver, prostate and connective tissue cancers (Fu et al. 2003; Simpson et al. 2006). This fusogenic property also translated into a significantly increased tumour cell killing as compared to previous versions of oncolytic HSV vectors (Fu et al. 2003; Simpson et al. 2006). In rodent tumour models, improved tumour shrinkage was seen with an approximately 5-10 fold reduction in the virus dose required to give equivalent tumour shrinkage or cure (Fu et al. 2003; Simpson et al. 2006).

1.4.2.4 Immunotherapy

The current therapy for some high risk patients with non-muscle invasive bladder cancer is intravesical BCG therapy. The bacteria are taken up into the bladder mucosa and this leads to an early immune response resulting in the release of several cytokines including TNFα, IL1, IL2, IL12 and interferon γ. These cytokines may exert their effects though the humoural or cellular immunity or through direct tumour cytotoxicity (Poppas et al. 1998). Some groups have sought to recreate this immune stimulation through gene therapy (Kausch et al. 2002). One such approach was to use the innate immunogenicity of an adenovirus which was engineered to carry an immunostimulatory gene IFNα. This was then used in combination with Syn3 to improve adenoviral transduction to human bladder cancer cells forming a tumour in nude mice (Benedict et al. 2004). High local concentrations of IFNα were achieved together with marked tumour regression and little toxicity to normal cells.

1.4.2.5 Antiangiogenesis

Inhibition of the process by which tumours develop a new blood supply, angiogenesis, is another potential approach to bladder cancer gene therapy. Work carried out by Shichionhe et al (Shichinohe et al. 2001) used a vector based on vesicular stomatitis virus (VSVG) to efficiently transduce bladder cancer cells with endostatin and

angiostatin *in vitro.* The vector demonstrated preferential transduction of the tumour cells over the endothelial cells.

1.4.2.6 RNAi and antisense technology

Most oncogenes can be targeted by antisense oligonucleotides in bladder cancer and it is a relatively established technique (Goodchild 2004). Antisense oligonucleotides have also been used before chemotherapy to help sensitize the tumour cells (Duggan et al. 2002). Hong et al. observed that certain cisplatin resistant cells lines which had elevated levels of BCL-2 became sensitive to cisplatin after treatment with an antisense BCL2 oligonucleotide (Hong et al. 2002).

The use of RNAi technology in bladder cancer is still relatively novel but is an exciting technology for gene therapy of bladder cancer (Bartz and Jackson 2005). RNAi is a process whereby double-stranded RNA molecules known as siRNAs promote specific and potent gene silencing by binding to the target gene and initiating degradation of the message before translation can occur. One study used siRNA to silence Cdc42 in two human bladder cancer cell lines, EJ and T24. RNAi mediated silencing led to a marked decrease in Cdc42 mRNA and protein expression but moreover induced apoptosis of EJ cells (Wu et al. 2008).

1.4.2.7 Combination therapies

The field of molecular cancer therapy using viral vectors embraces a range of technologies including direct attack on tumour cells using oncolytic viruses or prodrug activating systems, and enhancement of the immune response to tumour antigens. The combination of these new treatment modalities (such as the gene therapy with viral vectors) with the conventional therapies (chemotherapy, radiotherapy) might be a promising alternative therapeutic option.

Ionizing radiation and chemotherapy approaches to cancer therapy destroy dividing cells because tumour cells divide more rapidly than normal cells. These therapies have to be balanced between causing maximum damage to the cancer cells, while keeping toxicity in normal host cells within acceptable levels. The combination of conventional therapies and the gene therapy may further improve the efficacy. (Advani et al. 1997; Advani et al. 1999; Chahlavi et al. 1999; Stanziale et al. 2002; Toyoizumi et al. 1999).

It was previously shown that a replication-selective herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) was effective both *in vitro* and *in vivo* against human non-small cell lung cancer (NSCLC) cell lines but complete eradication of tumor has not been accomplished with a single viral treatment in a murine xenograft model (Toyoizumi et al. 1999). Mitomycin-C is a natural anti-tumor antibiotic, used clinically as a chemotherapy agent. The bioreductive activation of MMC allows for alkalization of guanine residues leading to DNA inter and intra-strand cross-linking (Paz et al. 1999). Toyoizumi et al evaluated the oncolytic activity of HSV-1716 in NCI-H460 cells in combination with mitomycin C (MMC). The oncolytic effect of HSV-1716 in combination with MMC was synergistic in two of five NSCLC cell lines. In the other three cell lines, the combined effect appeared additive and no antagonism was observed. The in vivo effect of this combination was then examined in a murine xenograft model. NCI-H460 flank tumors were directly injected with HSV-1716 followed by intravenous MMC administration 24 hours later. After 3 weeks, the mean tumor weight in the combined treatment group was significantly less than either individual treatment in an additive manner. The synergistic dose of MMC neither augmented nor inhibited viral replication in vitro and HSV-1716 infection did not upregulate DT-diaphorase, which is the primary enzyme responsible for MMC activation (Toyoizumi et al. 1999).
Mullerad et al tested the strain of HSV called NV1066 in combination with MMC in KU19-19 and SKUB cell lines and they found significantly improved cell kill for the combination treatment ($P<0.001$) (Mullerad et al. 2005). Based on the nine different combination ratios tested, it was determined that a ratio of 1:10 NV1066 to mitomycin-C conveyed the strongest synergistic effect, as the combination index achieved for this ratio was the lowest. The combination of the oncolytic replication competent herpes simplex virus type-1 (HSV) and mitomycin C as a chemotherapeutic agent demonstrated synergistic effect with about 90% estimated cell killing in an orthotopic murine model (Mullerad et al. 2005).

Other types of vector, in addition to herpes simplex viral gene therapies, were tested in combination with conventional therapies. *In vitro* treatment with an adeno-associated virus-2 vector encoding p53 gene treatment in combination with cisplatin showed a synergistic killing effect on the 5637 bladder cell line (Ruifa et al. 2006). The systemic administration of recombinant IL-2 in combination with ADV/RSV-HSV-tk gene

therapy with acyclovir was tested in a murine bladder tumor model and exhibited an enhanced antitumor effect (Terao et al. 2005). The combination of adenoviral vector containing wild-type human p53 gene (Ad5CMV-p53) and cisplatin revealed synergy which is a potential method for overcoming resistance (Pagliaro et al. 2003a).

Gene therapy combined with radiation represents another new approach for cancer treatment. An adenovirally directed, cytosine deaminase plus 5-fluorocytosine gene therapy was tested in combination with radiotherapy. This combination resulted in enhanced tumour cell killing of human bladder-cancer cells *in vitro* and enhanced tumour-growth inhibition in the KK47 subcutaneous tumour model (Zhang et al. 2003).

1.4.3 Vectors for bladder cancer gene therapy

1.4.3.1 Plasmids

The potential problem of inadvertent systemic release of viral vectors from the bladder has driven the search for non-viral vectors for gene transfer (Table 1.3). Plasmid vectors overcome this but have low efficiency of gene transfer. *In vitro* electroporation of HSVTK plasmid into chemically induced rat urinary bladder carcinomas cells results in between 50-70% cell death after administration of ganciclovir (GCV) (Shibata et al. 2003). A significant increase in apoptosis was also observed after a single electroporation *in vivo* followed by GCV i.p. administration (Shibata et al. 2003). BC-819 is a DNA plasmid that contains H19 gene regulatory sequences that drive the expression of an intracellular toxin. In patients with low grade superficial bladder cancer which expresses H19 intravesical BC-819 caused tumour ablation following intravesical administration (Sidi et al. 2008). The polycation poly(ethylenimine) was used to deliver plasmids coding for various combinations of caspases to Cox-2 overexpressing bladder and colon cancer cell lines. The combination of caspase 3 plus caspase 9 was the most effective and produced phosphatidylserine flipping in addition to fragmentation of genomic DNA (Zhang et al. 2009). In a recent study the authors used a nanocomplex consisting of the RB94 plasmid encapsulated by a cationic liposome. The potent tumor suppressor RB94 was delivered efficiently and it was effective for genitourinary and other cancers (Pirollo et al. 2008). Electroporation has also been tested as a method for gene transfer in a rat bladder model. For example a

plasmid encoding the neuronal nitric oxide synthase gene was injected into the bladder and electroporation was applied. The authors found a marked neuronal nitric oxide synthase immunoreactivity, and the released nitric oxide was significantly greater than in the control groups (Yoshida et al. 2008).

Table 1.3 DNA plasmid vectors using within bladder cancer models

Plasmid vectors	**Therapeutic gene**	**Permeability and Bioadhesion Agents**	**In vivo model**	**References**
pHSV*tk*	HSV TK + GCV	electrogene transfer	Chemically induced orthotopic tumour model	(Shibata et al. 2003)
Caspase plasmisds	combination of caspase genes	poly(ethylenimine)	In vitro only work	(Zhang et al. 2009)
RB94	RB110 tumour suppression	cationic liposome	Orthotopic bladder tumour model	(Pirollo et al. 2008)
pcDNA3.1-mouse nNOS	neuronal nitric oxide synthase gene	Electroporation	Orthotopic bladder model (smooth muscles)	(Yoshida et al. 2008)

1.4.3.2 Viral vectors for bladder cancer

A range of viral vectors have been used for gene therapy of bladder cancer. Within each subset of viral vectors work has been carried out on both non-replicating vectors (Table 1.4), which offer strong therapeutic gene expression, and on replication competent/oncolytic vectors (Table 1.5) which offer tumour selective kill due to viral replication and even stronger therapeutic gene expression due to multiple rounds of replication and promoter up regulation (Ring 2002).

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Viral vectors	Therapeutic gene	In vivo model	References
NRC retrovirus	CD40 ligand	Subcutaneously inoculated preinfected tumours (MBT2-CD40L) as vaccine.	(Kimura et al. 2003)
NRC retrovirus	prothymosin alpha (ProT)	Subcutaneously inoculated pre-infected tumours with and without prothymosin alpha (ProT)	(Shiau et al. 2001a)
NRC retrovirus	interferon-gamma (IFN-gamma)	Subcutaneously inoculated pre-infected tumours with and without interferon-gamma (IFN-gamma)	(Shiau et al. 2001a)
NRC Lentivirus	angiostatin and endostatin	In vitro only	(Shichinohe et al. 2001)
NRC Lentivirus	endostatin	Orthotopic bladder tumor model	(Kikuchi et al. 2004)
NRC Lentivirus	Mutant human telomerase	Kidney capsule athymic nude mouse model	(Li et al. 2004)
Adeno-associated virus-2	p53	In vitro only	(Ruifa et al. 2006)
NRC Adenovirus	p53	Orthotopic bladder tumor model	(Werthman et al. 1996)
NRC Adenovirus	Rb	Subcutaneously inoculated of pre-infected tumours with and without Rb (xenograph model)	(Xu et al. 1996)
NRC Adenovirus	p21	In vitro only	(Hall et al. 2000)
NRC Adenovirus	p53	Orthotopic bladder model Biosafety	(Perrotte et al. 2000)
NRC Adenovirus	p53	In vitro only	(Irie et al. 2001)
NRC Adenovirus	MMAC1/PTEN + doxorubicin	In vitro only	(Tanaka et al. 2000)
NRC Adenovirus	p16	In vitro only	(Lee et al. 2001)
NRC Adenovirus	p53 + cisplatin	Subcutaneous and orthotopic tumor models.	(Miyake et al. 2000)
NRC Adenovirus	p53 + cisplatin or doxorubicin	In vitro only	(Shirakawa et al. 2001)
NRC Adenovirus	thymidine kinase gene +GCV	Orthotopic bladder tumor model	(Sutton et al. 1997; Sutton et al. 2000)
NRC Adenovirus	thymidine kinase gene +GCV	Chemical induction of a orthotopic rat model	(Akasaka et al. 2001)
NRC Adenovirus	thymidine kinase gene +GCV +doxorubicin, cisplatin or mitomycin C or or methotrexate	In vitro only	(Freund et al. 2003)
NRC Adenovirus	thymidine kinase gene +GCV	Subcutaneous tumors model	(Freund et al. 2000b)
NRC Adenovirus	interferon–α2b + Syn 3	Intravesical rat non tumour model	(Connor et al. 2005)
NRC Adenovirus	interferon–α2b + Syn 3	Intravesical rat tumour model	(Tao et al. 2006)
NRC Adenovirus	interferon–α2b	Intravesical murine tumour model	(Adam et al. 2007)

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NRC Adenovirus	TRAIL gene	In vitro only	(Griffith et al. 2000)
NRC Adenovirus	TRAIL gene + Trichostatin A	In vitro only	(El-Zawahry et al. 2006)
NRC Adenovirus	connexin 26 (Cx26) gene + cisplatin	Subcutaneous nude mice models.	(Tanaka and Grossman 2001)
NRC Adenovirus	wild-type gelsolin	Orthotopic bladder cancer nude mouse model	(Sazawa et al. 2002)
NRC Adenovirus	anti-sense to IL-8	Subcutaneous nude mice models.	(Inoue et al. 2000)
NRC Adenovirus	anti-sense to IL-8	Subcutaneous nude mice models.	(Inoue et al. 2001)
NRC Adenovirus	human CD40 ligand +fluorouracil, cis-platin and mitomycin C	Subcutaneous nude mice models	(Vardouli et al. 2009)
NRC Adenovirus	Fas ligand gene fused to GFP	In vitro only	(Sudarshan et al. 2005)
ALVAC, NYVAC NRC vaccinia virus	beta-galactosidase and firefly luciferase	Mouse orthotopic bladder tumour model.	(Siemens et al. 2003)

Table 1.4 Non replication competent (NRC) viral vectors used within bladder cancer models

Viral vectors	Therapeutic gene	In vivo model	References
RC retroviral (RCR) vector	cytosine deaminase gene +5-FC	Orthotopic bladder tumor model	(Kikuchi et al. 2007b)
RC retroviral (RCR) vector	Escherichia coli purine nucleoside phosphorylase (PNP)	Orthotopic bladder tumor model	(Kikuchi et al. 2007b)
RC Adenovirus	-	Mouse orthotopic bladder tumour model.	(Wang et al. 2006)
wild-type VSV or a mutant Delta51M variant (AV3)	-	Orthotopic bladder cancer nude mouse model, tumor growth was quantified using bioluminescence imaging	(Hadaschik et al. 2008)
Wild type Reovirus	-	Intravesical rat tumour model	(Hanel et al. 2004)
RC vaccinia virus	p53	Mouse orthotopic bladder tumour model.	(Fodor et al. 2005)
RC pseudorabies virus	-	Mouse orthotopic bladder tumour model.	(Shiau et al. 2001b)
RC HSV-1 G207 and Nv1020	-	Mouse orthotopic bladder tumour model.	(Cozzi et al. 2001)
RC HSV-1 mutant HF10	-	Mouse orthotopic bladder tumour model.	(Kohno et al. 2005)
RC HSV-1	cytosine deaminase + fusogenic glycoprotein from gibbon ape leukaemia virus (GALV)	Rat orthotopic bladder tumour model.	See in this thesis.

Table 1.5 Replication competent (RC) viral vectors used within bladder cancer models

1.4.3.2.1 Retroviral vectors

In a recent study murine leukemia virus (MuLV)- based replication competent retroviral (RCR) vector was able to transduce a wide range of bladder cancer cells in a subcutaneous bladder tumour model (Bochner 2008). However the same group also found that intravesical administration of a replication competent retroviral vector in an orthotopic murine bladder tumour model led to low transduction efficiencies (20-60%). In contrast highly efficient transduction was observed (85%) in a KU-19-19 human tumour model using intravesically instilled replication competent retroviral vectors (Kikuchi et al. 2007b). Mice treated with replication competent retroviral vector containing cytosine deaminase gene plus 5-fluorocytosine showed a 50% survival rate compare to 0% survival rate in control groups over a 70 day period (Kikuchi et al.

2007b). In another *in vivo* model intratumoral injection of a replication-competent retroviral vector delivering Escherichia coli purine nucleoside phosphorylase (PNP) followed by systemically administered fludarabine phosphate, significantly inhibited the growth of pre-established KU-19-19 tumours (Kikuchi et al. 2007a). Antitumor immunity induced by a retroviral vector expressing CD40L was effective in a mouse vaccination model and suggested that immunogene therapy using CD40L may be a new strategy in the treatment of bladder cancer (Kimura et al. 2003). A replication-defective recombinant retroviral vector encoding prothymosin alpha (ProT), a putative thymic hormone, exhibited smaller tumour mass, lower tumor incidence and higher survival rates, as well as higher antitumor cytotoxic activities in a mouse model. ProT exerts antitumor effects through its immunomodulatory activities (Shiau et al. 2001b). Gene therapy based on the delivery of interferon-gamma (IFN-gamma) was also tested using retroviral vectors. A recombinant retroviral vector showed lower tumor incidence, lower tumor mass, and higher survival rate, as well as higher antitumor responses in a mouse model (Shiau et al. 2001a).

1.4.3.2.2 Lentiviral vectors

These have been shown to be capable of efficient and stable gene transfer to a variety of human bladder tumor cell lines. Using self-inactivating lentiviral vectors containing green fluorescent protein in combination with endostatin sustained long-term expression of endostatin was achieved in an orthotopic bladder tumour model and it was associated with decreased vascularisation and inhibition of tumor growth (Kikuchi et al. 2004). Long-term expression and secretion of angiostatin and endostatin was also found in lentivirus transduced T24 cells, which resulted in significant inhibition of cellular proliferation on co culture with endothelial cells (Shichinohe et al. 2001). These reports suggest the potential utility of antiangiogenic gene therapy with lentiviral vectors for the treatment of cancer. In a recent study a lentiviral system was used to efficiently overexpress a mutant-template human telomerase RNA to add mutant DNA to telomeres in cancer cells. The overexpression of this mutant-template human telomerase RNA inhibited cell growth and induced apoptosis in telomerase-positive precancerous or cancer cells but not in telomerase-negative cells (Li et al. 2004).

1.4.3.2.3 Adeno-associated virus-2

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Mutations in the p53 tumor suppressor gene are the most common molecular genetic abnormalities in bladder cancer and p53 gene transfer in the human bladder cancer cell line by adenoviral or other vectors has been demonstrated to be cytotoxic. AAV encoding the p53 gene has been shown to enter bladder cancer cells and express its gene products which are cytotoxic to bladder cancer cells. Additionally in vitro treatment with a adeno-associated virus-2 vector encoding p53 gene treatment in combination with cisplatin showed synergistic killing effect on the 5637 bladder cell line (Ruifa et al. 2006).

1.4.3.2.4 Adenoviral vectors

It was first shown in the 1990's that replication-defective adenoviral vector can infect and express transgenes within bladder cells, offering possibility for therapeutic gene transfer (Bass et al. 1995; Morris et al. 1994; Watanabe et al. 2000; Werthman et al. 1996) . Using adenoviral vectors cell cycle related genes, including wild type p53, p21, MMAC1/PTEN, p16 and N-terminal truncated retinoblastoma (RB) protein, were strongly expressed in bladder cancer models *in vitro* and *in vivo* but with limited efficacy (Hall et al. 2000; Irie et al. 2001; Lee et al. 2001; Perrotte et al. 2000; Tanaka et al. 2000; Werthman et al. 1996; Xu et al. 1996). Efficacy of such cell cycle related genes within adeno vectors has been shown to improve in the presence of chemo agents such as cisplatin or doxorubicin (Miyake et al. 1998; Miyake et al. 2000; Shirakawa et al. 2001; Tanaka et al. 2000). Phase 1 studies using replication deficient adenovirus containing wild type p53 gene showed safe administration in locally advanced bladder cancer patients (Kuball et al. 2002; Pagliaro et al. 2003b).

A wide range of suicide genes have also been expressed by replication-defective adenoviral vectors within bladder cancer. These include the thymidine kinase gene (Akasaka et al. 2001; Freund et al. 2000a; Freund et al. 2000b; Freund et al. 2003; Sutton et al. 1997). The interferon alpha protein has antitumour effect through prolongation of the cell cycle, inhibition of biosynthetic enzymes and apoptosis. The intravesical delivery of the interferon–α2b gene with a replication deficient adenoviral vector led to a strong increase in interferon concentration in the bladder (Connor et al. 2005). In interferon-alpha infected cells vascular endothelial growth factor was decreased and anti-angiogenic interferon-gamma inducible protein 10 was up-regulated.

This led to an increased angiostatic activity of the bladder cancer microenvironment (Adam et al. 2007). Using a mouse superficial bladder cancer model it was shown that the efficacy of a single intravesical treatment with an adenovirus encoding human interferon alpha plus Syn 3 (gene transfer enhancing agent) is dose dependent and the efficacy was also correlated with the level of interferon produced in the urine after treatment (Tao et al. 2006).

TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily of cytokines that induces apoptosis in a variety of cancer cells. Introduction of the human TRAIL gene into TRAIL-sensitive tumor cells using an adenoviral vector led to the rapid production and expression of TRAIL protein, and subsequent death of the bladder tumor cells *in vitro* (Griffith et al. 2000). In a recent study a histone deacetylase inhibitor trichostatin A treatment enhanced the efficacy of an adenovirus that expresses TNF-related apoptosis-inducing ligand (AdTRAIL). Trichostatin A enchances the adenovirus efficacy by restoring CAR expression and by generating a more pro-apoptotic phenotype that would facilitate bystander activity of TRAIL (El-Zawahry et al. 2006). The modulation of CAR expression by inhibition of histone deacetylation (ie. valproic acid) has proved useful in improving the efficacy of adenoviral therapy (Bochner 2008). Ipriflavone and plant isoflavones were also found to exhibit the ability to induce the CAR gene in combination with a histone deacetylase inhibitor (FK228)(Pong et al. 2006). The addition of butyrate was found to increase the transduction rate of adenovirus and increase the antitumour effect of adenovirus-p16 in bladder cancer cell lines with little CAR expression. (Lee et al. 2001)

The connexin 26 (Cx26) gene encodes a protein involved in gap junctional intercellular communication and is a putative tumour suppressor. Infection with a Cx26 expressing adenoviral vector suppressed the growth of human bladder cancer cell lines such as UM-UC-3, UM-UC-6, UM-UC-14, and T24 *in vitro* and prevented tumour formation *in vivo* (Tanaka and Grossman 2001). This adenoviral vector was also shown to have dramatic synergy *in vivo* with cisplatin (Tanaka and Grossman 2001). Gelsolin is an actin regulatory protein that is undetectable or reduced in human bladder tumours compared with normal epithelial cells. Over expression of wild-type gelsolin from an adenoviral vector inhibits tumour growth in an orthotopic bladder cancer nude mouse

model by causing cells to arrest or delay at the G2/M phase of the cell cycle (Sazawa et al. 2002).

Replication-defective adenoviral vectors offer a stable platform for anti-sense expression to target genes within bladder cancer cells. Down regulation of basic fibroblast growth factor or IL-8 has been shown resulting in endothelial cell apoptosis and significant tumour growth inhibition in vivo within a bladder model A replication defective recombinant adenoviral vector expressing human CD40 ligand (RAd-hCD40L), (a tumour necrosis factor receptor) caused anti-proliferative and pro-apoptotic effects on human bladder carcinoma cell lines *in vitro* and suppressed cancer growth in a mouse xenograft tumour model (Vardouli et al. 2009). A replication-deficient adenovirus containing a modified Fas ligand gene fused to green fluorescent protein (AdGFPFasL) was cytotoxic to bladder cancer cells that would otherwise be considered Fas resistant (Sudarshan et al. 2005). A recent study investigated the therapeutic effect of a double-restricted oncolytic adenovirus (AxdAdB-3 containing a mutant E1A and an E1B-55KD deletion) on human bladder cancer cell lines and on a mouse orthotopic bladder tumour model. AxdAdB-3 was found to be effective in tumour growth inhibition, leading to significantly prolonged survival (Wang et al. 2006).

1.4.3.2.5 Vesicular stomatitis virus (VSV)

VSV is an oncolytic, enveloped, negative-sense RNA virus that selectively replicates in IFN-resistant cells, but is strongly suppressed in IFN-responsive normal tissues. Human bladder cancer cell lines were treated with either wild-type VSV or a mutant Delta51M variant (AV3). Both viruses could kill the more aggressive IFN-nonresponsive bladder cancer cell lines, whereas IFN-responsive cells were less susceptible. In a mice bladder tumour model VSV instillation therapy showed promising antitumor activity and safety (Hadaschik et al. 2008).

1.4.3.2.6 Reovirus

Reovirus is a non-pathogenic virus in humans, which is found in the respiratory and enteric tracts. Reovirus causes oncolysis through the overactivation of Ras signalling cascade of target cells (Coffey et al. 1998; Strong et al. 1998). In untransformed cells

the reovirus activated protein kinase blocks viral protein translation. In tumours with activated Ras this protein kinase activation is inhibited, allowing viral replication and oncolysis (Hanel et al. 2004). In an *in vitro* co-culture spheroid bladder cancer model reovirus selectively destroyed the transformed cells by lysis or induction of apoptosis (Kilani et al. 2003). In a rat orthotopic bladder tumour model AY-27 cells were susceptible to reovirus oncolyis. The animal survival was dose dependent and a survival rate of 90% was found in the reovirus group compared to 50% survival rate in the BCG treated group (in a 100 day period) (Hanel et al. 2004). Systemic administration of reovirus in immunocompetent animals provokes an antiviral immunity that limits the efficacy of reovirus efficacy (Hirasawa et al. 2003), but intravesical administration of reovirus may avoid this limitation.

1.4.3.2.7 Poxviruses

Poxviruses have several advantages as potential agents for cancer therapy. They can be used as an oncolytic vector and as vectors for gene delivery. The vaccinia virus can replicate in mammalian cells, is highly cytolytic for a broad range of tumour types, and is genetically stable and safe. The vaccinia virus can be genetically modified and has a large capacity for the delivered genes (Harrington et al. 2008). Recombinant vaccinia virus is effective in introducing foreign antigens locally into tumors *in vivo*, supporting its use in clinical immunotherapy (Lee et al. 1994). A recombinant vaccinia virus expressing human p53 can induce the death of MB-49 bladder tumor cells *in vivo*, not only through the lytic effect of the virus, but also through expression of the death inducing p53 transgene (Fodor et al. 2005). The avipox virus is not able to replicate in mammalian cells although they have been used as a means of delivering immunogenic genes. The canarypox virus (ALVAC) can infect mammalian cells, but it is restricted to avian species for replication. It has been shown to be a safe and effective vector in humans (Taylor et al. 1991). The attenuated vaccinia virus (NYVAC) is made nonlytic in mammalian cells. The efficacy of gene transfer by ALVAC, NYVAC and replication competent adenovirus was compared and the ALVAC and NYVAC was found better than adenovirus *in vitro* and in an orthotopic murine bladder cancer model (Siemens et al. 2003). A phase I trial using intravesical vaccinia virus in patients with muscle invasive transitional cell carcinoma has been conducted. Bladder histology samples

after cystectomy showed evidence of viral infection in tumor and normal urothelial cells and there were no signs of vaccinia related toxicity except mild dysuria. This study demonstrates that vaccinia virus can be administered safely into the bladder (Gomella et al. 2001).

1.4.3.2.8 Pseudorabies virus

A mutant pseudorabies virus (YP2) which was carrying glycoprotein D and herpes simplex virus type 1 thymidine kinase genes was recently studied on human bladder, mouse bladder, and hamster oral cancer cells *in vitro*. The aim of this study was to target HER-2/neu-overexpressing cancer cells. YP2 replicated selectively in and lysed the HER-2/neu-overexpressing cells. In a mouse orthotopic bladder tumour model YP2 retarded MBT-2 bladder tumour growth by more than 50% and more than half of the treated mice survived for over 50 days whereas control mice survived less than 30 days (Shiau et al. 2007).

1.4.3.2.9 Herpes simplex virus

Herpes simplex virus will be discussed later in section 1.6.

1.5 Oncolytic viruses

1.5.1 Historical background of oncolytic viruses

It is likely that the use of viruses in the treatment of cancer was the result of the observation that clinical remission was occasionally seen in patients who contracted an infectious disease. These early reports were almost exclusively related to haematological malignancies which are associated with significant immune suppression. As early as 1904 there were reports of the beneficial effects seen in patients with leukaemia who developed influenza (Dock 1904). More recent clinical reports have described the regression of leukaemia, Hodgkins's disease, and Burkitt's lymphoma in association with measles infection (Bluming and Ziegler 1971; Gross 1971; Pasquinucci 1971; Taqi et al. 1981; Zygiert 1971). From these reports investigators have deduced the following: occasionally, certain viruses can destroy cancer cells without causing undue harm to the patient, this is most often seen in patients with a suppressed immune

system, and these virally-induced remissions are short-lived and incomplete (Kelly and Russell 2007).

Formal evaluation of viral therapy began in 1949 with a study looking at hepatitis in 22 patients suffering from Hodgkin's disease (Hoster et al. 1949). 7 of 13 patients, who developed hepatitis, showed improvement lasting over a month or more. Over the next twenty years a large number of different human pathogens were assessed in clinical trials. Early trials focused on West Nile virus, but limited responses and concerns regarding neurological side effects meant that its development was dropped (Southam and Moore 1952). Attention then turned to adenoviruses and herpes viruses. Adenoviruses were initially viewed with enthusiasm having limited side-effects and dramatic responses after intra-tumoural injection. However, it became clear that adenoviral infection was rapidly cleared by the immune system, especially in those with pre-existing exposure to adenovirus (Kelly and Russell 2007). The lack of significant efficacy coupled with the risks of using non-attenuated human pathogens led to a temporary hiatus in oncolytic virotherapy research.

Methods to limit the pathogenicity of oncolytic viruses were attempted as early as the 1950s. The concept of viral adaptation, whereby the oncolytic potential of a virus was enhanced by numerous passages in a given tumour, had been proven by Moore et al (Moore 1952). They rightly assumed that viruses would develop mutations that would enhance replication in the cells in which they were propagated. However, it was not until the advent of recombinant DNA technology in the early 1990s that the prospect of altering the selectivity of viruses became possible. The first studies to use this approach was published in 1991 by Martuza et al. and Maclean et al. using a genetically modified Herpes Simplex virus (HSV) (MacLean et al. 1991; Martuza et al. 1991). They developed HSV mutants that lack thymidine kinase or ICP 34.5 and these viruses were only able to replicate in dividing cells. When these modified viruses were used in a nude mouse glioma model they caused tumour growth inhibition and improved survival. Since then a large number of human pathogens have been genetically modified to increase their specificity for cancer cells and enhance their oncolytic capability.

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1.5.2 Properties of oncolytic viruses

Viruses can transfer their genes from one host cell to another very efficiently and this has led to the widespread use of viruses as genetic vectors. For safety reasons, such virus vectors are generally rendered replication deficient but, unfortunately, this has limited the efficiency of treatment by restricting the number of cells to which the therapeutic gene is delivered. Therefore, the use of replication-competent viruses has been proposed, since virus replication would be expected to lead to amplification and spread of the therapeutic genes *in vivo*, however such replicating virus vectors must have some kind of tumour selectivity in their growth. They are dependent upon the host for replication, and differences in the cellular processes of normal host cells and tumour cells provide the potential for tumour targeting while sparing normal cells.

Recent advances in molecular biology and cell biology and a better understanding of cancer and the replication and pathogenesis of viruses have allowed the production of new agents that have been engineered to enhance their anti-tumour potential. Herpesviruses and adenoviruses are among the vectors that have been genetically engineered and developed as new agents against cancer, but there are many inherited tumour selective viruses that have been characterised including reovirus, Newcastle disease virus and vesicular stomatitis virus. Each of these agents has shown tumour selectivity *in vitro* and *in vivo*. Different viral vectors used for bladder cancer were discussed in detail above in this thesis.

The most important factor in defining oncolytic vectors is whether they can replicate in and destroy human tumour cells. Receptors for viral entry must be expressed on the target tumour cells. Ideally the wild-type virus should cause no disease or a mild, well characterised human or animal disease. The availability of drug treatment is also an advantage. Recombination with wild type viruses in the environment should not lead to a new pathogen. It has been suggested that non-integrating vectors are potentially safer. A genetically stable virus is desirable from the point of view of safety and manufacture. Finally, the virus vector must be amenable to high titre production and purification under good manufacturing practice.

1.6 Herpes simplex virus

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The Herpesviridae consist of three sub families, the α, β, and γ herpes viruses, differing in their pathology, tropisms and characteristics of latent infection. Human pathogen members include: the herpes simplex viruses HSV-1, HSV-2 and varicella-zoster virus (VZV) that represent the *alphaherpesvirinae*. Cytomegalovirus (CMV) is the prototype for *betaherpesvirinae* and also includes, both human herpes virus 6 and 7 (HHV-6, HHV-7). Epstein Barr virus (EBV) and Kaposi's Sarcoma associated virus (human herpes 8, HHV-8) are examples of *gammaherpesvirinae*. All viruses within herpesviridae have one characteristic that links them together, their ability to establish and maintain latent infection for the lifetime of the host, from which they can re-activate and cause disease long after the primary infection.

HSV-1 virus infection is highly prevalent worldwide, serum studies having also shown that antibody prevalence increases with age (Nahmias et al. 1990). The natural route of HSV-1 infection involves uptake of the virus by skin epithelial cells, whereupon the virus lytic cycle genes are expressed and the virus replicates (Roizman 2001). Following lytic replication the virus infects sensory nerve terminals and is transported retrogradely to the nerve cell body. The virus can then either undergo a further round of replication or the virus can enter latency. Sensory neurons are the natural site of herpes latency and as such the virus is thought of as neurotropic. However, *in vitro* and *in vivo* HSV-1 has been shown to infect a broad range of cell types as well as neurons. The lytic, replicative cycles of HSV-1 and HSV-2 are responsible for the symptoms of oral and genital herpes, respectively (Whitley 2001). HSV-1 can also infect the central nervous system to cause encephalitis, but this is very rare, only occurring in approximately 1-2 cases per million, per year (Kennedy 1984).

1.6.1 Herpes Simplex Virus-1

HSV-1 is a double stranded DNA virus with a genome of 152kb, encoding over 80 polypeptides (Roizman 2001). The HSV genome comprises two unique regions, short and long, each of which is flanked by inverted terminal repeats. The genes contained within the inverted repeats are present in two copies per viral genome. The unique segments can invert relative to each other, thus yielding four possible isomers. Approximately half of the 80 proteins encoded by HSV are essential for virus replication *in vitro*, the rest being necessary for full pathogenesis in the host.

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1.6.2 Properties of Herpes Simplex virus 1, relevant to oncolytic virus therapy

1. **Mutation of a number of individual genes allows tumour selective virus replication and blocks virulence.** Because wild type HSV-1 can infect replicating or quiescent cells, certain genes can be deleted to render the virus non-virulent, which also results in selective replication in tumour cells. (MacLean et al. 1991; Martuza et al. 1991; McKie et al. 1996; Rampling et al. 2000; Todo et al. 2001).
2. **Ease of manipulation**. Foreign DNA can be inserted into the HSV genome by homologous recombination techniques (Roizman and Jenkins 1985). This property is not exclusive to HSV, but the lytic nature of HSV-1 aids fast plaque purification. An alternative method used by some groups is to employ the bacterial artificial chromosome (BAC), a single–copy plasmid that can stably retain a large size (300kb) DNA as an insert (Shizuya et al. 1992).
3. **Oncolytic HSV can infect a broad range of human tumour cell types.** This replication competent virus can replicate and spread in situ and exhibit oncolytic activity through a direct cytopathic effect (Ring 2002). The following studies have shown that oncolytic HSV infects and replicates *in vitro* and *in vivo* in a wide range of human tumour cell types: glioma (Andreansky et al. 1997; Andreansky et al. 1996; Chambers et al. 1995; Detta et al. 2003; Samoto et al. 2002), colon carcinoma (Carroll et al. 1996), retinoblastoma (Nicolo and Chiocca 1998), epithelial ovarian cancer (Coukos et al. 1999), colorectal cancer (Kooby et al. 1999; Reinblatt et al. 2004), prostate cancer (Cozzi et al. 2002; Walker et al. 1999), non-small cell lung cancer (Toyoizumi et al. 1999), gallbladder carcinoma (Nakano et al. 2001), head and neck squamous cell carcinoma (Wong et al. 2001), esophageal adenocarcinoma (Stiles et al. 2003), breast cancer (Liu and Rabkin 2005; Pin et al. 2004a; Teshigahara et al. 2004), thyroid cancers (Yu et al. 2004), hepatocellular carcinoma (Pin et al. 2004b) and rhabdomyosracoma (Currier et al. 2005).
4. **HSV-1 is a highly lytic virus, resulting in tumour cell death.** Viral plaques can be detected in less than 16 hours.

5. **HSV does not integrate into the host genome**. By not integrating, use of HSV avoids the possibility of activating proto-oncogenes in the host genome, as has recently been described with integrating vectors (Fischer et al. 2002).
6. **Ease of production.** Oncolytic HSV can easily be grown in culture to the titres required for clinical use.
7. **Large insert capacity.** HSV allows the insertion of up to 30 kb of exogenous DNA (Longnecker 1988). This capacity allows the insertion of multiple therapeutic genes such as prodrug activating genes, immune-stimulatory genes and fusogenic membrane glycoprotein genes to increase tumour killing.
8. **Viral replication control.** Most versions of oncolytic HSV ((1716, (MacLean et al. 1991))(G207, (Mineta et al. 1995))(OncoVex, (Liu et al. 2003))) retain an intact thymidine kinase gene. As a result, many anti-herpes drugs, which require the thymidine kinase gene for activity, [e.g. Ganciclovir (GCV), Acyclovir (ACV), 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracyl (FIAU)) are available to prevent virus replication if this were thought to be necessary in a patient.

1.6.3 HSV-1 mutations giving tumour selective replication

During development of different oncolytic HSV vectors several types of mutations were investigated (nucleotide metabolism mutants, ICP34.5 mutant, ICP34.5+ICP6 mutant, HSV-1/HSV-2 intertypic recombinants, ICP34.5+US2 mutant, ICP34.5+US12 mutant, ICP34.7+ICP47 mutant). In this thesis we discuss the role of those mutations that are found in the Oncovex GALV/CD virus, which was tested by our group.

1.6.3.1 Single mutation to the ICP34.5 neurovirulence gene

The existence of a neurovirulence locus in the long repeat region of the HSV genome is well documented (MacLean et al. 1991; Taha 1989). This phenotype has been specifically assigned to the RL-1 gene (Chou et al. 1990; Dolan et al. 1992) and its encoded protein ICP34.5 (Chou et al. 1990). Expression of this protein facilitates viral replication in non-dividing cells, such as adult neurons (Robertson et al. 1992; Whitley et al. 1993). ICP34.5- mutant viruses are also avirulent (Chou et al. 1990; MacLean et al. 1991; Taha 1989). The LD_{50} of many strains of wild type HSV-1 is less than 300 pfu

following intracranial delivery. In contrast upwards of 10^6-10^9 pfu of ICP34.5 mutant viruses have been safely injected intracranially into mouse, rat, non-human primate, and human brains (Chou et al. 1990; Hunter et al. 1999; MacLean et al. 1991; Markert et al. 2000; Mineta et al. 1995; Rampling et al. 2000; Simpson et al. 2006; Sundaresan et al. 2000). An ICP34.5- mutant (1714) was identified in studies of spontaneous mutants of wild type HSV that had lost their neurovirulence properties. The 1714 virus had a number of deletions and mutations, including a 759bp deletion in ICP34.5. When this particular deletion was introduced into 17syn+ backbone, creating virus strain 1716, a loss in neurovirulence was observed (MacLean et al. 1991) demonstrating the RL1 gene to be responsible.

The function of HSV ICP34.5 is to disrupt the host anti-viral defence mechanisms. However, ICP34.5 doesn't target protein kinase R (PKR) itself but instead forms a complex with protein phosphatase 1 which is then directed to dephosphorylate eIF-2α, promoting translation of viral transcripts and subsequently inhibiting the induction of apoptosis and promoting infection (He et al. 1997; Roizman and Markovitz 1997). The carboxyl-terminal domain of ICP34.5 is homologous to the corresponding domain of a conserved mammalian protein called growth arrest and DNA damage 34 protein (GADD34). GADD34 can substitute for the corresponding domain in ICP34.5 blocking the effects of the PKR/ eIF-2α pathway (Brown et al. 1997).

ICP34.5- mutants in which both copies of the gene are mutated are incapable of replicating in neurons, but can replicate in and destroy glioma cells *in vitro* and *in vivo* (Andreansky et al. 1996). This suggests that deletion of the ICP34.5 gene somehow allows the virus to specifically target cancer cells while sparing normal tissue (Andreansky et al. 1996; Rampling et al. 2000). The precise mechanism for growth of ICP34.5 mutants in each tumour type is not fully understood, but it is known from knockout mouse studies that deletions and mutations in PKR and the IFN receptors allow ICP34.5 mutant growth (Leib et al. 1999; Leib et al. 2000), and that these mutations and deletions have been found in a number of tumour types (Haus 2000). Work from 2001 hypothesized that the higher levels of Ras activation found in transformed cells as compared to normal cells inhibits or reverses eIF-2α phosphorylation, thereby allowing viral protein synthesis and virus replication (Farassati et al. 2001). However, more recently this hypothesis has been disproved as no

correlation has been found between Ras status and virus susceptibility in various panels of tumour cells (Mahller et al. 2006; Sarinella et al. 2006). The early work leading to the Ras hypothesis was carried out in mouse cells over-expressing the Ras oncogene, a phenotype which does not accurately model human tumour cells and probably explains the misleading results obtained. Smith et al (Smith et al. 2006) went on to show a correlation between viral growth and activation of MEK which together suggests that the deregulation of different and/or multiple pathways in different tumour types allows tumour selective growth of ICP34.5 mutants.

It is important to note that oncolytic HSV containing only a deletion in both copies of the ICP34.5 gene such as R3616 (Chou et al. 1990) and 1716 (MacLean et al. 1991) are now considered relatively primitive oncolytic viruses because they fail to replicate in a number of tumour cell types (Mohr 2005), unlike more advanced multiply mutated viruses in development now. The reason for this is that 34.5 mutants cannot take advantage of the other HSV-1 genes that also act on the IFN/ PKR/ eIF-2α pathway, US11 (Mohr 2005). 1716, a single deletion oncolytic HSV vector, showed clinical safety, as well as indications of efficacy, in human phase I trials (MacKie et al. 2001; Papanastassiou et al. 2002; Rampling et al. 2000).

1.6.3.2 ICP34.5-, ICP47 double mutants

When an ICP34.5 deleted virus was serially passaged in tumour cells a novel mutant appeared, which exhibited dramatically improved growth properties in tumour cells (Mohr 1996). This so called suppressor mutant virus (SUP) contained an additional mutation that overcomes the protein synthesis block by altering the expression profile of US11, which encodes a viral RNA binding protein, from a late gene to an immediate early gene (Cassady et al. 1998a; Mohr 1996; Mulvey et al. 1999). The altered regulation of US11 in the SUP mutant takes place because of a deletion in the US12 gene encoding ICP47, which places US11 under the control of the ICP47 immediate early promoter. Accumulation of US11 at early times during infection inhibits the activation of the cellular PKR kinase and allows protein synthesis to proceed in the absence of the ICP34.5 gene product (Cassady et al. 1998b; Mulvey et al. 1999). A physical complex between US11 and PKR has been observed in infected cells, and this protein-protein interaction may also play a role in inhibiting PKR activation (Cassady et

al. 1998a). The neurovirulence of the SUP mutant was tested by intracerebral injection of immuno-competent mice and rats and the virus showed that, like the ICP34.5 single mutant, it was severely attenuated (Mohr et al. 2001; Simpson et al. 2006).

Wild type HSV-1 infection causes down regulation of major histocompatibility complex (MHC) class I expression on the surface of infected cells (Hill et al. 1995; Jennings et al. 1985). The binding of ICP47 to the transporter associated with antigen presentation (TAP) blocks peptide transport in the endoplasmic reticulum and loading of MHC class I molecules (Fruh et al. 1995; Hill et al. 1995; York et al. 1994). Consequently human tumour cells infected with ICP47- mutants express high levels of MHC class I on a their surface compared to wild type HSV infected cells (Liu et al. 2003; Todo et al. 2001). This would be expected to improve any anti-tumour immune response following intra-tumoral injection of the virus due to the presentation of tumour antigens at much higher levels on the surface of both tumour cells and HSV infected antigen presenting cells. Deletion of ICP47 has been included in G207 (Mineta et al. 1995) to give G47Delta. G47Delta has been demonstrated to give both enhanced antigen expression and enhanced anti tumour activity due to increased expression of US11 (Taneja et al. 2001; Todo et al. 2001).

In order to develop oncolytic HSV with greater tumour selective replicative ability, clinical isolates (BL1, JS-1) were tested for their ability to replicate in and kill human tumour cell lines as compared to the previously used laboratory strains (Liu et al. 2003). Both clinical isolates showed greater tumour cell killing than serially passaged laboratory strains, suggesting that they provided a better starting point for the development of an oncolytic virus. ICP34.5 and ICP47 were then deleted from one of these clinical virus strains resulting in tumour selectivity, the expression of US11 gene as an IE rather than a L gene to further increase tumour replication (Taneja et al. 2001), and increased antigen presentation (Hill et al. 1995; Liu et al. 2003; Todo et al. 2001). Both the use of the clinical isolate, and the increased expression of US11 were shown to increase tumour shrinkage in mouse tumour models (Liu et al. 2003). Finally, the gene encoding granulocyte macrophage colony-stimulating factor (GM-CSF) was inserted into this virus in place of ICP34.5, and this was demonstrated to increase the anti-tumuor immune response generated such that un-injected as well as injected tumours could be cured in mouse models. This virus was called OncoVEX^{GM-CSF} (Liu et al.

2003). Expression of GM-CSF has previously been shown to induce myeloid precursor cells to proliferate and differentiate, is a recruiter and stimulator of dendritic cells and has shown promise in pre-clinical and clinical trials in cancer (Andreansky et al. 1998; Bennett et al. 2001; Parker et al. 2000; Toda et al. 2000; Wong et al. 2001).

A phase I clinical trial has been completed using OncoVEX^{GM-CSF} by intra-tumoral injection in patients with cutaneous or sub-cutaneous deposits of breast, head and neck and gastrointestinal cancer and malignant melanoma (Hu et al. 2006). The virus was generally well tolerated with local inflammation, erythema and febrile responses being the main side effects seen, which were expected from previous studies with oncolytic viruses. Virus replication, and GM-CSF expression were observed, as was considerable tumour necrosis including in tumours adjacent to those which had been injected with the virus (Hu et al. 2006). Some evidence of a more distant, potentially immune-mediated effect, was observed as in some cases distant tumours became inflamed. Following these promising results, OncoVEX^{GM-CSF} is currently in a number of Phase II studies in individual tumour types.

1.6.4 Herpes Simplex Virus against bladder cancer

Intravesical oncolytic viral therapy using attenuated, replication-competent herpes simplex viruses G207 and Nv1020 were studied in an orthotopic syngeneic mice bladder tumour model (Cozzi et al. 2001). Animals treated with a single instillation of G207 showed a significant reduction in tumor weight compared to the control group. No bladder tumor was found in G207 treated animal group examined macroscopically and microscopically at necropsy, whereas control animals exhibited signs of excessive tumor burden, including weight loss, hematuria, and a palpable mass (Cozzi et al. 2001). Animals tolerated the treatment well with no adverse events and no deaths. Both G207 and NV1020 were highly effective when administered weekly for 3 weeks. Treatment was well tolerated with no adverse events and no treatment-related deaths. Tumor weight was also significantly reduced between groups treated with virus when compared with control (Cozzi et al. 2001). There was no statistically significant difference between viruses and similarly, there was no difference when comparing NV1020 and BCG or G207 and BCG (Cozzi et al. 2001). In conclusion both viruses were effective at infecting, replicating within, and achieving subsequent cell lysis for

human bladder cancer cell lines and murine transitional cell carcinoma cells. Both viruses were found effective in the orthotopic mice tumour model, where single and even more multiple instillations were effective at reducing tumour burden. (Cozzi et al. 2001).

A herpes simplex virus (HSV) type 1 mutant HF10 was studied in both human and murine bladder cancer cells. HF10 replicated well in both T24 and MBT-2 cells, and it induced extensive cell lysis. Treatment with HF10 mutant significantly prolonged the survival periods and increased the survival rates through its oncolytic activity in an immunocompetent orthotopic mouse bladder model (Kohno et al. 2005).

1.6.5 OncoVex$^{GALV/CD}$

OncoVex$^{GALV/CD}$ was derived from HSV-1 strain JS-1 and has two deletions, that of the genes encoding ICP34.5 and ICP47. Properties of the OncoVEX$^{GALV/CD}$ virus due to these deletions were discussed above.

OncoVEX$^{GALV/CD}$ expresses the envelope of GALV minus the R– peptide under the CMV promoter and OncoVEX^{CD} expresses Fcy::Fur under the RSV promoter (Figure 1.6). OncoVex$^{GALV/CD}$ was developed and tested recently by Simpson et al. in 2006. OncoVex$^{GALV/CD}$ combines GALV expression with prodrug conversion, which gave improved therapeutic effect compared with the use of either approach alone. Unlike when using, for example, adenovirus vectors to deliver the *GALV* gene (Diaz et al. 2000), virus replication was not inhibited, which may be due to the faster replication time of HSV.

The combined expression of a highly potent prodrug activating gene (CD=cytosine deaminase fusion to uracil phosphoribosyltransferase) and the fusogenic glycoprotein from gibbon ape leukaemia virus (GALV) further increased the local tumour control on several cancer cell lines (other than bladder cancer cell lines) (eg. COLO 205, HCT 116, CAPAN-1, HT 1080, U87-MG, CALU-1) *in vitro* (Simpson et al. 2006).

Simpson et al. have showed *in vivo* on a rat model that the effectiveness of the individual approaches gave 57% tumor cure for Fcy::Fur and 83% tumor cure for GALV expression, compared with only 16.6% with the backbone vector. Using OncoVex$^{GALV/CD}$ with the double approach they reached a statistically significant improvement with 100% tumor cure (Simpson et al. 2006).

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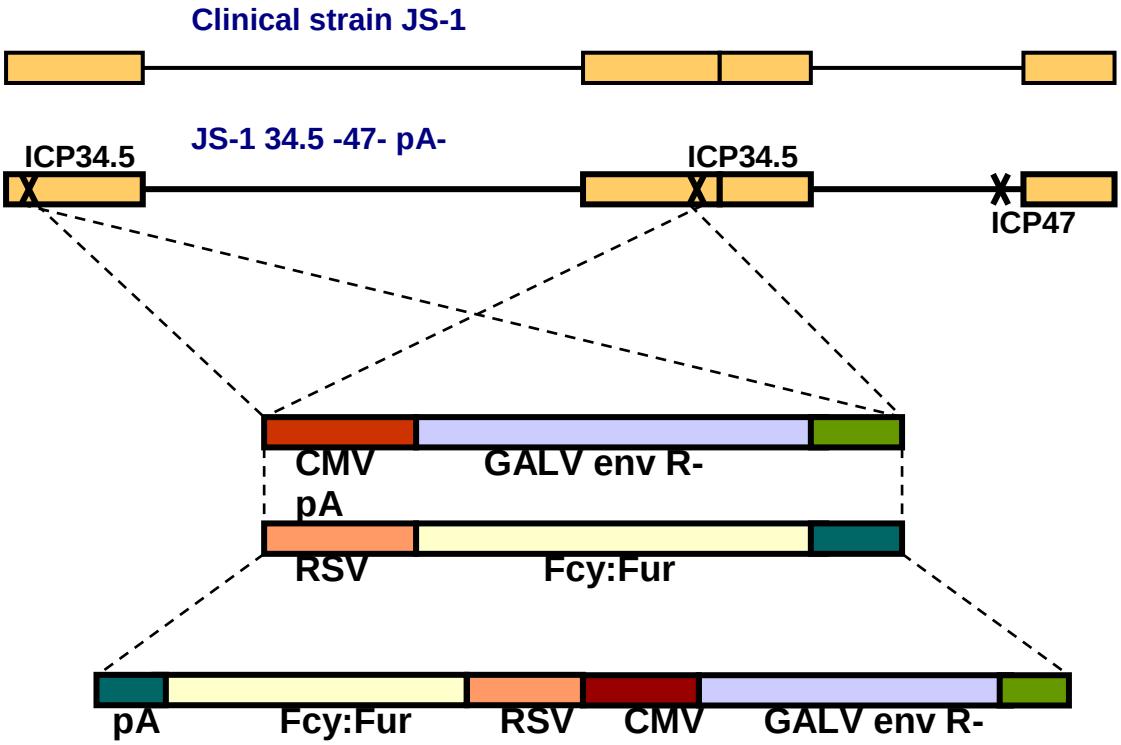

Figure 1.6 Schematic representation of virus vectors used in this study. HSV-1 strain JS-1 was isolated, by taking a swab from a cold sore of an otherwise healthy volunteer (Liu *et al.*, 2002). JS-1 34.5- 47- pA- has two deletions. The first involves removal of the coding region of the ICP34.5 gene (nucleotides 124948-125713 based on the sequence HSV-1 strain 17+). The second involves a 280bp deletion of ICP47 (nucleotides 145570-145290 based on the sequence HSV-1 strain 17+) (Liu *et al.*, 2002). JS-1 34.5-GALV *env* R-47- pA- expresses the retroviral envelope of gibbon ape leukaemia virus – the R- peptide (Genbank NC_001885, 5552-7555bp) (Bateman *et al.*, 2000, Galanis *et al.*, 2001) under the control of the CMV promoter. JS-1 34.5- RSV Fcy:Fur 47- pA- expresses the enzyme prodrug activator yeast cytosine deaminase fusion to uracil phospho-ribosyltransferase (Invivogen) under the control of the RSV promoter. JS-1 34.5- GALV *env* R-/ Fcy:Fur 47- pA- combines both fusogenic retroviral envelope and enzyme prodrug activator.

1.7 Animal bladder tumour models

An animal tumour model that resembles human disease both histologically and in behaviour is essential for preclinical evaluation of new treatment modalities. The ideal animal bladder tumour model should include the following characteristics:

- The tumour should grow intravesically (orthotopically), such that the tumour can be directly exposed to antitumour drugs in its natural environment.
- The tumour should be of pure TCC origin, with different stages of disease progression (CIS, papillary and invasive diseases) and, as for the human disease, the majority of the tumours should be superficial, but not progressive.
- The animal host should be immunocompetent and reasonably large, so it can be treated by various antitumour modalities.
- The tumour should be technically easy to develop within a reasonable time period, and highly reproducible with respect to its natural history.

Soloway (Soloway 1977) showed that bladder tumour cells (MBT-2) could be implanted on the murine bladder mucosa by intravesical tumour inoculation, if the bladder mucosa was pretreated with *N*-methyl-*N*-nitrosourea (MNU). He reported about 60% tumour takes. Ibrahiem and colleagues (Ibrahiem et al. 1983) injected rat tumour cells into rat bladder muscularis to develop an invasive bladder tumour model, because of tumour cell growth failure when inoculated on the bladder mucosa using Soloway's procedure. Their model did not closely mimic the human counterpart because the tumour was actually invasive and covered with normal bladder mucosa (Ibrahiem et al. 1983; Iinuma et al. 1995). Chin et al (Chin et al. 1991) established an orthotopic mouse bladder tumour model by implanting MBT-2 tumour cells on bladder mucosa, which was pretreated with mild HCl/KOH. Compared with Soloway's procedure, the one used by Chin et al was more convenient. Carcinogen could be avoided and higher tumour takes (75–80%) were achieved (Chin et al. 1991). The advantages and limitations of previous animal bladder tumour models were reviewed by Oyasu (Oyasu 1995). In mice, most of the deeply invasive bladder tumours were squamous cell carcinomas, as opposed to TCC. The carcinomas observed in mice tended to invade into the perivesical organs by direct extension, and seldom showed metastasis to regional lymphnodes or lung as observed in humans.

However, the rat model, because of its size, is easier to work with for intravesical therapies and, furthermore, it may parallel the human disease processes better than a mouse model (Ohtani et al. 1986; Oyasu 1995).

In our experiments we used a technique to develop a rat ortothopic bladder tumour model that was described by Xiao at al in 1999 (Xiao et al. 1999). Using the AY-27 cell

line they have developed and characterized a highly reproducible, transplantable, purely TCC rat orthotopic bladder tumour model. The procedures are not technically complicated, are well tolerated by the animals and result in minimal morbidity associated with occasional mild haematuria subsequent to tumour cell instillation. The fact that tumour cells used for intravesical instillation originated from cell cultures rather than directly from solid tumour fragments, might contribute to the high tumour establishment observed. Tumour cells prepared from tumour fragments may also contain stromal cells, lymphocytes, etc., and their viability might be compromised due to mechanical or enzymatic treatment.

To facilitate tumour seeding, the bladder mucosa was conditioned with an acid rinse. The conditioning consisted of intravescal administration of hydrochloric acid and neutralized with potassium hydroxide. Histologically, HCl/KOH instillation elicited focal urothelial denudation with slight submucosal blood vessel dilatation. Immediately after bladder conditioning, the AY-27 cells were instilled and left indwelling for at least 1 hour. If single-cell suspensions of AY-27 cells were instilled in normal (unconditioned) bladder it did not elicit tumour establishment.

According to Xiao's results prior to 13 days post-inoculation, all tumours detected were superficial (T1, CIS). In a group of animals sacrificed on days 16–17 post-inoculation, the tumour establishment was 97%, with 65% being superficial (T1, CIS), 29% superficially invasive (T2), and 6% deeply invasive (T3) disease. With time, there was progression of the disease process with invasive tumours detected in 66% of the animals followed 21–50 days post-tumour inoculation.

To determine the starting point of treatment in an orthotopic rat bladder model is an important question. Ideally all rats should have formed tumours before the treatment starts. Xiao et al. described three stages of tumour growth, i.e. early tumour establishment (1–13 days), mid-stage intravesical progression (14–21 days), and advanced intravesical progression and extravesical spread (22–50 days) (Xiao et al. 1999). Thus 14–16 days post-inoculation seems to be the most suitable time for intravesical therapies.

Hendricksen at al. showed tumour cell growth and extension along the basement membrane only at 3 and 4 days and it appeared as a patchy or Pagetoid subtype of CIS. From 6 days on, 40% of rats had early progression to muscle-invasive disease. The fast

progression toward muscle-invasive growth leaves a narrow window for the start of experimental intravesical treatment against NMIBC, preferably at 5 days after tumour cell inoculation (Hendricksen et al. 2008).

1.7.1 Assesment of tumour growth

1.7.1.1 Cystoscopy

In human the gold standard for the detection of bladder cancer is cystoscopy, which is an invasive and relatively expensive method. Evaluation of an orthotopic rat bladder urothelial cell carcinoma model by cytoscopy was recently described by Hendricksen et al. (Hendricksen et al. 2008). The authors performed pneumocystoscopy using a fibre-optic needle arthroscope of 1.0 mm diameter with a miniature straight 0º telescope, which resulted in excellent bladder visualization. With serial cystoscopy to 17 days they were able to compare *in vivo* macroscopy with the
histology of rats. Overall, tumour establishment was >80%, with predominantly carcinoma in situ preceding or concomitant with invasive tumour growth. All tumours were formed at 3–5 days, and remained non-muscle-invasive up to 5 days. From 6 days, tumours progressed to muscle-invasive disease in 40% of the rats. The tumours were apparent in >90% of rats from 5 days on, with a specificity and sensitivity of >90%. But cystoscopy could not differentiate NMIBC from muscle-invasive bladder cancer in that bladder model.

1.7.1.2 Urine cytology

In human voided urine cytology is a highly specific, noninvasive adjunct to cystoscopy. Urine cytology is the interpretation of a pathologist concerning the nature of cells disaggregated from their environment in the urothelium. Urinary specimens do not always contain a representative sample of the bladder and may not contain tumour cells even when a tumour is present. Therefore the diagnostic yield of urine cytology is increased if at least 3 samples are obtained. The accuracy of cytology depends upon the reviewer's expertise. The endpoint of urine cytology is to identify the tumor cells themselves, therefor the predictive value of a positive result is very high. It has good

sensitivity and specificity for detecting high grade bladder tumors, but has poor sensitivity to detect low grade disease.

Cytology was used in a rat orthotopic superficial bladder cancer model to assess tumour growth in an experiment where the authors evaluated the effect of whole-bladder photodynamic therapy (PDT) against bladder cancer. Cytology of the urine sediment failed to detect half the tumours in the treatment groups resulting in poor correlation with actual bladder cancer formation (Gronlund-Pakkanen et al. 2003).

1.7.1.3 Tumor markers

There are several types of bladder cancer markers and tests that are available in humans. These tumour markers can be divided into 2 groups, soluble urine markers (BTA-Stat, BTA-TRAK, NMP-22, BLCA-4, BLCA-1, Survivin, Cytokeratins, HA-HAase Test) and cell-associated markers (Microsatellite Analysis, Telomerase, DD23, Quanticyt Nuclear Karyometry, Multi-target Multi-color FISH Assay (UroVysion Test)), depending upon whether urine specimens or exfoliated cells in urine are used in the assay. All of these tumour markers are used in human and have no major role in animal bladder cancer models.

1.7.1.4 Ultrasound

Ultrasound scan is an important tool in human, which can detect bladder tumours with at least 5 mm size. The accuracy of ultrasound depends upon the examiner's expertise. Rooks et al. (Rooks et al. 2001) evaluated the use of ultrasound in an orthotopic bladder tumour model in mice treated with TNP-470 (an angiogenic inhibitor). They found intraabdominal tumours as small as 1.5 mm with ultrasonography. Ultrasound can provide accurate intermediate end points for monitoring experimental intraabdominal tumor growth and response to therapy in the mouse model.

1.7.1.5 Magnetic resonance imaging (MRI) scan

Magnetic resonance imaging has an important role not in the diagnosis but in the staging of bladder cancer in human. Mazurchuk et al. showed that MRI as an imaging technique is feasible to construct tumour growth curves (Mazurchuk et al. 1997), but the technique is difficult for small early lesions, and is relatively complicated (Chin et al.

1991). Xiao *et al.* reported little benefit from MRI, due to the tumour detection limit of >2 mm, and the high reproducibility of their model (Xiao et al. 1999).

1.7.1.6 Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (QRT-PCR) is a modification of the polymerase chain reaction used to rapidly measure the quantity of DNA, complementary DNA or ribonucleic acid present in a sample. Like other forms of polymerase chain reaction, the process is used to amplify DNA samples, via the temperature-mediated enzyme DNA polymerase. PCR was usefull to examine transduction and vectors spread of different therapeutical viral vectors in an orthotopic mice bladder tumour model (Kikuchi et al. 2007b).

1.7.1.7 Bioluminescence imaging

Bioluminescence imaging has become a very popular tool for noninvasive monitoring of fundamental biological and molecular processes in small living subjects. The non-invasive IVIS camera detects the luciferase activity of the tumour. Luciferase is a light-emitting enzyme that can generate light (known as bioluminescence) after reacting with specific substrates (eg. tumour cells). These enzymes are isolated from various organisms, conveniently modified for expression in mammalian cells, and are extensively used in molecular biology and cell culture experiments (Ray 2007). The emitted light is used as a detection system for luciferase activity. At selected time intervals tumour implantation an intravenous injection of luciferin should be given and the animal should be placed within a light-tight chamber for imaging. The light-tight chamber has a vertically-mounted highsensitivity cooled CCD camera. This operates in single photon-counting mode, since the luminescence signal is extremely weak. The animal is placed within the cabinet, attached to an anaesthetic machine, and image collection takes place over a period of 5 minutes. Bioluminescence imaging *in vivo* is a powerful new optical tool for monitoring the response of tissue (in this case malignant tumour) to treatments (Wilson 2003).

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2 Objectives

- evaluate the effectiveness of the OncoVex$^{GALV/CD}$ virus in the treatment of bladder cancer
- test the efficacy of Oncovex$^{GALV/CD}$ *in vitro* on several transitional cell human bladder tumour cell lines (EJ, RT112, T24, VMCUB-I, TCCSUP-G, 5637, KU19-19) compared to the backbone virus (Oncovex^{GFP})
    - test for HSV infection alone in these cells and then elucidate whether expression of fusogenic glycoprotein from this virus increases cytotoxic cell killing within these cells
    - test *in vitro* the efficacy of Oncovex$^{GALV/CD}$ in the presence of 5-fluorocytosine on these human bladder tumour cell lines compared to the backbone virus
- test *in vitro* the efficacy of Oncovex$^{GALV/CD}$ in combination with conventional chemotherapies such as mitomycin, cisplatin, gemcitabine on human bladder tumour cell lines (EJ, T24, TCCSUP-G, KU19-19).
- set up a stable and useful rat orthotopic bladder tumour model that is suitable to evaluate the effectivity of different therapeutical options (eg. Oncovex$^{GALV/CD}$ treatment)
    - test whether AY-27 rat bladder tumour cells are susceptible for HSV entry
    - due to the lack of HSV receptor on the surface of AY-27 cells we aimed to stably transfect AY-27 cells with the herpesvirus entry receptor (HVEM) and select a clone of these cells that support infection with Herpes Simplex Virus
    - test the *in vitro* efficacy of Oncovex$^{GALV/CD}$ on the new AY-27 HVEM cell line with the fusion assay and also test the *in vitro* efficacy

of Oncovex$^{GALV/CD}$ in the presence of 5-fluorocytosine with the prodrug assay

- assess the the rate of tumour growth on a flank tumour model and compare the new AY-27 HVEM cell line to the original AY-27 cells
- set up a stable rat orthotopic bladder tumour model with the AY-27 HVEM cell line
- assess the effectiveness of QRT-PCR in detecting tumour growth using urine and tissue samples
- stably transfect AY-27 HVEM cell clone with a plasmid encoding the luciferase enzyme and select a clone of these cells that show luciferase activity for bioluminescence imaging
- evaluate the effectivity of this bioluminescence imaging on the rat orthotopic bladder tumour model

- evaluate *in vivo* the efficacy of Oncovex$^{GALV/CD}$ on our previously developed rat orthotopic bladder tumour model

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3 Materials and Methods

3.1 Cell culture

Cell culture preparations were carried out under sterile conditions in a laminar flow safety cabinet class II. Tissue culture plasticware was obtained from *Nunc*, and media and supplements were supplied by *Sigma*.

3.1.1 Cell lines

Human bladder carcinoma cells (EJ, T24, RT112) and baby hamster normal kidney cells (BHK-21) were purchased from American Tissue Culture Collection (ATCC). Other human bladder carcinoma cells (VMVUB-I, TCCSUP-G, 5637, KU19-19) were kindly given by Professor Margaret Knowles (Cancer Research UK Clinical Centre, Leeds). The rat bladder carcinoma cell line (AY-27) was kindly given by Dr. Ronald B. Moore (University of Alberta).

Table 3.1 Mammalian Cell Lines used in following studies

Cell line	Species Source	Tissue	Hystological type	ECACC or ATCC No	Media
BHK-21 (clone 13)	Baby Syrian Hamster	Kidney normal	-	85011433	DMEM
EJ	Human	Bladder carcinoma	TCC	85061108	DMEM
T24	Human	Bladder carcinoma	TCC	85061107, HTB-4	McCoy's
RT112	Human	Bladder carcinoma	TCC	85061106	MEME
VMCUB-I	Human	Bladder carcinoma	TCC	University Leeds	DMEM
TCCSUP-G	Human	Bladder carcinoma	TCC	University Leeds HTB-5	DMEM

5637	Human	Bladder carcinoma	TCC	University Leeds HTB-9	RPMI
KU19-19	Human	Bladder carcinoma	TCC	University Leeds	RPMI
AY-27	Rat	Bladder carcinoma	TCC	University Alberta	RPMI

3.1.2 Cell Handling

BHK-21, EJ, VMCUB-I, TCCSUP-G cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) and 5637, KU19-19 cell lines were grown in RPMI-1640 (both Sigma).

T24 cells were grown in McCoy's 5A Medium and RT112 cells were grown in Minimum Essential Medium Eagle. All media was supplemented with 2mM GlutaMAX-1 supplement (Invitrogen), 100 units/ml penicillin, and 100 units/ml streptomycin (Sigma) and either 10% (v/v) foetal calf serum (FCS) for routine passage or 2% (v/v) FCS for experimental work. Cell culture work was carried out in a sterile environment provided by a laminar flow hood with double HEPA filter. Cell lines were regularly tested for mycoplasma infection. Passage of cell cultures was carried out when cells were approaching confluence: every 3-7 days depending on the cell line. Cells were washed with Hanks Balanced Salt Solution (HBSS) to remove any foetal calf serum and then 0.05% trypsin (Sigma-Aldrich) was added. Once the cells had been mobilised fresh medium containing foetal calf serum was added to stop the trypsinisation. The cell suspension was then centrifuged at 1500rpm for 3 minutes. The cell pellet was resuspended in the appropriate medium and plated out in the required dilution. Cells were incubated at 37°C and either 5% or 10% CO_2 depending on the cell line.

3.1.3 Cell line storage

In order to maintain stocks of the various cell lines, cells were regularly frozen down. Cells in log phase of growth were pelleted and resuspended at 10^6-10^7cells/ml in complete medium and an equal volume of 20% DMSO in foetal calf serum added. 1ml

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aliquots were transferred to labelled cryotubes which were then placed in a 1°C freezing container and stored overnight in a -80°C freezer. The isopropanol in these containers allows for slow freezing at approximately 1°C/minute. Cells were then transferred to liquid nitrogen storage the following day.

Recovery of cells from liquid nitrogen storage was performed by rapid thawing in a 37°C water bath. Thawed cells were washed in 10ml of medium, harvested by centrifugation (1500rpm for 3 minutes) and were then transferred to 75cm^2 flasks containing fresh culture medium.

3.1.4 OncoVex GFP and OncoVex GALV/CD stocks

OncoVex GFP (backbone virus) and OncoVex GALV/CD stocks were supplied by BioVex Inc. (34 Commerce way, Woburn, MA, 01801, USA). The OncoVex GFP and OncoVex GALV/CD stocks were stored at -80°C in 1000µl aliquots.

OncoVEX was derived from HSV-1 strain JS-1 (Liu et al. 2003) and has two deletions, that of the genes encoding ICP34.5 (nucleotides 124948-125713 based on the sequence of HSV-1 strain 17+) and ICP47 (nucleotides 145570-145290). OncoVEX^{GALV} expresses the envelope of GALV minus the R– peptide (Genbank accession no. NC_001885; 5,552-7,555 bp) under the CMV promoter. OncoVEX^{CD} expresses Fcy::Fur under the RSV promoter. OncoVEX$^{GALV/CD}$ expresses both the GALV and Fcy::Fur proteins (Simpson et al. 2006) (Figure 1.6).

3.1.5 Viral titre assay (Plaque assay).

Virus stock titer and virus stability was measured by standard plaque assay. BHK cells were plated at 1.25 x10^5 cells per well of a 24 well tray and incubated overnight (80% confluent). A 1:10 serial dilution of virus suspension from 1x10^{-3} – 1x10^{-7}ml was prepared, 200µl of which was placed on the cells. The cells were then incubated for 1 hour at 37^{0}C/5%CO_2. The media was then removed and replaced with 1mls of 1:2 of 1.6% (v/v) carboxymethyl cellulose. The cells were then incubated for a further 48 hours at 37^{0}C/5%CO_2 and the wells were then assayed for the number of plaques in each well in order to determine titre of virus. Virus not expressing reporter gene were visualised by fixing and staining the plaques. (See section 3.1.8). The cells were then digitally photographed (Figure 3.1) using an inverted microscope (Nikon Eclipse

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TE200) and Lucia Image (MV-1500 version 4.6). Virus expressing green fluorescent protein marker gene were visualised under an inverted fluorescent microscope (*Nikon* Eclipse TE200) at wavelength 520nm. The titre of virus was measured in plaque forming units per ml (pfu/ml).

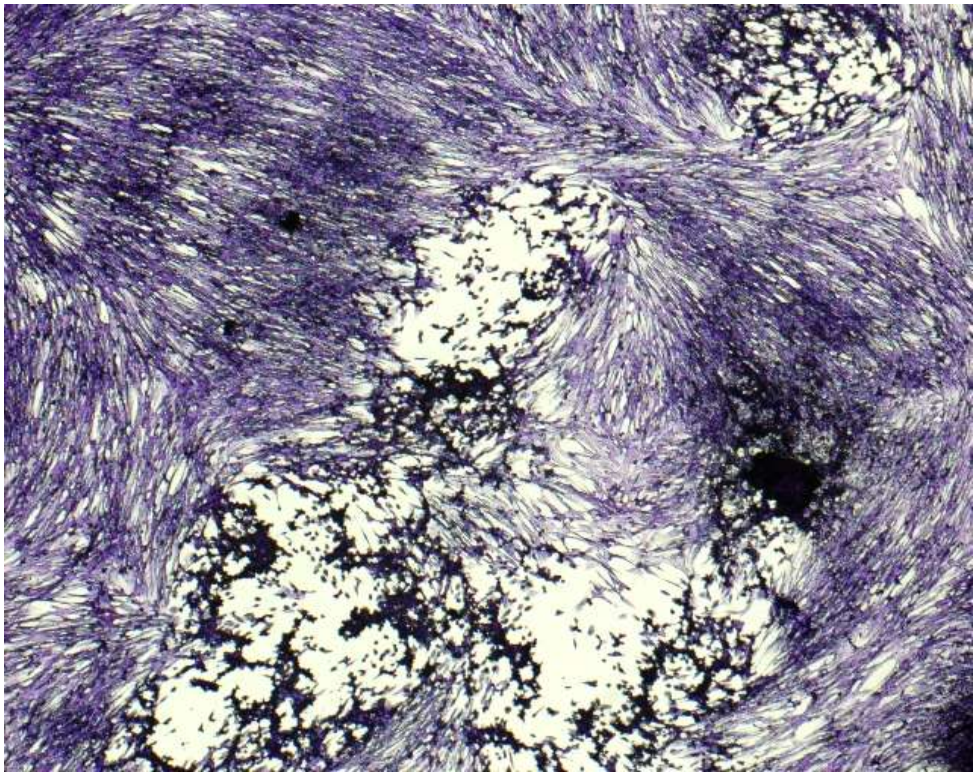

Figure 3.1 Plaque visualized by Crystal Violet

3.1.6 Fusion assay (GALV dose response assay)

Test cells were plated at 1.25 x 10^5 per well of a 24-well tray or at different amounts ($1.5x10^4$, $2x10^4$, $2.5x10^4$, $3x10^4$) per well of a 96-well tray and incubated at 37°C/5% CO_2 o/n. The FGM was removed, and 200 µL of $OncoVex^{GALV/CD}$ or $OncoVex^{GFP}$ at MOIs of 0.0001, 0.001, 0.01, and 0.1 in RPMI with 2% FCS were added. This was then incubated at 37°C/5% CO_2 for 1 hour. The virus dilutions were removed and replaced with 1 mL FGM. This was then incubated at 37°C for 48 hours. From the 24 well trays the cells were washed and fixed in glutaldehyde, then stained with Crystal Violet for digital photographs. (See section 3.1.8). From the 96 well trays the cells were measured by the MTS assay.

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3.1.7 Prodrug-activating assay

Test cells were plated at 1 x 10^5 per well of a 24-well tray and incubated at 37°C/5% CO_2 overnight. The cells were infected with OncoVEX^{GFP} and OncoVEX$^{GALV/CD}$ at different MOIs (1, 0.1, 0.01) according to the fusion assay results (in 200 µL RPMI with 2%FCS) and no virus control. After 30 minutes at 37°C/5% CO_2, the virus was removed, and 1 mL of FGM containing 5-FC ($C_4H_4FN_2O$; Sigma) at different concentrations (600-800-1000-1200-1400 µmol/L) was added and incubated for 48 hours at 37°C/5% CO_2. The cell supernatant was transferred into a fresh tube, and the cell debris was removed by spinning at 1,500 rpm (340 x *g*) for 5 minutes at 4°C. The supernatants were added to a fresh tube and then incubated at 60°C for 10 minutes, to inactivate the virus. The resulting supernatants were allowed to cool to room temperature. Test cells were plated at 1 x 10^4 per well of a 24-well tray or 1 x 10^3 per well of a 96-well tray and were incubated at 37°C/5% CO_2 overnight. The heat-treated supernatants were added to the fresh test cells and incubated at 37°C/5% CO_2 for 72 hours. From the 24 well trays the cells were washed and fixed in glutaldehyde, then stained with Crystal Violet for digital photographs. (See section 3.1.8). From the 96 well trays the cells were measured by the MTS assay.

3.1.8 Fixing and staining protocol for cells or viral plaques

The cells were washed twice with PBS and then incubated with 2ml of 0.1% Glutaldehyde (*Sigma*) in PBS for 10 minutes at RT. After another two washes with PBS the cells were stained with 1ml of 0.1% w/v Crystal Violet solution (in 20% Ethanol) for 10 min in order to visualise the cells or plaques. Excess stain was removed with H_2O, and the plates were allowed to dry. The cells were then digitally photographed.

3.1.9 MTS Assay

Cell viability was quantified using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Sigma-Aldrich). This assay is based on the reduction of the tetrazolium salt, MTS, to a coloured formazan compound by living cells in culture. This assay is similar to the MTT assay with the advantage that the formazan product of MTS reduction is soluble in

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cell culture medium, unlike MTT which has to be dissolved in DMSO. Metabolism in living cells produces "reducing equivalents" such as NADH or NADPH. These reducing compounds pass their electrons to an intermediate electron transfer reagent that can reduce MTS into the aqueous, formazan product. When the cell dies, it rapidly loses the ability to reduce tetrazolium products. Therefore the production of the coloured formazan compound is proportional to the number of viable cells in culture. The cell viability was quantified using the CellTiter 96 AQueous One Solution Cell Proliferation Assay reagent 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS; Promega) according to manufacturer's instructions. Briefly, 20µl of MTS reagent in 180µl of fresh medium was added to each well. Following incubation at 37^{o}C for 1-4 hours, absorbance was measured at 495nm. Survival was calculated as a percentage compared to untreated cells using the formula:

$$\% \text{ Cell Survival} = \frac{(\text{Treated Value} - \text{background})}{(\text{Untreated Value} - \text{background})} \times 100$$

3.2 Cell cloning techniques

3.2.1 Linearisation of DNA.

It was important to linearise the plasmid DNA to be transfected, as this increases the efficiency of integration. Ideally, the enzyme site chosen should be at least 200bp from either end of the promoter/poly A of the gene of interest since some bases can be removed from the plasmid during integration. 10µg of linearised DNA were prepared per cell type to be transfected.

The following digest was set up:

3µg DNA, 10µl restriction enzyme buffer (buffer E, buffer D), 5µl appropriate enzyme (10units/µl) (SspI, NotI), ddH_20 to 100µl, Incubate for 4 hours at the appropriate temperature

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The resulting DNA was then purified using the GFX PCR DNA and Gel Band Purification Kit (*Amersham Biosciences*) as per manufacturer's instructions. The DNA was then eluted in a final volume of 50µl.

The Pc DNA3 HVEM plasmid and the pGAL CMV Luc Hygo plasmid were used in this study (Figure 3.2, Table 3.2)

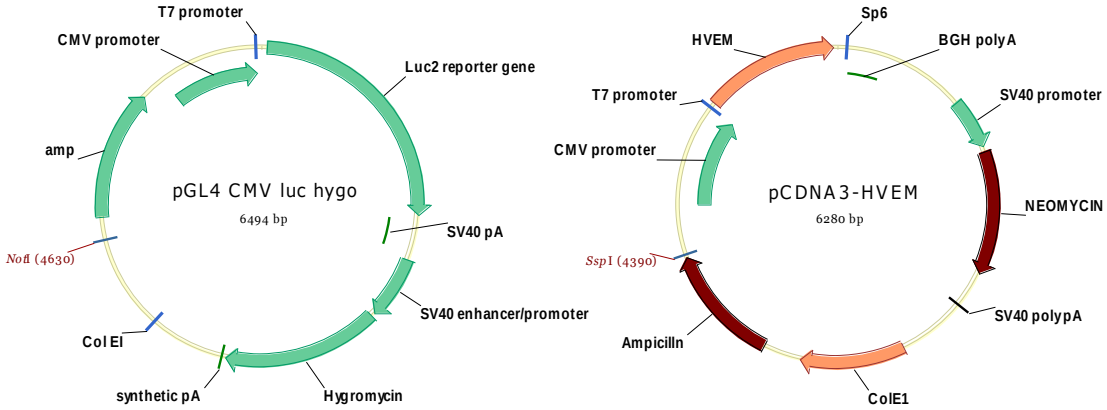

Figure 3.2 Pc DNA3 HVEM and the pGAL CMV Luc Hygo plasmids

Table 3.2 Pc DNA3 HVEM and the pGAL CMV Luc Hygo plasmids

Plasmids	**Linearisation restriction enzyme**
Pc DNA3 HVEM	Ssp I
pGAL CMV Luc Hygo	Not I

3.2.2 Antibiotic killing curves

The aim of the antibiotic killing curve was to determine the minimum concentration of antibiotic (G418 (Gibco), hygromycin (Sigma)) required to give complete cell death after 7 days of treatment. Cells were plated at $5x10^4$-$1x10^4$ cells per well of a 24 well tray and incubated overnight. The selection media was added to the wells at range of concentrations (e.g. for G418 which was used in this study at a concentration of ~300µg/ml we tried 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000µg/ml). The plates were observed every day for 7 days, each time estimating the level of cell death. It was necessary to change the selecting media every 2 days. After 10 days the cells were fixed and stained as above.

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3.2.3 Transfection of cells to produce luciferase/HVEM expressing cell lines

AY-27 cells were plate at $6x10^5$ cells per well of 6 well tray (using media without antibiotics) and incubate at 37^0C o/n. 1μg of linearised plasmid (pcDNA3 HVEM, pGAL CMV Luc Hygo) was diluted in 100μl of RPMI (without FCS) per transfection. The lipofectin (Invitrogen) was diluted in RPMI (without FCS). Both were let stand at room temperature for 30-45 minutes. The diluted DNA and lipofectin were combined, gently mixed and incubated at room temperature for 15 minutes. The growth media from the AY-27 cells was removed and the cells were washed with 2ml of RPMI without FCS. 800μl of RPMI without FCS was added to DNA/ lipofectin mix and then added to the cells. The cells were incubated at 37^0C for 7hrs. After this incubation the DNA/ lipofectin mix was removed, 2ml of FGM was added and incubated at 37^0C o/n. After 48hrs the cells were split into 90mm culture dishes (x9) and incubated at 37^0C o/n. After 24 hours FGM was removed and FGM containing the appropriate antibiotic was added.

3.2.4 Selection and scale up of transfected clones

48 hours after transfection cells were split into 90mm culture dishes (x9) culture dishes and the appropriate selection media was added 24 hours later (Table 3.3). The concentrations of antibiotic used to treat the cells were pre-determined by carrying out an antibiotic killing curve (See section 3.2.2).

The cells were observed every day and the selection media was changed every 2-3 days (especially if there is a high level of cell death). Individual cell colonies were visualised between 5-20 days of selection (depending on the cell line and compound). Once the colonies were clearly observable, they could be transferred to individually wells of a 96-well tray containing 100μl of selection media. This was achieved by picking the colonies, with a p20 gilson, directly from the plate whilst observing it under an inverted microscope (*Nikon* Eclipse TE200). The cells were then transferred to one well of a 96-well tray and pipetted up and down a few times to break up the cells. The individual cell

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lines took between 2-14 days to produce a confluent monolayer in a well of a 96-well tray. Once confluent, the cell lines were transferred to one well of a 24-well dish containing 500μl selection media/well, using 50μl trypsin versene to remove the cells from the 96-well dish. The cell lines were grown from 24 well stage to a 6 well stage and finally up to a 25cm^2 flask. At all times the cell lines were exposed to selection media. Once confluent, the flask of cells was frozen down into 3 ampoules, one of which was kept as a master stock. Once a suitable cell line was identified, it was scaled up and frozen stocks were produced.

Table 3.3 Concentration of selection agents used in this study

Antibiotics	**AY-27 cells**
G418 *(Gibco*) dissolve in H_2O	300μg/ml
Hygromycin *(Sigma)* dissolve in H_2O	100μg/ml

3.2.5 Sub-cloning cell lines

This protocol was carried out after a suitable cell line had been identified from a relevant screening process in order to ensure that the cell line was derived from an individual transfected cell. 1x10^3 cells per well in 200μl fresh FGM with appropriate concentrations of selection antibiotics were added to every well in row A of 96-well tray. 100μl selection media was added to rows B-H of the dish. Then a 1:2 serial dilution of cells suspension was carried out from row A to row H. The trays were incubated until the wells containing 1 cell become confluent. The new sub-clones were grown up and re-screened to identify positives.

3.2.6 Infection screening assay for AY-27 HVEM

To identified clones of AY-27-HVEM that support replication of HSV-1 test cells were plated at 1x10^{5}cells per well of a 24 well tray and incubated at 37^{o}C/5% CO_2 o/n. The media was then removed and the cells infected with OncoVex^{GFP} virus at MOI 0.1 (in a volume of 200μl RPMI) and incubated at 37^{o}C/5% CO_2 for 1Hr. The virus was removed and 1ml of fresh FGM media was added. The infection was incubated at 37^{o}C/5% CO_2 for 72 hrs. Infection of OncoVex^{GFP} virus expressing green fluorescent protein marker

gene were visualised under an inverted fluorescent microscope (*Nikon* Eclipse TE200) at wavelength 520nm.

3.2.7 Luciferase activation screening assay.

AY-27 HL cells were plated at $5x10^5$ cells per well of 6 well tray and incubated at 37^0C o/n. After this incubation the FGM was removed and the cells were washed (3x) with 1.5ml ice cold PBS. The PBS was removed and 120μl of cell 1x culture lysis reagent (Sigma 5x cell culture lysis Reagent, C 4707) was added. The lysate was incubated at room temperature for 15 minutes after which the cells were scrape off and spun at 12,000g at 4^0C for 1 minute. The supernatant was removed and store on ice. The lyophilized luciferase assay substrate (sigma L 0407) was resuspended in 10ml of luciferase assay buffer (Sigma L 0532). The luciferase substrate and the tested cell lysate were allowed to equilibrate to room temperature before use. 20μl of the cell lysate was added to 100μl of the luciferase substrate assay buffer. Light emission was read after 10 seconds incubation at room temperature using Beckman colterDTX 880. (Luciferase assay kit MB-260)

3.3 Quantitative Reverse Transcrpition Polymerase Chain Reaction (QRT-PCR)

3.3.1 RNA extraction

Urine and bladder tissue samples were screened using QRT-PCR to detect expression of HVEM gene as a marker for the presence of tumour.

RNA extraction was performed using Qiagen RNeasy Plus Micro Kit (cat no 74034). Prior to starting, 10μl of 6-mercaptoethanol was added for every 1ml of buffer RLT Plus used. Rat urine was spun at 16K for 10 minutes at 4 Cº tp produce a cell pellet. These cell pellets from the urine were lysated with 350μl buffer RLT, then the lysate was vortexed for 1minute. Bladder tissue was homogenized with a tissue ruptor and cells were lysated with 350ul RTL, then vortexed for 1minute. The lysate was homogenized by passing at least 5 times through a 21 gauge needle. The urine or bladder tissue lysate was transferred to gDNA Eliminator spin column placed in a 2ml

collection tube and centrifuged for 30 seconds at 8,000rpm. The column was discarded and 350μl of 70% ethanol added to the flow through and mixed. 700μl of the sample was then transferred to an RNeasy MinElute spin column placed in a 2ml collection tube and centrifuged for 15 seconds at 8,000rpm. The flow through was discarded and 700μl of buffer RW1 added to the RNeasy MinElute spin column and centrifuged at 8,000rpm for 15 seconds. The flow through was discarded and 500μl of buffer RPE added to the spin column and centrifuged for a further 15 seconds. The flow through was discarded and 500μl of 80% ethanol added to the spin column and centrifuged for 2 minutes at 10,000rpm. The RNeasy MinElute spin column was placed in a new 2ml collection tube and centrifuged at 10,000rpm for 5 minutes. The spin column was then placed in a 1.5ml collection tube and 14μl of RNase-free water added directly to the column membrane. This was then centrifuged for 2 minutes at 10,000rpm to elute the RNA. The RNA was quantified using Nano-drop spectrophotometer and stored at -80°C.

3.3.2 cDNA formation and QRT-PCR

RNA was reverse-transcribed as described below. 0.5μg of the target RNA was first denatured by heating to 65°C for 5 min then place on ice. The resulting RNA was incubated in a volume of 20μL at 50°C for 1 h with final concentrations of 0.1M of DTT, 10 mM of deoxynucleotide triphosphate mix (dNTP) , as well as 100 μg/μL of poly-T primers, 5x cDNA synthesis buffer, 15 units of reverse transcriptase (Invitrogen), and 40 units of RNaseOUT (Invitrogen). The cDNA synthesis reaction was terminated by placing tubes at 85°C for 5 min. 180μl of water was added to each cDNA sample and 5μl of cDNA was added for a Q-PCR.

Quantitative reverse transcription-PCR was done using the Stratagene MX4000 real-time PCR machine. The Stratagene MX4000Note: measures PCR product accumulation during the exponential phase of the reaction, prior to the amplification becoming vulnerable to limited reagents and cycling variability. Fluorescence increases in accordance with increasing levels of PCR product. 5μl of cDNA was added to 1μl of primer mix, 6.25μl of water, 0.25μl reference dye and 12.5μl of SYBR green and run in the PCR machine.

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3.4 Synergy testing

3.4.1 Assessment of Synergy

The effect of combination of agents on cell proliferation was assessed by calculating combination index (CI) values using CalcuSyn software (Biosoft). Derived from the median-effect principal of Chou and Talalay, the CI provides a quantitative measure of the degree of interaction between two agents.

Analysis using the CalcuSyn programme performs optimally when data is collected from a constant ratio combination design experiment. Typically the combination agents are chosen at their equipotent ratio (eg at the ratio of their IC_{50}s) and mixtures of the agents at 4x, 2x, 1x, 0.5x and 0.25x IC_{50} are prepared and tested, (Table 3.4, 3.5.).

Table 3.4 Synergy testing protocol

Agent 1							
Agent 2		0	0.25x $(ED_{50})_1$	0.5x $(ED_{50})_1$	1x $(ED_{50})_1$	2x $(ED_{50})_1$	4x $(ED_{50})_1$
	0	Control $(fa)_0$	$(fa)_1$	$(fa)_1$	$(fa)_1$	$(fa)_1$	$(fa)_1$
	0.25x $(ED_{50})_2$	$(fa)_2$	$(fa)_{1,2}$				
	0.5x $(ED_{50})_2$	$(fa)_2$		$(fa)_{1,2}$			
	1x $(ED_{50})_2$	$(fa)_2$			$(fa)_{1,2}$		
	2x $(ED_{50})_2$	$(fa)_2$				$(fa)_{1,2}$	
	4x $(ED_{50})_2$	$(fa)_2$					$(fa)_{1,2}$

Because the ratio is constant, each mixture can be treated as a single agent to obtain the Dm (median effect dose or concentration – ED50, IC50 or LD50 or TD50 if appropriate), m parameter (exponent signifying the sigmoidicity i.e. shape of the dose-effect curve), construct an automated Fa-CI (fraction affected-combination index) table and plot classic and conservative isobolograms.

In situations where the ratios are random or arbitrary or one concentration changes and the other is fixed, CalcuSyn can still be used to calculate combination index (CI) values for each data point provided the m and Dm parameters for each single agent are

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available. Fa-CI plot simulations are not available, although the combination data plots can be placed on the Fa-CI plot

Broadly speaking, a CI value of 1 is additive, < 1 is synergistic and > 1 is antagonistic. This is rather over simplified and the CalcuSyn authors suggest

Range of CI	Symbol	Description
<0.1	+ + + + +	very strong synergism
0.1-0.3	+ + + +	strong synergism
0.3-0.7	+ + +	synergism
0.7-0.85	+ +	moderate synergism
0.85-0.90	+	slight synergism
0.90-1.10	+/-	nearly additive
1.10-1.20	-	slight antagonism
1.20-1.45	- -	moderate antagonism
1.45-3.3	- - -	antagonism
3.3-10	- - - -	strong antagonism
>10	- - - - -	very strong antagonism

3.4.2 *In vitro* synergy assay

The effect of the combination of OncoVex GALV/CD and chemotherapy (cisplatin, gemcitabine) on bladder tumour cell (EJ, TCCSUP-G, T24) proliferation was assessed by calculating combination-index (CI) values using CalcuSyn software (Biosoft, Ferguson, MO). Derived from the median-effect principle of Chou and Talalay (Chou and Talalay 1984) the CI provides a quantitative measure of the degree of interaction between two or more agents. A CI of 1 denotes an additive interaction, >1 antagonism and <1 synergy.

Cisplatin stock was at 1mg/ml (=1g/l), Mwt = 290.1g/mol

1g/l ÷ 290.1g/mol→ 0.003447M = 3.447mM = 3447μM

Gemcitabine stock was at 40mg/ml (=40g/l), Mwt = 299.66g/mol

40g/l ÷ 299.66g/mol→ 0.133484M = 133.48mM

Test cells were plated at 1×10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex GALVCD and chemotherapy (cisplatin or gemcitabine) in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100μL of appropriate dilution was added to each well as shown in Table 2.6 and incubate 48hr. After 2 days the cells were measured by the MTS assay.

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Table 3.5 Prepared 96 well tray for synergy testing

Cells only			Cisplatin 1xED_{50}
Oncovex 0.25xED_{50}	M E D I U M		Cisplatin 2xED_{50}
Oncovex 0.5 xED_{50}			Cisplatin 4xED_{50}
Oncovex 1xED_{50}			Oncovex 0.25xED_{50} Cisplatin 0.25xED_{50}
Oncovex 2xED_{50}			Oncovex 0.5 xED_{50} Cisplatin 0.5 xED_{50}
Oncovex 4xED_{50}			Oncovex 1xED_{50} Cisplatin 1xED_{50}
Cisplatin 0.25xED_{50}			Oncovex 2xED_{50} Cisplatin 2xED_{50}
Cisplatin 0.5 xED_{50}			Oncovex 4xED_{50} Cisplatin 4xED_{50}

3.5 Orthotopic rat bladder tumour model

All procedures were approved by United Kingdom Home Office and institutional boards. All animal experiments were repeated at least three times. Fischer F344 female rats were purchased from B&K Universal Ltd and from HARLAN.

3.5.1 Anaesthesia and analgesia of the rats

Female Fischer F344 rats were anesthetised either with intraperitioneal injections of Domitor and Ketaset or with balanced inhalation of Isoflurane. The used doses of Domitor and Ketaset is shown in Table 3.6. After the procedure the same amount Antisedant as Domitor was given to each animal to terminate the anaesthesia.

Table 3.6 Anaesthetic drug doses used in this study

Anaesthetic	**Concentration of the stock**	**Diluted stock 5x dilution in PBS ***	**Used dose**	**Dilute drug volume per 100g**
Domitor (Medotomidine)	1mg/ml	0.20mg/ml	0.25mg/kg ip	0.125ml/100g
Ketaset Ketamine	100mg/ml	20mg/ml	35mg/kg ip	0.175ml/100g

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At the induction part of balanced Isoflurane anaesthesia the average dose of Isoflurane was 20.25 ml/hour and after 2 minutes the average dose was turned to 6.75 ml/hour of Isoflurane (Figure 3.3)

Figure 3.3 Inhalation anaesthesia with Isoflurane and the shared inhalation tube

There was no need to use painkillers (Vetergesic) during and after the procedures in this study.

3.5.2 Bladder tumour implantation

Rats were placed in a supine position (on a homeothermic blanket). A 18-gauge plastic intravenous cannula (BD Venflon ^{TM} Pro 393207) was used as a catheter. The catheter was inserted into the bladder via the urethra. To facilitate the tumour seeding the bladder mucosa was damaged by instillation of 0.4 ml 0.1 N hydrochloric acid for 15 seconds followed by a 15-second rinse with 0.4ml of 0.1 N sodium hydroxide for neutralization. The bladder was then drained and washed five times with 0.6ml PBS (pH 7.4). A suspension of freshly harvested AY-27 cells (1.5-2.5x10^6 cells in 0.4ml of medium) was then instilled and maintained in the bladder for 1 hour on homeothermic blanket to maintain. The rats were turned 90° laterally every 15 minutes to ensure

exposure of the entire bladder wall to the tumour cells. After 1 hour the catheters were removed, and the rats were allowed to void spontaneously.

3.5.3 Assessment of tumour growth

3.5.3.1 Quantitative reverse transcription polymerase chain reaction

The frequent catheterisation to collect urine samples for the various assays may cause more damage and pain for the animals. We collected urine samples by holding the rats in a metabolization cage (Figure 3.4) for 1 hour (on day 0,4,7,11,14 after tumour implantation). Quantitative reverse transcription polymerase chain reaction (QRT-PCR) was performed on the collected urine samples to evaluate whether tumour growth is detectable by QRT-PCR.

Figure 3.4 Metabolization cage

3.5.3.2 Bioluminescent imaging using IVIS camera

Fischer F344 rats were anaesthetised and $1x10^6$ AY-27 HL-S cells in 100ul RPMI (without FCS) were injected subcutaneously. Luc expression was quantitated by real-time bioluminescent imaging using the IVIS System 200 series (Xenogen Biosciences) D-Luciferin Firefly Potassium salt (15 mg/mL; Xenogen Biosciences) was administered at 150 mg/kg and imaging was performed after 5 min. 30s exposures were obtained at

high sensitivity using the machine default settings (f/stop, 1; emission filter, open; field of view d, 12 cm; and subject height, 1.5 cm). The Xenogen software provides visual images of bioluminescence detection using a coloured overlay on a photographic image taken immediately before luminescence measurements. The areas shown in colour represent the regions of bioluminescence detected. Results were analyzed using Living Image 2.60.1 software (Xenogen Biosciences). Areas of Luc expression were defined using the autocontouring tool and the photon flux and average radiance (photons/cm^2/s) were estimated over the defined area.

3.5.4 In vivo treatment of orthotopic rat bladder tumour model

Female Fischer F344 rats were anesthetised and place in a supine position on a homeothermic blanket. A catheter was inserted into the bladder via the urethra with the use of an 18-gauge plastic intravenous cannula (BD Venflon ^{TM} Pro 393207). The rats were randomly assigned to 3 treatment groups. Group A was treated with Oncovex GALV/CD + Prodrug, Group B was treated with Oncovex GALV/CD + PBS, Group C was treated with PBS + Prodrug. On day 7,14 and 21 rats in Group A and B were treated with Oncovex GALV/CD, in Group C with PBS. On day 7-8, 15-16 and 22-23 rats in Group A and C were treated with prodrug, in Group B with PBS. (Figure 3.5)
All kind of treatments were instilled transurethrally through the catheter and were maintained in the bladder for 40 minutes. The rats were turned 90° laterally every 10 minutes to ensure exposure of the entire bladder wall. 9x10.7 pfu Oncovex GALV/CD virus was administered in 600µl PBS (Virus stock: 1.8x10.5pfu/µl). 600µl of 5-Fluorocytosine (5-FC) in RPMI was then instilled (Concentration of stock: 15.0mg/ml). As control 600µl PBS (pH 7.4) was added. After 1 hour the catheters were removed, and the rats were allowed to void spontaneously.

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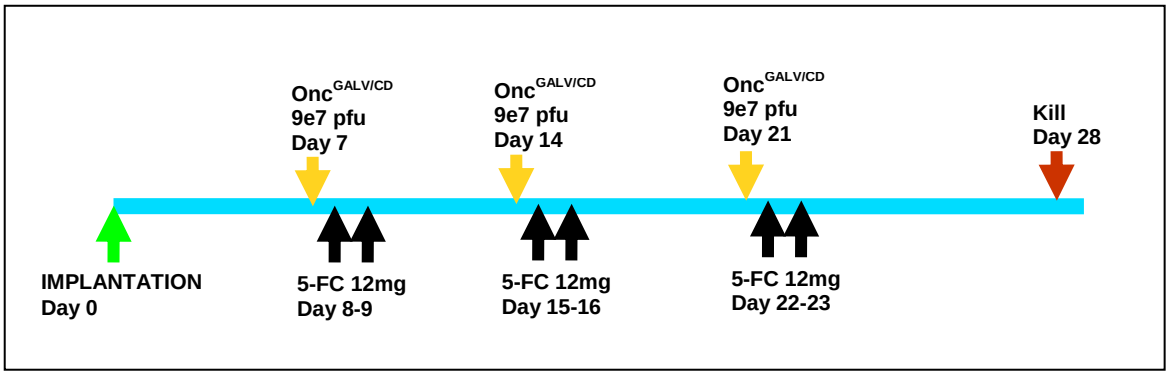

Figure 3.5 Protocol for in vivo studies

After the treatment the animals were monitored daily for general health status and for signs symptoms of advanced bladder cancer (weight loss, abdominal mass, haematuria). The rats were sacrificed after a 28 days. There bladders were removed and assessed for tumour abundance. The harvested bladders were weighed, then opened up and the bladder surface which macroscopically contained tumour was measured with a caliper. Other organs within peritoneal cavity (liver, kidneys, spleen) were also checked during the autopsy for signs of macroscopic metastasis. The removed bladders were stored either in RNA later (for q-PCR) or either fixed in 10% phosphatebuffered formalin (for histological evaluation).

3.6 Statistics

The values of the experiments were presented as the mean ± standard deviation and analyis of variance was measured with using unpaired Student's t-test. Statistical significance was determined at P value less than 0.05. The survival curves were generated using the Kaplan-Meier method and compared using log-rank test.

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4 Results

4.1 Testing Oncovex$^{GALV/CD}$ on bladder tumour cells *in vitro* with Fusion assay

A panel of human bladder cancer cell lines was infected with OncoVex^{GFP} and OncoVEX$^{GALV/CD}$ to compare plaque morphology, which may change with expression of the fusogenic glycoprotein from Gibbon Ape Leukaemia Virus. All the bladder cells infected with OncoVex^{GFP} showed a classic HSV-1 cytopathic effect. In contrast, four out of the seven cell lines infected with OncoVEX$^{GALV/CD}$ showed fusion in the form of large multinucleated syncytia which were then surrounded with cells showing the more classic HSV-1-mediated effect, (EJ cells, T24 cells, VMCUB-I cells, and 5637 cells). To study whether the formation of multinucleated syncytia increases cytopathic effect of this virus (OncoVEX$^{GALV/CD}$) when compared to backbone vector (OncoVex^{GFP}) *in vitro* MTS assays were carried out. Bladder tumour cells were infected with both viruses at a range of MOI (10,1, 0.1, 0.01, 0.001, 0), incubated for 48 hrs and the resulting infected cells were then tested for MTS activity, which measures the activity of mitochondrial enzymes needed for cell proliferation/cell survival, HSV replication should inhibit cell proliferation and therefore MTS activity (Promega). Lower levels of MTS activity was seen with OncoVEX$^{GALV/CD}$ than OncoVex^{GFP} on infected EJ (42-54% decrease in cell survival), T24 (35-45%) VMCUB-I (36-37%), 5637 (35%) cells. This suggests that the presence of GALV *env* R- results in a higher cytotoxicity and therefore increased tumour cell killing (Figure 4.1.1-4). In contrast RT112, TCCSUP-G, and KU19-19 human bladder tumour cells did not show a fusogenic plaque morphology when infected with OncoVEX$^{GALV/CD}$, or an increase in tumour cell killing in the presence of fusogenic glycoprotein from Gibbon Ape Leukaemia Virus (Figure 4.1.5).

In summary all bladder tumours cells tested showed oncolysis in the presence of HSV. The presence of fusogenic glycoprotein enhanced the tumour cell killing by up to 54% in four out of seven bladder tumour cell lines (Table 4.1.1).

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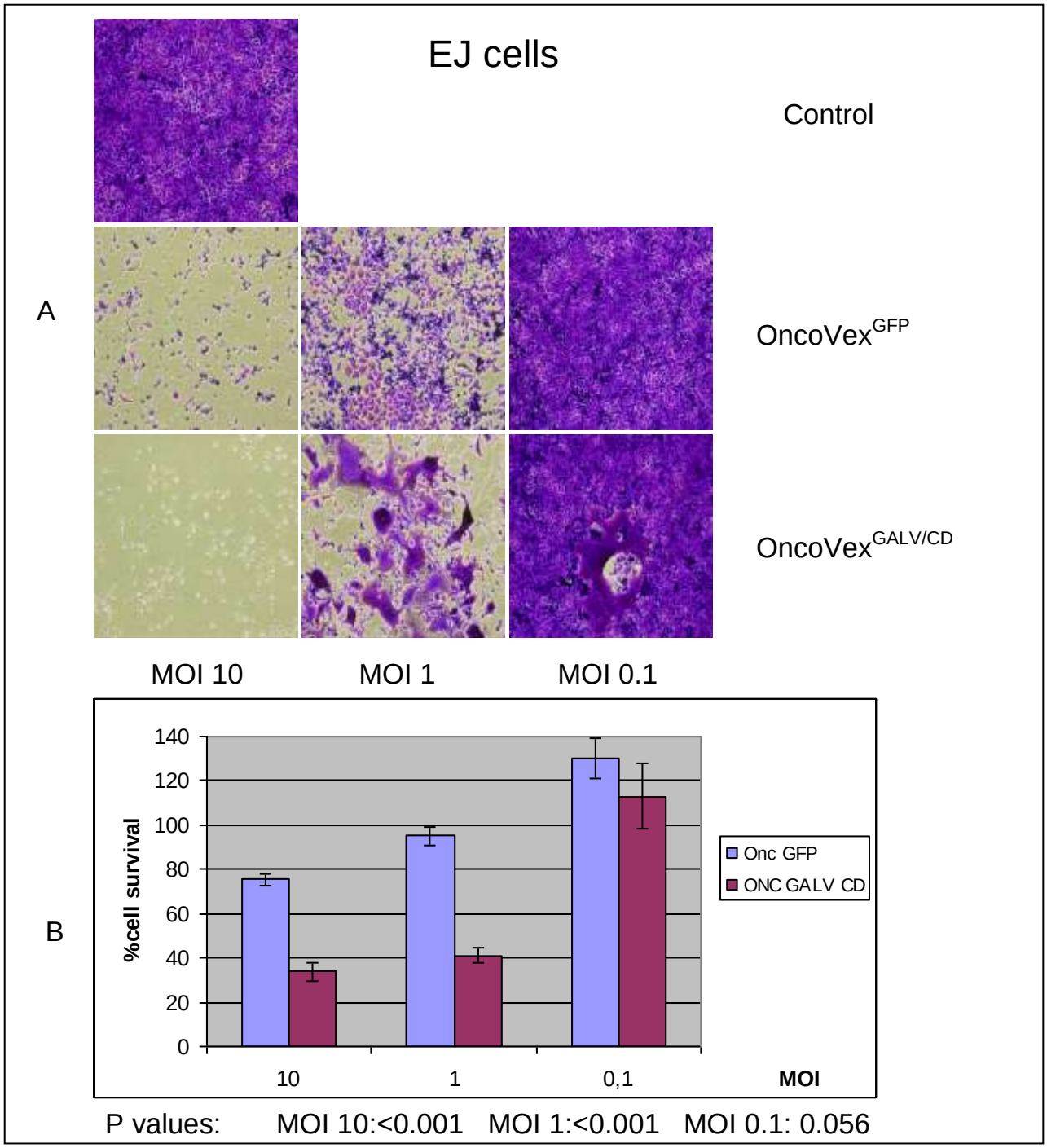

Figure 4.1.1 $2x10^4$ EJ cells were infected with OncoVex^{GFP} or OncoVex$^{GALV/CD}$ at MOIs of 10, 1, 0.1 according to fusion assay protocol. Then assayed by Crystal Violet staining (magnification 100x)(A) or MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells (B). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations. **A:** In the mid row cells infected with OncoVex^{GFP} show a classic HSV-1 cytopathic effect, where tumour cell killing is increasing (from right to left) by the increasing levels (MOI) of virus due to its oncolytic activity. In the lower row cells infected with OncoVEX$^{GALV/CD}$ show fusion (large multinucleated syncytia)

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surrounded with cells showing the classic HSV-1 cytopathic effect and higher levels of cell killing compared to the mid row.

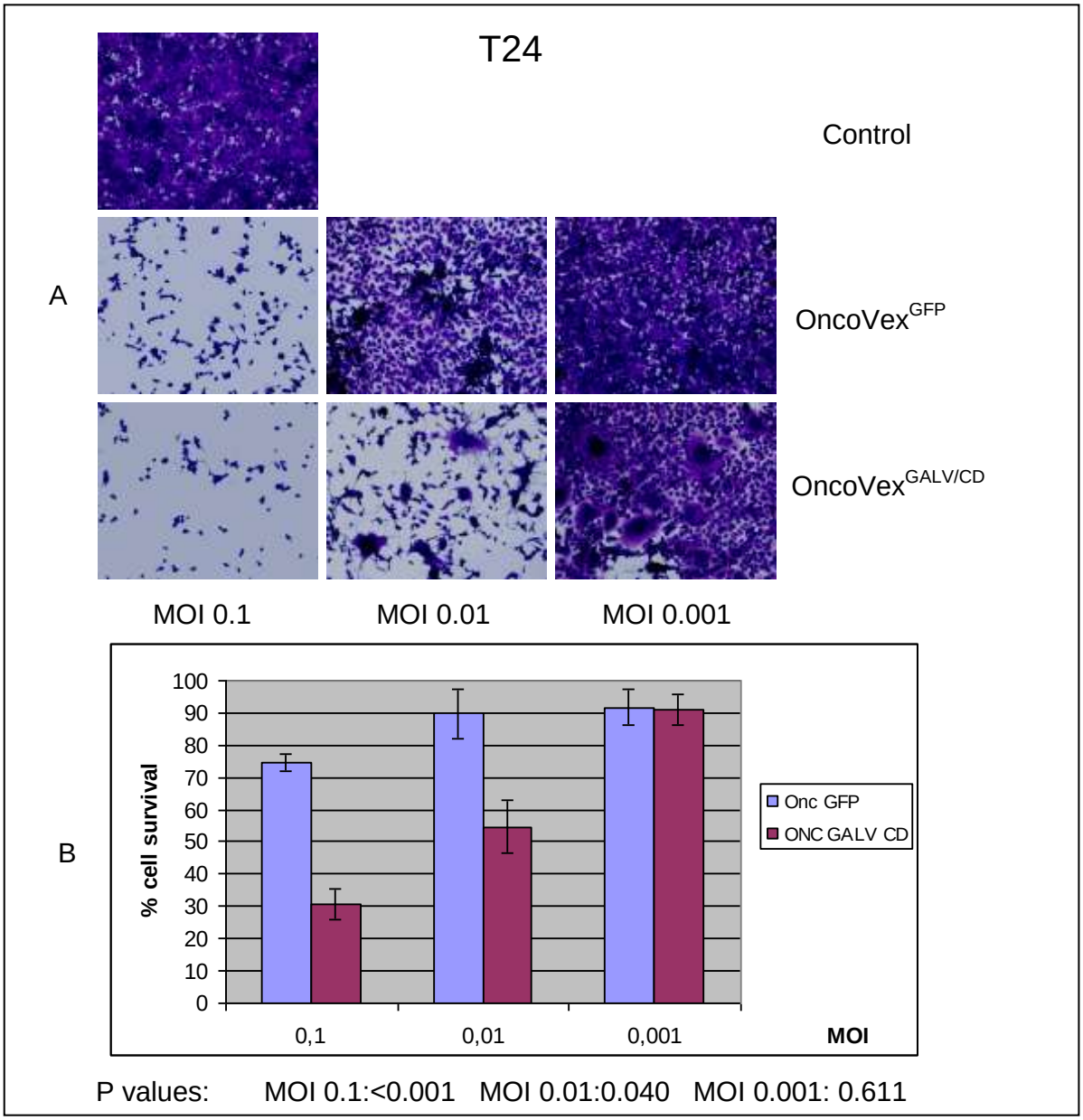

Figure 4.1.2 2x10^4 T24 cells were infected with OncoVex^{GFP} or OncoVex$^{GALV/CD}$ at MOIs of 0.1, 0.01, 0.001 according to fusion assay protocol. Then assayed by Crystal Violet staining (magnification 100x)(A) or MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells (B). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations. **A:** In the mid row cells infected with OncoVex^{GFP} show a classic HSV-1 cytopathic effect, where tumour cell killing is increasing (from right to left) by the increasing levels (MOI) of virus due to its oncolytic activity. In the lower row cells infected with OncoVEX$^{GALV/CD}$ show fusion (large multinucleated syncytia) surrounded with cells showing the classic HSV-1 cytopathic effect and higher levels of cell killing compared to the mid row.

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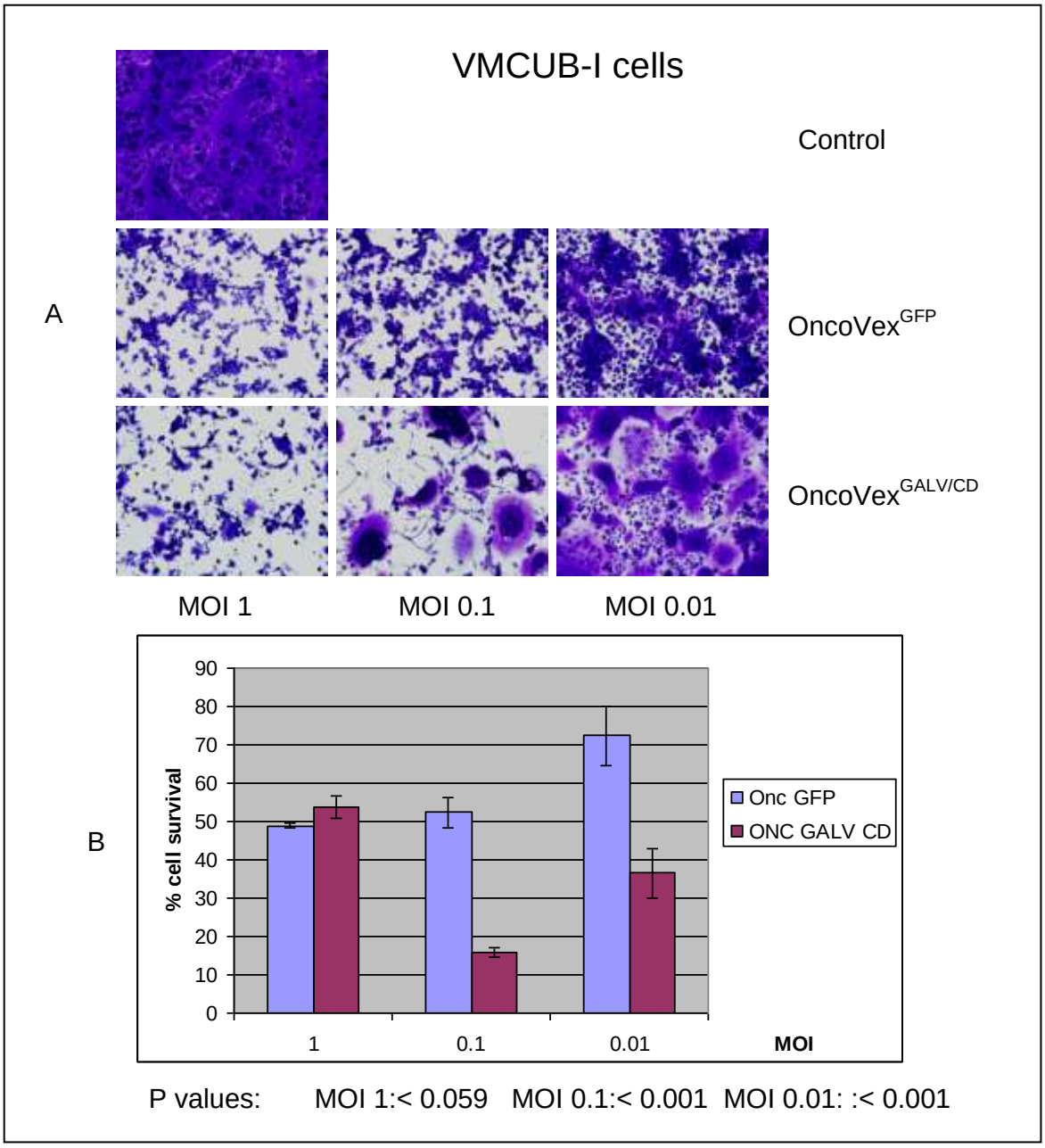

Figure 4.1.3 $2x10^4$ VMCUB-I cells were infected with OncoVex^{GFP} or OncoVex$^{GALV/CD}$ at MOIs of 1, 0.1, 0.01 according to fusion assay protocol. Then assayed by Crystal Violet staining (magnification 100x)(A) or MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells (B). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations. **A:** In the mid row cells infected with OncoVex^{GFP} show a classic HSV-1 cytopathic effect, where tumour cell killing is increasing (from right to left) by the increasing levels (MOI) of virus due to its oncolytic activity. In the lower row cells infected with OncoVEX$^{GALV/CD}$ show fusion (large multinucleated syncytia) surrounded with cells showing the classic HSV-1 cytopathic effect and higher levels of cell killing compared to the mid row.

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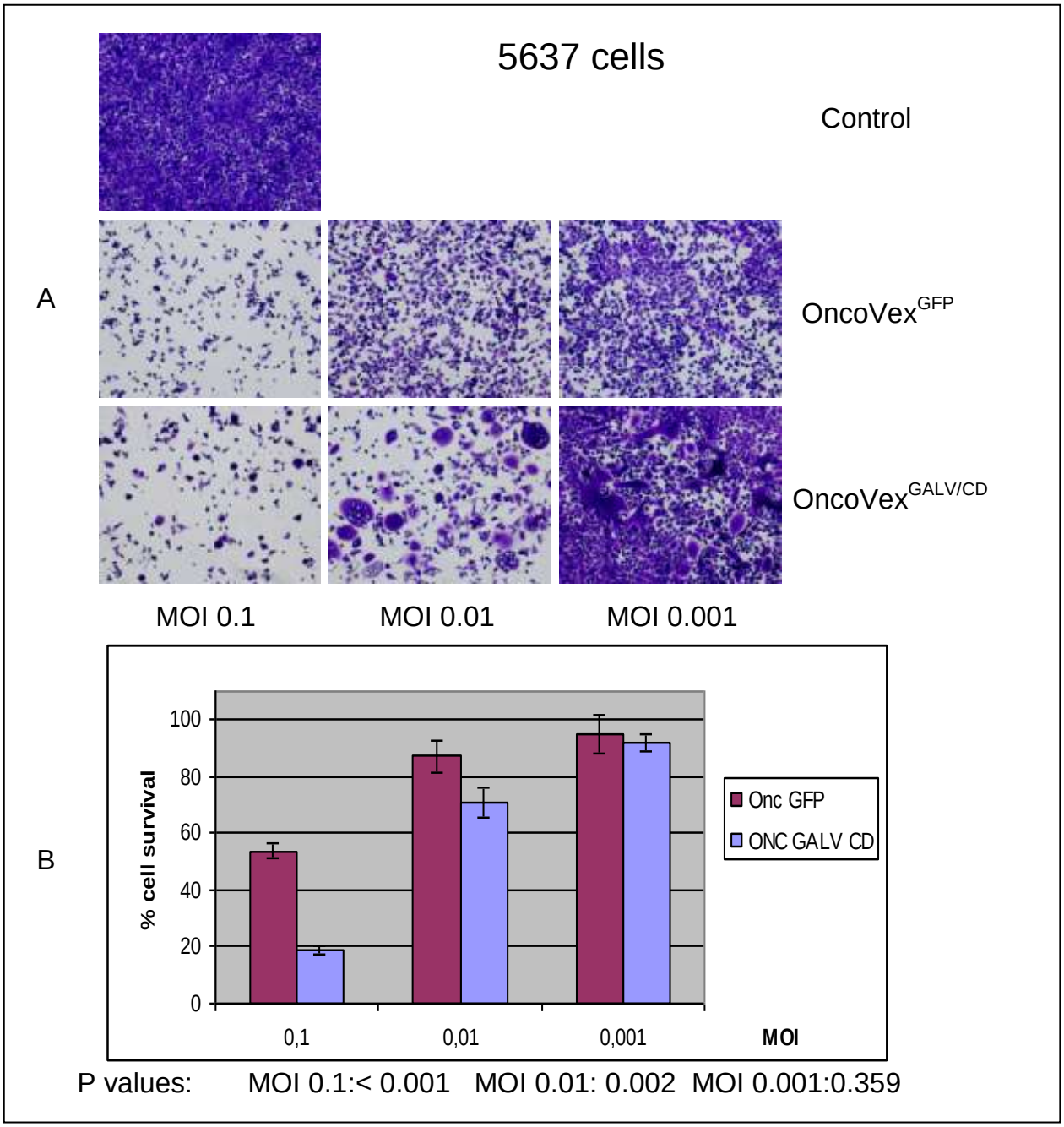

Figure 4.1.4 $2x10^4$ 5637 cells were infected with OncoVex^{GFP} or OncoVex$^{GALV/CD}$ at MOIs of 0.1, 0.01, 0.001 according to fusion assay protocol. Then assayed by Crystal Violet staining (magnification 100x)(A) or MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells (B). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations. **A:** In the mid row cells infected with OncoVex^{GFP} show a classic HSV-1 cytopathic effect, where tumour cell killing is increasing (from right to left) by the increasing levels (MOI) of virus due to its oncolytic activity. In the lower row cells infected with OncoVEX$^{GALV/CD}$ show fusion (large multinucleated syncytia) surrounded with cells showing the classic HSV-1 cytopathic effect and higher levels of cell killing compared to the mid row.

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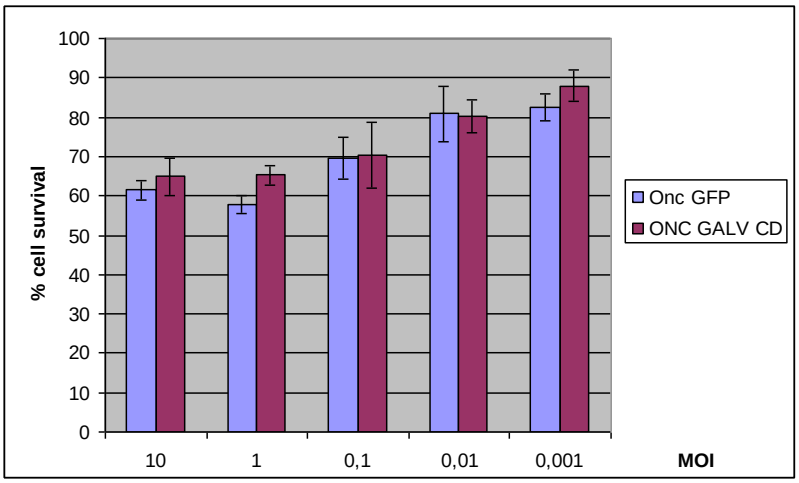
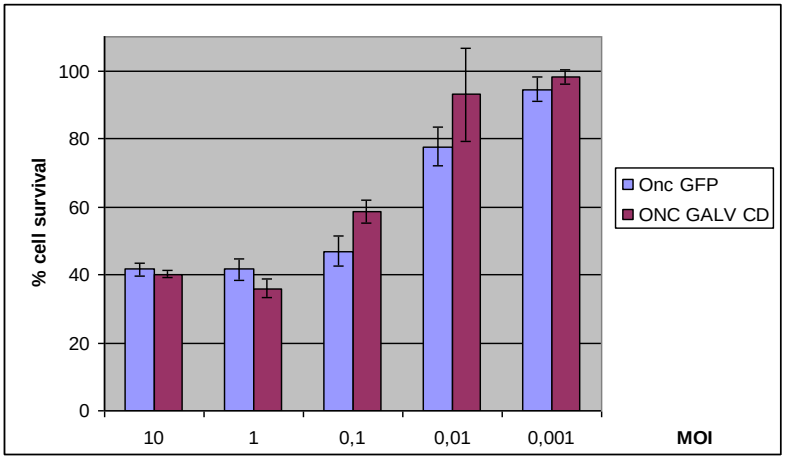
RT112



TCCSUP-G



KU19-19

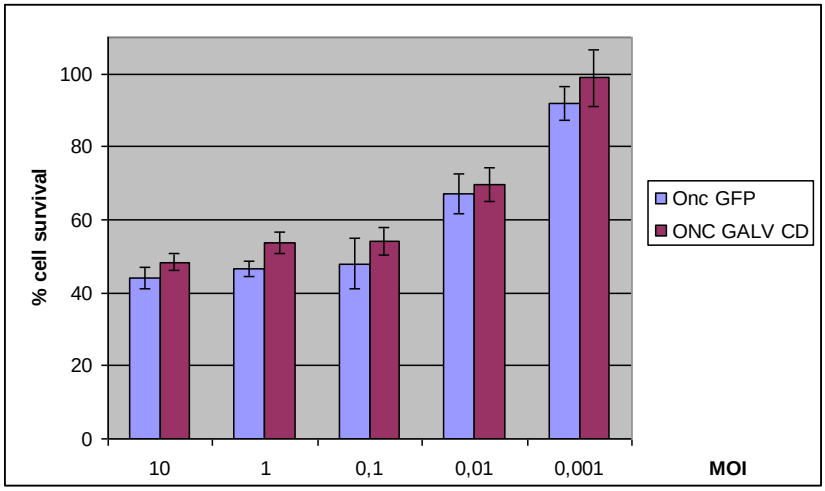


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Figure 4.1.5 $2x10^4$ RT112, TCCSUP-G and KU19-19 cells were infected with $OncoVex^{GFP}$ or $OncoVex^{GALV/CD}$ at MOIs of 10, 1, 0.1, 0.01, 0.001 according to fusion assay protocol. MTS assay (Promega) did not show increase in cell killing with $OncoVEX^{GALV/CD}$ compared $OncoVex^{GFP}$ backbone alone (P was > 0.05). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

Table 4.1.1 Summary of fusion assay results on human cell lines

Human bladder tumour cell lines	Histological type	HSV-1 CPE	Increase efficacy due to GALV *env* R-
EJ	TCC	+	**42-54%**
T24	TCC	+	**35-45%**
RT112	TCC	+	**0%**
VMCUB-I	TCC	+	**36-37%**
TCCSUP-G	TCC	+	**0-6%**
5637	TCC	+	**35%**
KU19-19	TCC	+	**0%**

4.2 Testing $Oncovex^{GALV/CD}$ on bladder tumour cells *in vitro* with Prodrug assay

Fcy::Fur is a fusion of two genes *CD* and *UPRT*, which metabolizes 5-FC more efficiently than either gene alone. To study the cell killing effects of HSV-expressing *Fcy::Fur* gene in the presence of 5-FC human bladder tumour cells were infected with $OncoVEX^{GALVCD}$ or $OncoVEX^{GFP}$ in the presence or absence of 5-FC. The cell supernatants were then heat inactivated to neutralize the virus, allowing the effects of any 5-FU produced to be determined on fresh tumor cells (Figure 4.2.1).

In the presence of supernatants from cells infected with $OncoVEX^{GFP}$ no cell death was seen with or without 5-FC (Figure 4.2.2A). However, in the presence of the supernatant from $OncoVEX^{GALVCD}$ infected cells displayed effective cell killing in the presence of 5-FC (Figure 4.2.2A). Results were similar in a range of human bladder tumor cell lines, including EJ cells, RT112 cells, TCCSUP-G cells, 5637 cells, KU19-19 cells (Figure 4.2.2-6). In contrast no observed tumour cell killing was seen with T24 or VMCUB-I human bladder tumour cells suggesting that either the cells are not sensitive to 5-FU metabolites or that the cells did not allow the active metabolism of 5-FC into 5-FU (Figure 4.2.7). MTS assays have allowed us to quantitate the effects of prodrug

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activation therapy on bladder tumour cells. EJ cells are the most susceptible for the prodrug activation therapy. A 55% decrease in tumour cell survival was detected on EJ cells at the concentration of 100µm 5-FC, which decreased further to 78% at 600µm 5-FC. RT112 and KU19-19 cells belong to the mid group in terms of sensitivity for OncoVEX$^{GALV/CD}$ prodrug activation therapy. At between 600-1000µm 5-FC these cell lines showed around 70% decrease in tumour cell survival. Finally TCCSUP-G and 5637 cells showed the lowest sensitivity for this prodrug activation therapy. They showed around 53% decrease in tumour cell survival at 1000µm 5-FC.

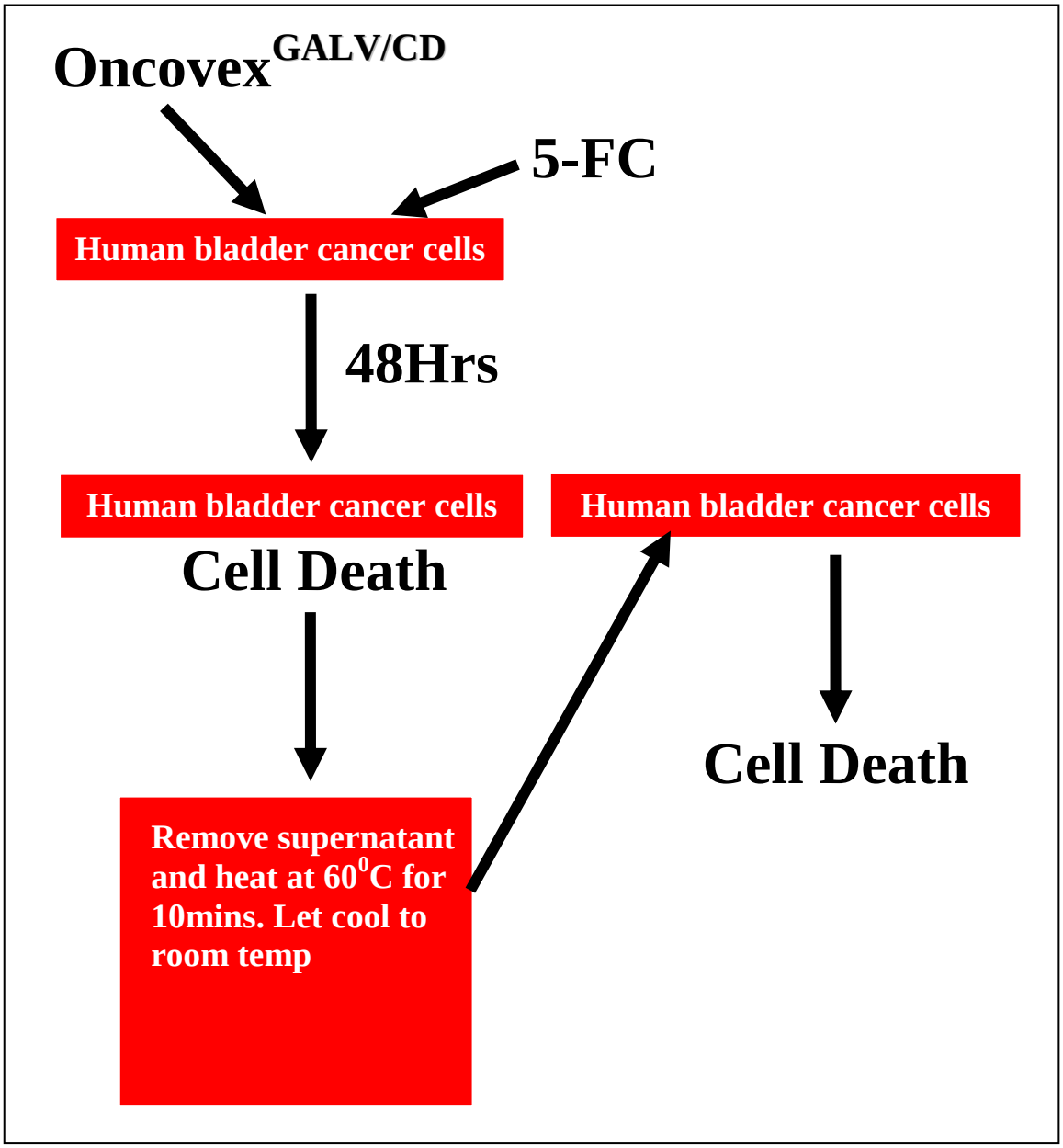

Figure 4.2.1 Outline of the protocol for studying the effects of Fcy:Fur, expressed in HSV-1 backbone vector, in presence of 5-FC.

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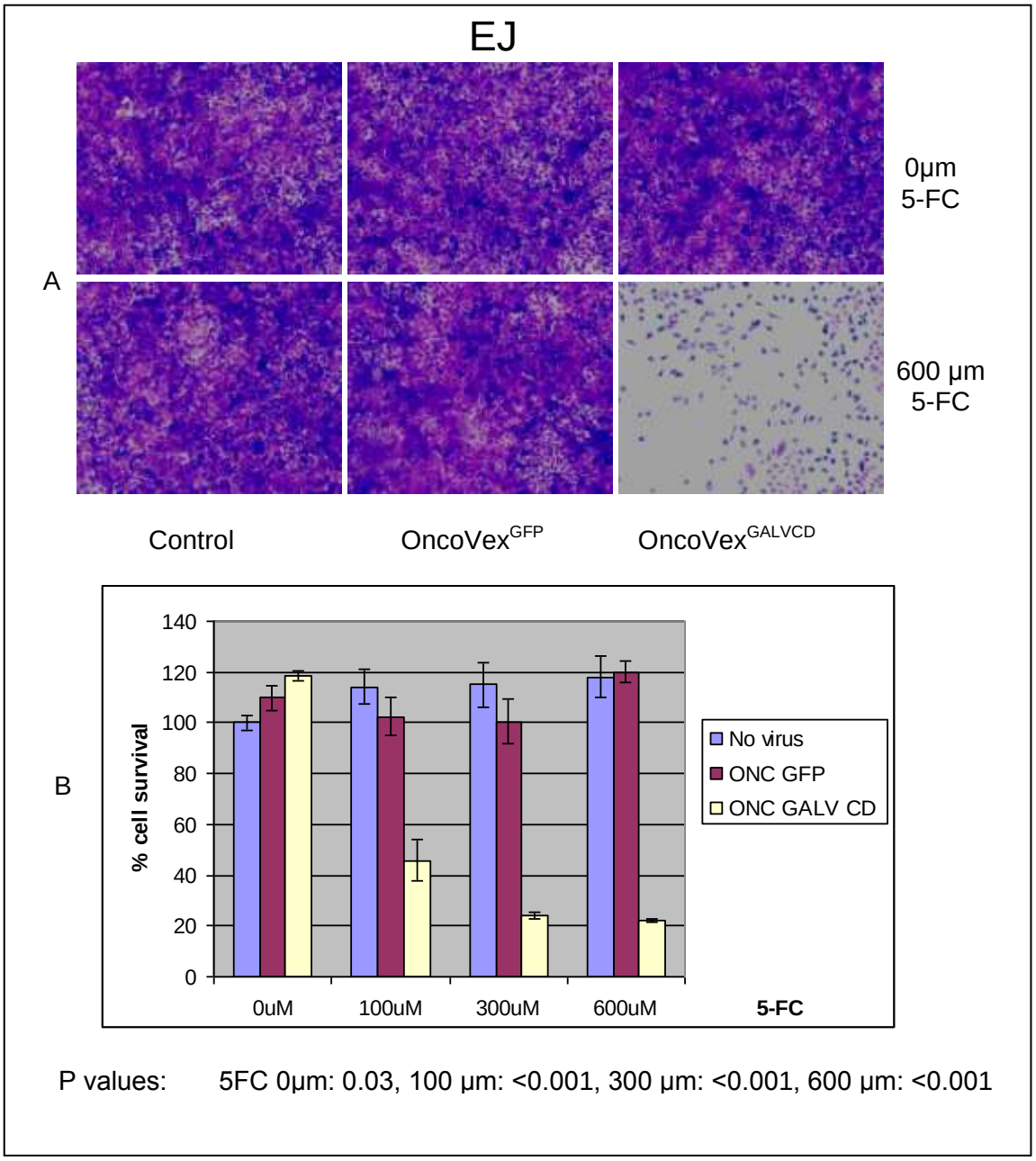

Figure 4.2.2 1 x 10^5 EJ cells were infected with OncoVex^{GFP} and OncoVex$^{GALV/CD}$ at MOI of 1 according to the fusion assay results and no virus control. According to prodrug assay protocol FGM containing 5-FC at different concentrations (0-100-300-600 µmol/L) was added. Assayed by Crystal Violet staining (magnification 100x)(A) or MTS assay (Promega)(B) was performed. Average cell survival was calculated as a percentage compared to untreated cells. All results showed enchanced tumour killing in the presence of OncoVEX$^{GALV/CD}$ and 5-FC (**A:** Low amount of living cell is observed in lower right picture). Quantification of the prodrug studies showing that in all cases the differences were significant (P <0.05). (P values: GALV/CD+0µm Vs GFP+0µm =0.03, GALV/CD+100µm Vs GALV/CD+0µm =<0.000, GALV/CD+300µm Vs GALV/CD+0µm =<0.000, GALV/CD+600µm Vs GALV/CD+0µm =<0.000) Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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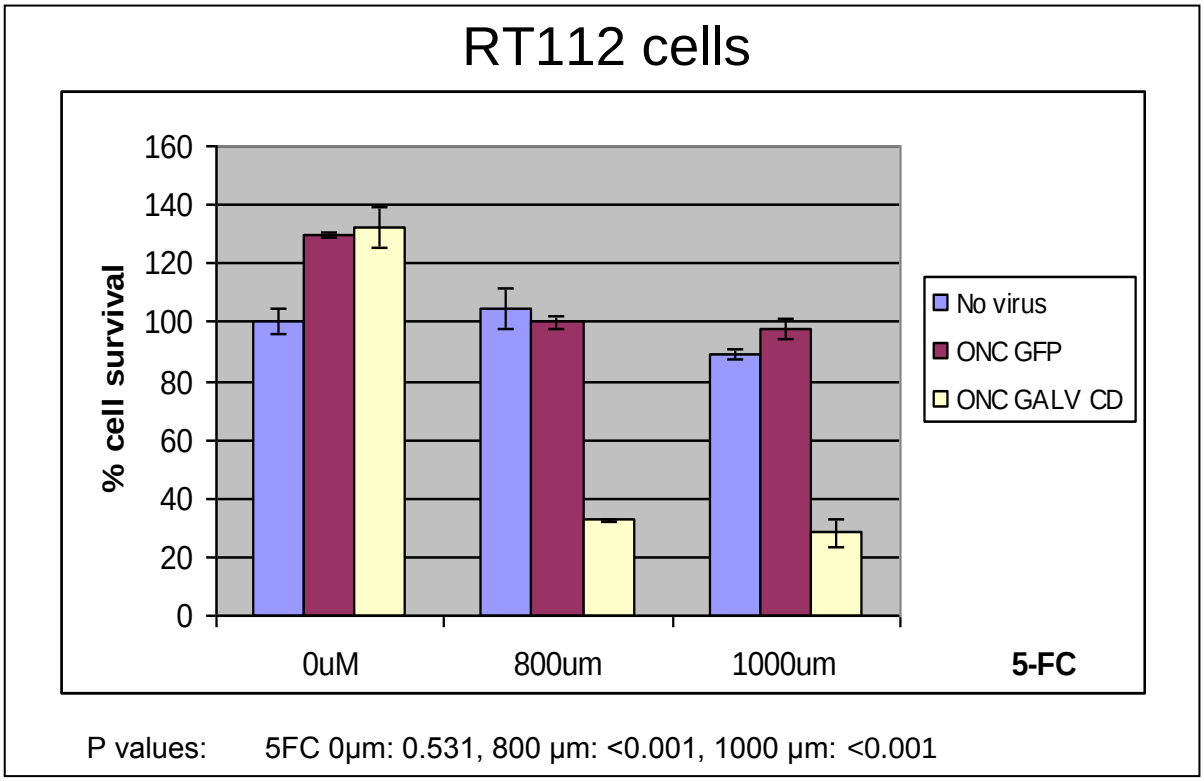

Figure 4.2.3 1 x 10^5 RT112 cells were infected with OncoVex^{GFP} and OncoVex$^{GALV/CD}$ at MOI of 0.1 and no virus control. According to prodrug assay protocol FGM containing 5-FC at different concentrations (0-800-1000 µmol/L) was added and assayed by MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells. All results showed enchanced tumour killing in the presence of OncoVEX$^{GALV/CD}$ and 5-FC. Quantification of the prodrug studies showing that in all cases the differences were significant (P <0.05). (P values: GALV/CD+0µm Vs GFP+0µm =0.531, GALV/CD+800µm Vs GALV/CD+0µm =<0.000, GALV/CD+1000µm Vs GALV/CD+0µm =<0.000) Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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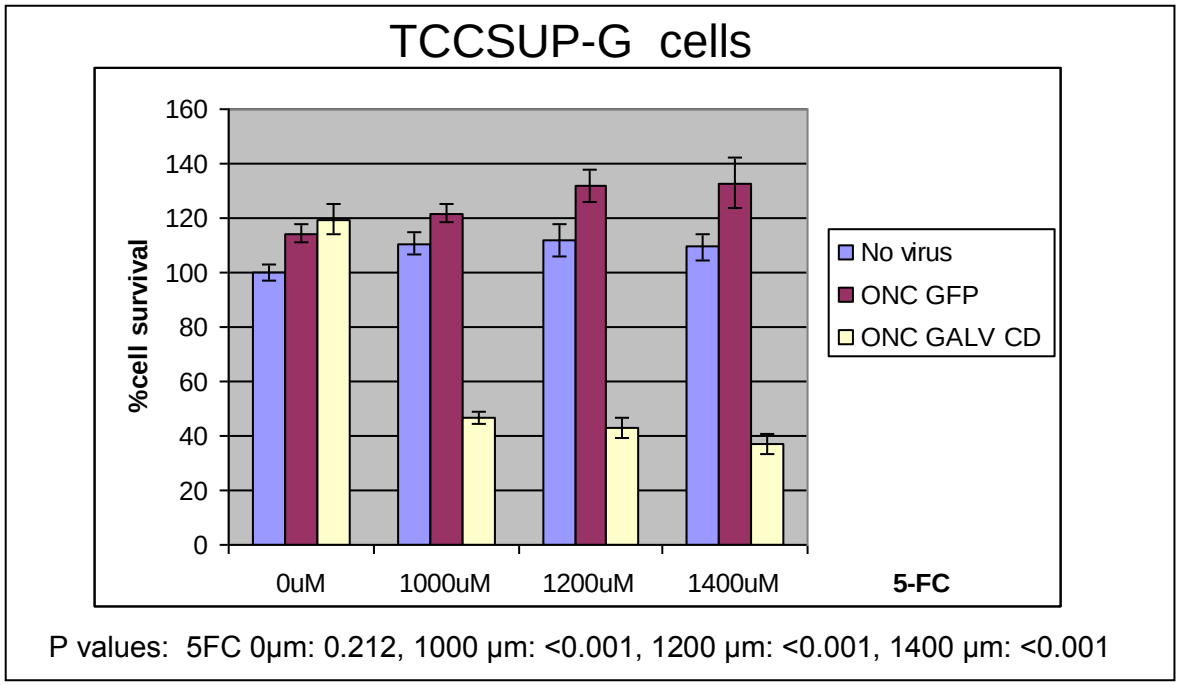

Figure 4.2.4 1 x 10^5 TCCSUP-G cells were infected with OncoVex^{GFP} and OncoVex$^{GALV/CD}$ at MOI of 0.1 and no virus control. According to prodrug assay protocol FGM containing 5-FC at different concentrations (0-1000-1200-1400 µmol/L) was added and assayed by MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells. All results showed enchanced tumour killing in the presence of OncoVEX$^{GALV/CD}$ and 5-FC. Quantification of the prodrug studies showing that in all cases the differences were significant (P <0.05). (P values: GALV/CD+0µm Vs GFP+0µm =0.212, GALV/CD+1000µm Vs GALV/CD+0µm =<0.000, GALV/CD+1200µm Vs GALV/CD+0µm =<0.000, GALV/CD+1400µm Vs GALV/CD+0µm =<0.000) Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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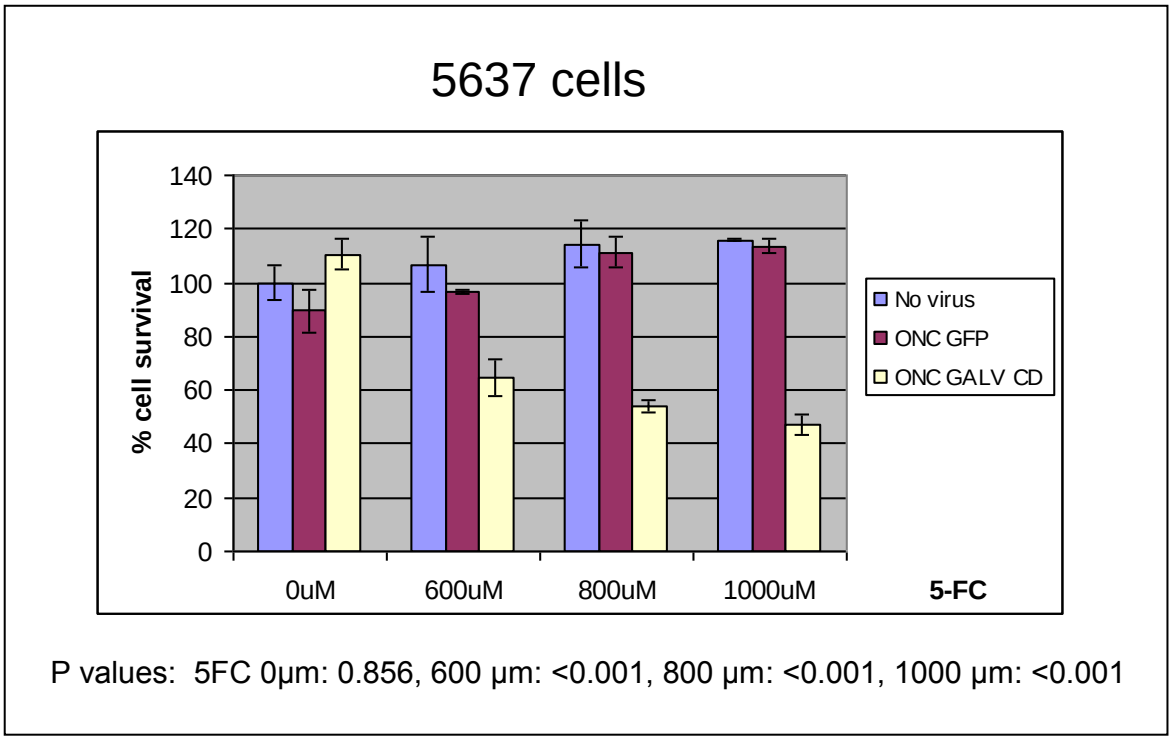

Figure 4.2.5 1 x 10^5 5637 cells were infected with OncoVex^{GFP} and OncoVex$^{GALV/CD}$ at MOI of 0.1 according to the fusion assay results and no virus control. After 30 minutes at 37°C/5% CO_2, the virus was removed, and 1 mL of FGM containing 5-FC ($C_4H_4FN_2O$; Sigma) at different concentrations (0-600-800-1000 µmol/L) was added and incubated for 48 hours at 37°C/5% CO_2. The cell supernatants were then heat inactivated and added to 1 x 10^4 fresh 5637 cells and incubated at 37°C/5% CO_2 for 72 hours and assayed by MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells. All results showed enchanced tumour killing in the presence of OncoVEX$^{GALV/CD}$ and 5-FC. Quantification of the prodrug studies showing that in all cases the differences were significant (P <0.05). (P values: GALV/CD+0µm Vs GFP+0µm =0.856, GALV/CD+600µm Vs GALV/CD+0µm =<0.000, GALV/CD+800µm Vs GALV/CD+0µm =<0.000, GALV/CD+1000µm Vs GALV/CD+0µm =<0.000) Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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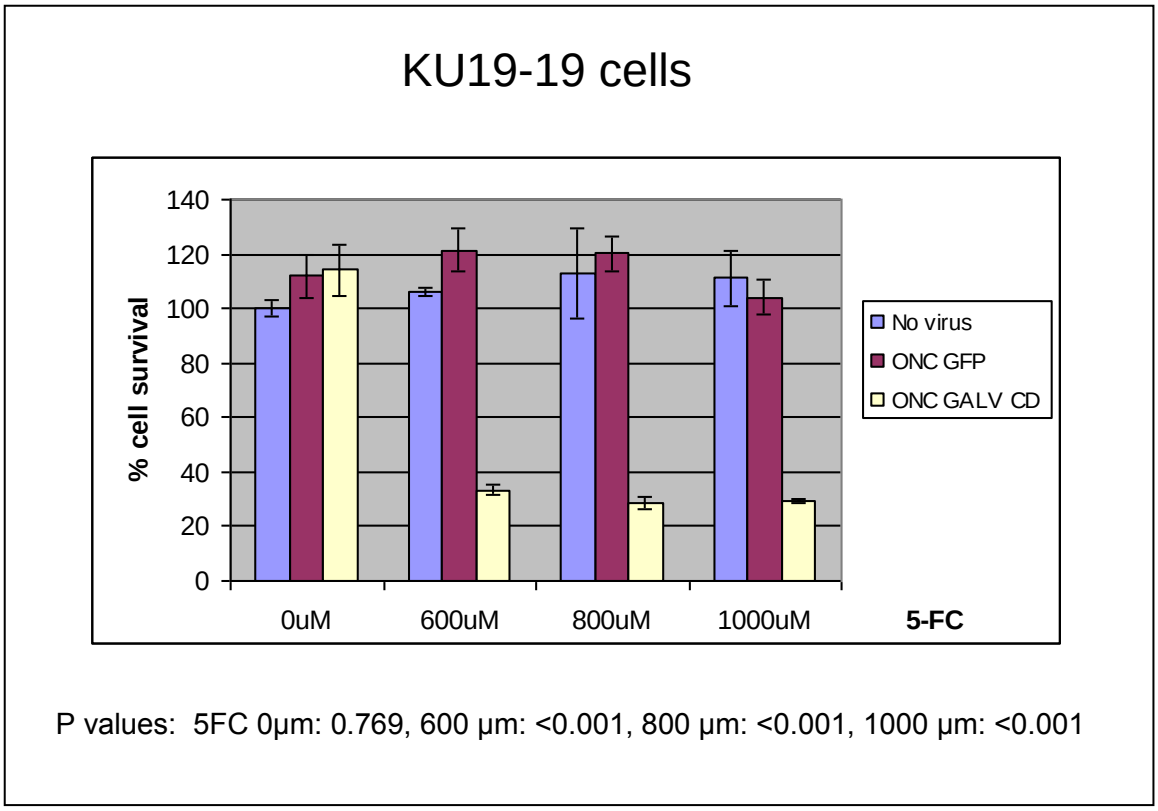

Figure 4.2.6 1 x 10^5 KU19-19 cells were infected with OncoVex^{GFP} and OncoVex$^{GALV/CD}$ at MOI of 0.1 and no virus control. After 30 minutes at 37°C/5% CO_2, the virus was removed, and 1 mL of FGM containing 5-FC ($C_4H_4FN_2O$; Sigma) at different concentrations (0-600-800-1000 µmol/L) was added and incubated for 48 hours at 37°C/5% CO_2. The cell supernatants were then heat inactivated and added to 1 x 10^4 fresh KU19-19 cells and incubated at 37°C/5% CO_2 for 72 hours and assayed by MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells. All results showed enchanced tumour killing in the presence of OncoVEX$^{GALV/CD}$ and 5-FC. Quantification of the prodrug studies showing that in all cases the differences were significant (P <0.05). (P values: GALV/CD+0µm Vs GFP+0µm =0.769, GALV/CD+600µm Vs GALV/CD+0µm =<0.000, GALV/CD+800µm Vs GALV/CD+0µm =<0.000, GALV/CD+1000µm Vs GALV/CD+0µm =<0.000) Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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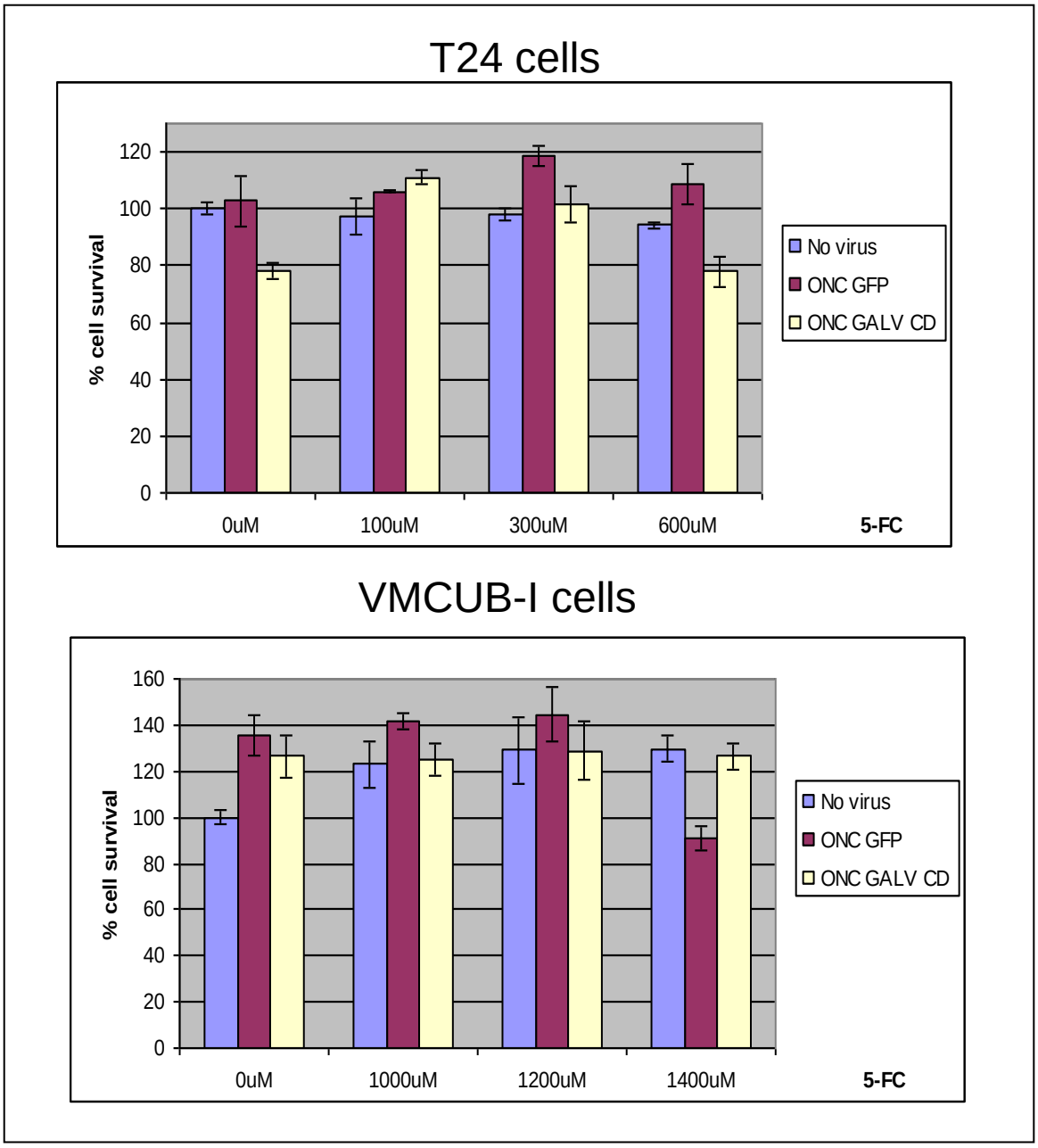

Figure 4.2.7 1 x 10^5 T24 and VMCUB-I cells were infected with OncoVex^{GFP} and OncoVex$^{GALV/CD}$ at MOI of 0.1 and no virus control. After 30 minutes at 37°C/5% CO_2, the virus was removed, and 1 mL of FGM containing 5-FC ($C_4H_4FN_2O$; Sigma) at different concentrations (0-100-300-600-800-1000-1200-1400 µmol/L) was added and incubated for 48 hours at 37°C/5% CO_2. The cell supernatants were then heat inactivated and added to 1 x 10^4 fresh T24 and VMCUB-I cells and incubated at 37°C/5% CO_2 for 72 hours. MTS assay (Promega) did not show enchanced tumour killing in the presence of OncoVEX$^{GALV/CD}$ and 5-FC (P was > 0.05). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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In summary five out of seven bladder tumour cell line showed increased tumour cell killing *in vitro* when infected with OncoVEX$^{GALV/CD}$ virus in the presence of 5-FC (Table 4.2.1). We have tried to calculate IC50's of each cell line, but we do not have enough data yet to accurately calculate an IC50. We would need to repeat our experiments with a bigger spread of 5-FC concentration.

Table 4.2.1 Summary of prodrug assay results on human bladder tumour cell lines.

Cell type	Effective dose of 5-FC.	% Decrease cell survival
EJ	600uM	78%
T24	600uM	~ 0%
RT112	800uM	68%
VMCUB-I	1400uM	0%
TCCSUP-G	1000uM	54%
5637	1000uM	53%
KU19-19	600uM	67%

We can also conclude from the summary of results (Table 4.2.2) that all human bladder tumour cell lines tested are susceptible to HSV oncolysis and showed enhanced tumour cell killing in at least one type (fusion or prodrug) of the assays when infected with OncoVEX$^{GALV/CD}$ virus.

Table 4.2.2 Summary of fusion and prodrug assay results on human bladder tumour cell lines.

Cell type	Hystologic type	HSV-1 CPE	Fusion	Prodrug
EJ	**TCC**	+	+	+
T24	**TCC**	+	+	-
RT112	**TCC**	+	-	+
VMCUB-I	**TCC**	+	+	-
TCCSUP-G	**TCC**	+	-	+
5637	**TCC**	+	+	+
KU19-19	**TCC**	+	-	+

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4.3 Testing Oncovex$^{GALV/CD}$ on bladder tumour cells *in vitro* in combination with conventional chemotherapies

The effect of the combination of OncoVex$^{GALV/CD}$ and chemotherapy (mitomycin, cisplatin, gemcitabine) on bladder tumour cell (EJ, TCCSUP-G, T24, KU19-19) proliferation was assessed by calculating combination-index (CI) values using CalcuSyn software (Biosoft, Ferguson, MO). Derived from the median-effect principle of Chou and Talalay (Chou and Talalay 1984), the CI provides a quantitative measure of the degree of interaction between two or more agents. A CI of 1 denotes an additive interaction, >1 antagonism and <1 synergy. Analysis using the CalcuSyn programme performs optimally when data is collected from a constant ratio combination design experiment. Typically the combination agents are chosen at their equipotent ratio (eg at the ratio of their IC_{50}s). The human bladder cancer cell lines were infected with OncoVEX$^{GALV/CD}$ or treated with one of the chemotherapeutical agents (mitomycin, cisplatin, gemcitabine) or both in combination where the equipotent ED ratio of each agent was mixed for combination. The cells were measured by the MTS assay after 2 days. Our results showed synergistic effect when OncoVEX$^{GALV/CD}$ was combined with mitomycin in EJ, T24 and KU19-19 cells (Figure 4.3.1-3). Whereas the combination effect of OncoVEX$^{GALV/CD}$ with cisplatin or gemcitabine was antagonistic in EJ, T24 and TCCSUP-G cells (Figure 4.3.4-9) (Table 4.3.1). In order to evaluate whether this antagonistic effect is related to the Herpes Simplex Virus instead of to the implanted GALV and CD genes we have tested the backbone OncoVEX^{GFP} in combination with cisplatin or gemcitabine in EJ cells. Results showed antagonistic effect both with cisplatin or gemcitabine (Table 4.3.1).

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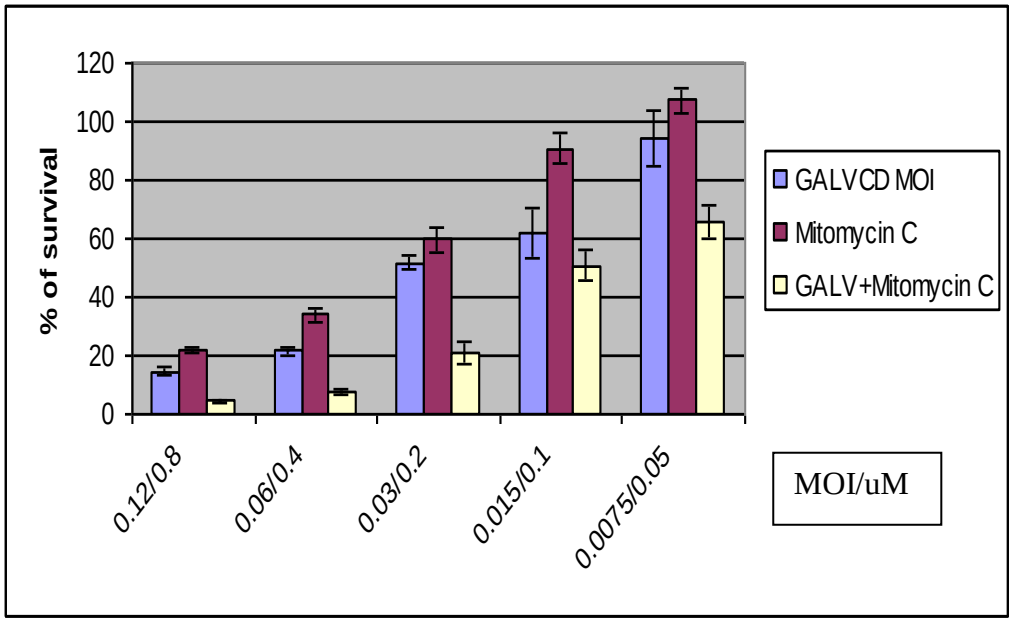

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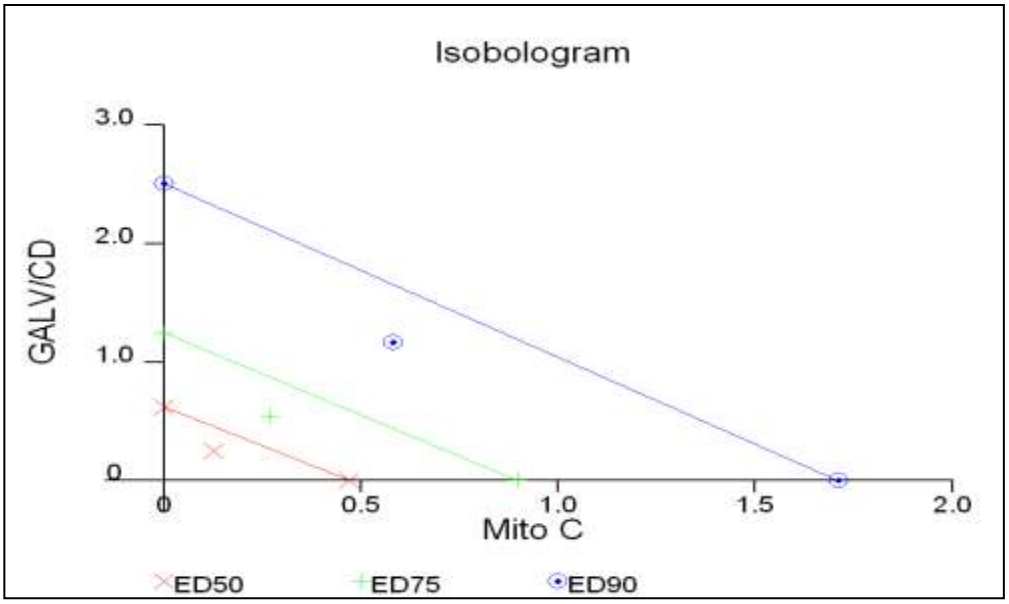

	Combination index
ED50	0.67
ED75	0.73
ED90	0.8

Figure 4.3.1 EJ cells were plated at 1 x 10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex^{GALVCD} and mitomycin in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100µL of appropriate dilution was added to each well and incubate 48hr. After 2 days the cells were measured by the MTS assay (A). The isobologram curves (B) and the combination indexes were prepared by using CalcuSyn programme (Biosoft, Ferguson, MO). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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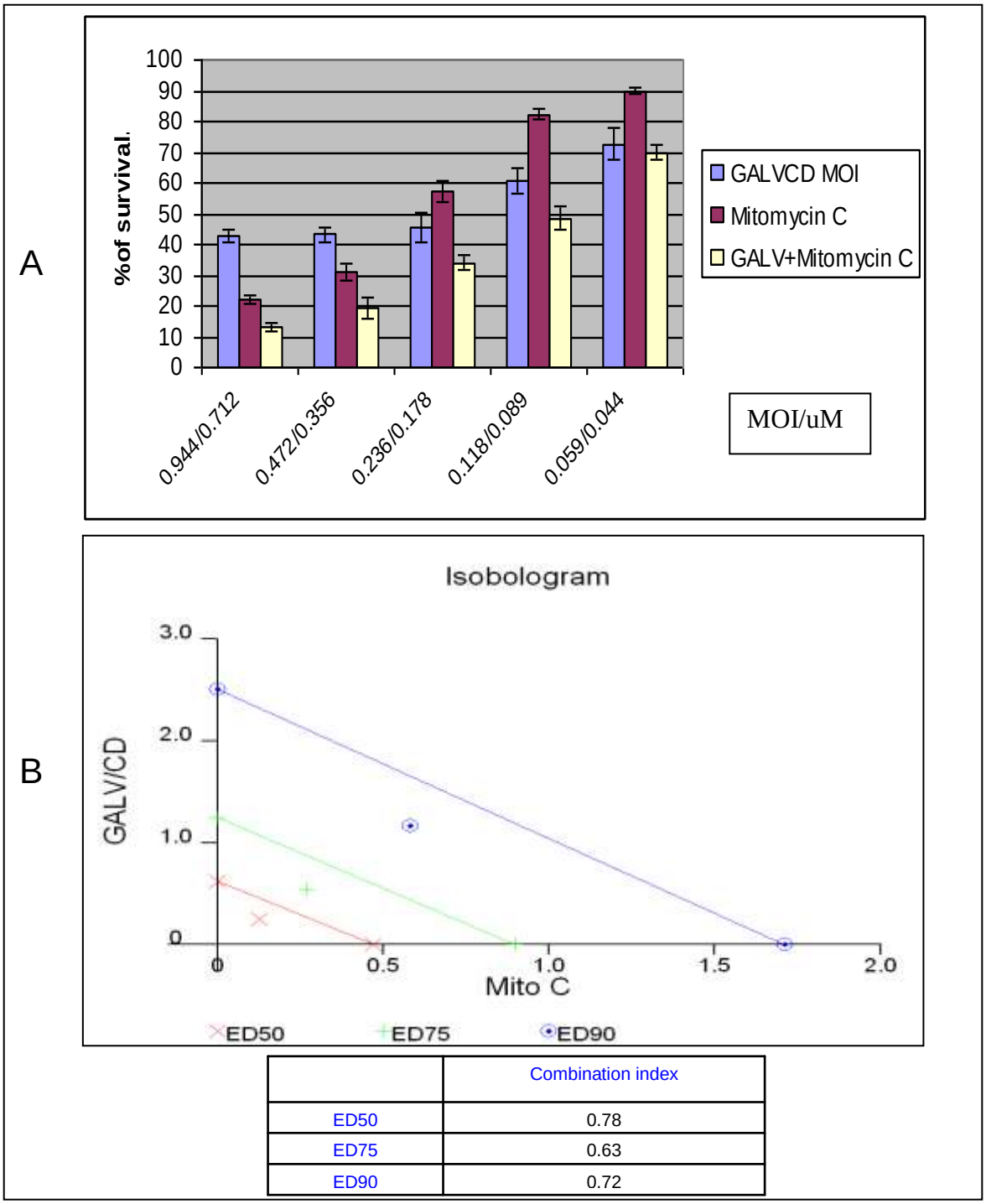

	Combination index
ED50	0.78
ED75	0.63
ED90	0.72

Figure 4.3.2 T24 cells were plated at 1 x 10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex^{GALVCD} and mitomycin in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100µL of appropriate dilution was added to each well and incubate 48hr. After 2 days the cells were measured by the MTS assay (A). The isobologram curves (B) and the combination indexes were prepared by using CalcuSyn programme (Biosoft, Ferguson, MO). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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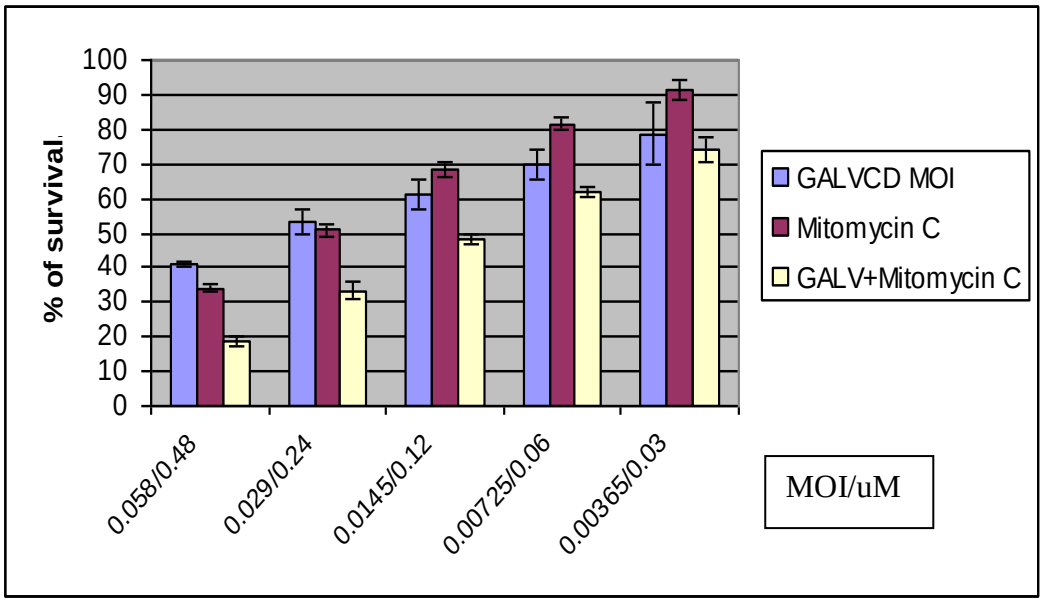

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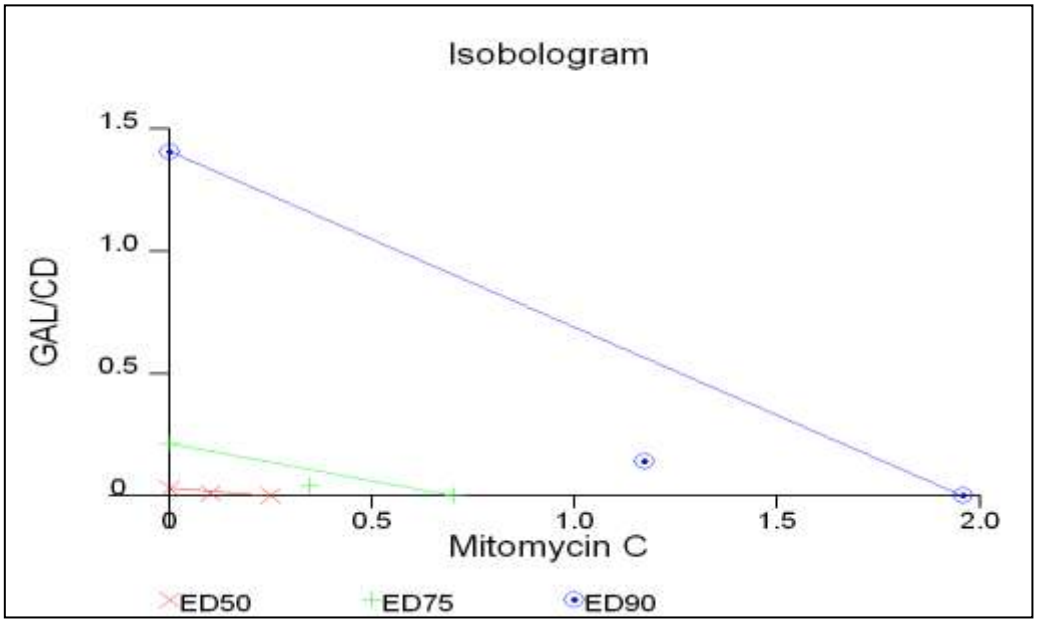

	Combination index
ED50	0.78
ED75	0.68
ED90	0.69

Figure 4.3.3 KU19-19 cells were plated at 1 x 10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex^{GALVCD} and mitomycin in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100µL of appropriate dilution was added to each well and incubate 48hr. After 2 days the cells were measured by the MTS assay (A). The isobologram curves (B) and the combination indexes were prepared by using CalcuSyn programme (Biosoft, Ferguson, MO). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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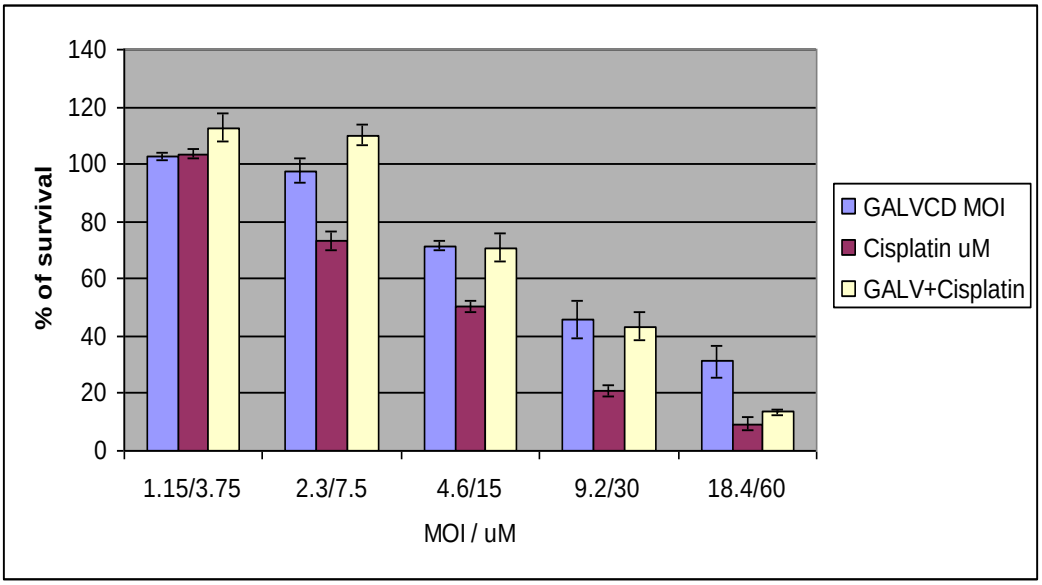

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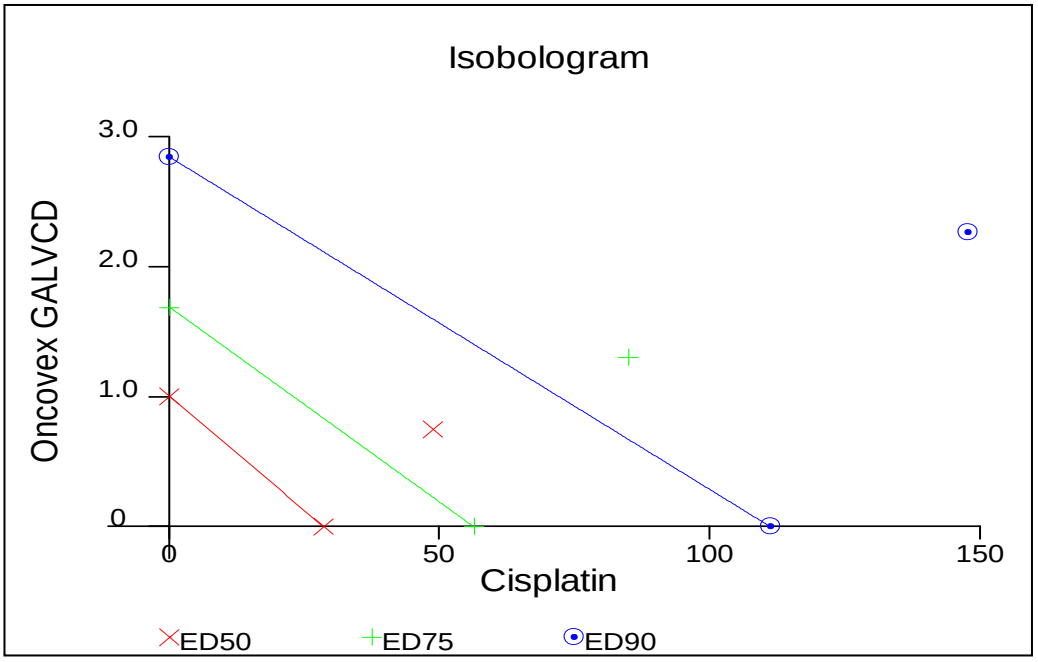

	Combination index
ED50	2.45
ED75	2.27
ED90	2.12

Figure 4.3.4 EJ cells were plated at 1 x 10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex^{GALVCD} and cisplatin in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100µL of appropriate dilution was added to each well and incubate 48hr. After 2 days the cells were measured by the MTS assay (A). The isobologram curves (B) and the combination indexes were prepared by using CalcuSyn programme (Biosoft, Ferguson, MO). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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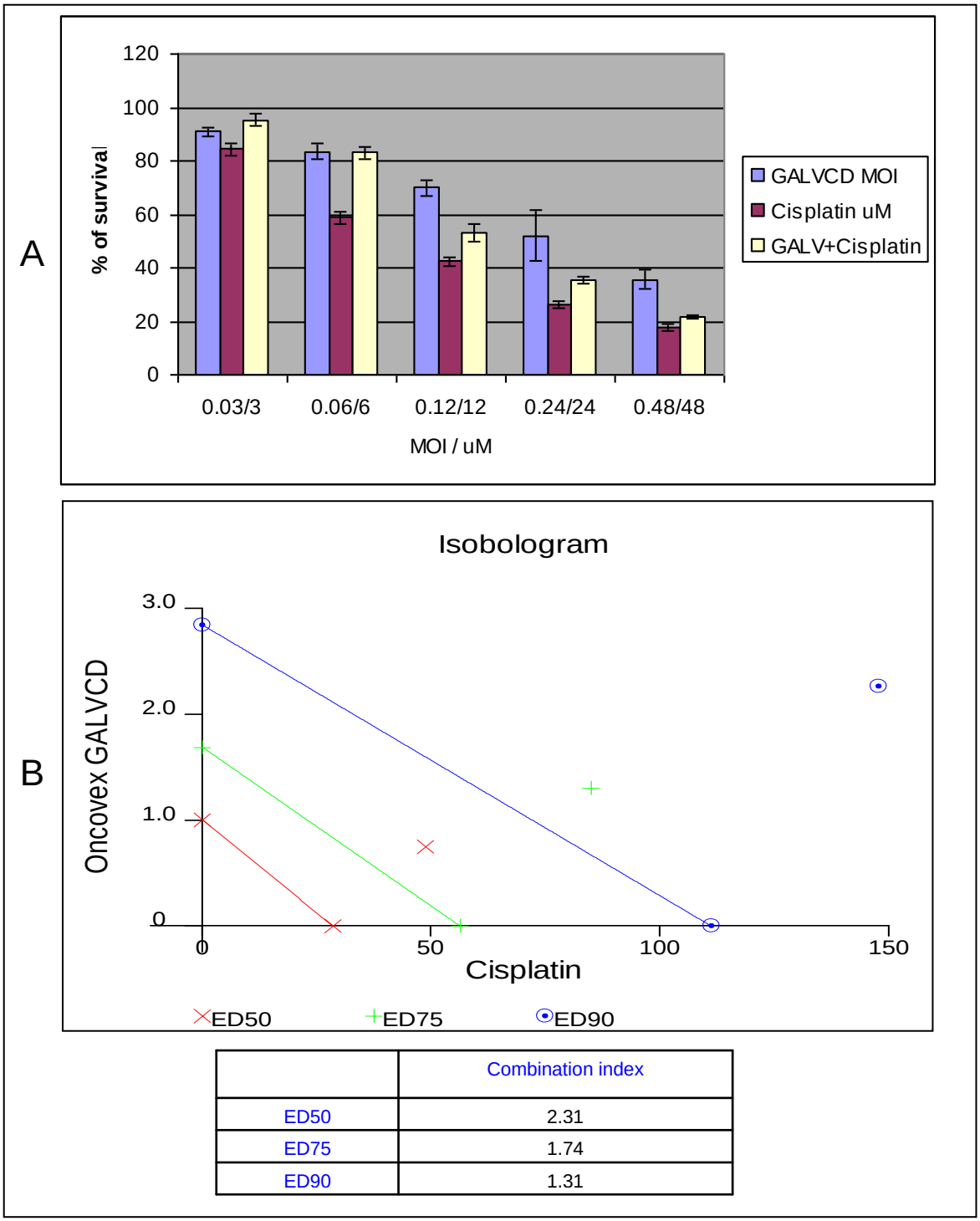

	Combination index
ED50	2.31
ED75	1.74
ED90	1.31

Figure 4.3.5 T24 cells were plated at 1 x 10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex^{GALVCD} and cisplatin in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100µL of appropriate dilution was added to each well and incubate 48hr. After 2 days the cells were measured by the MTS assay (A). The isobologram curves (B) and the combination indexes were prepared by using CalcuSyn programme (Biosoft, Ferguson, MO). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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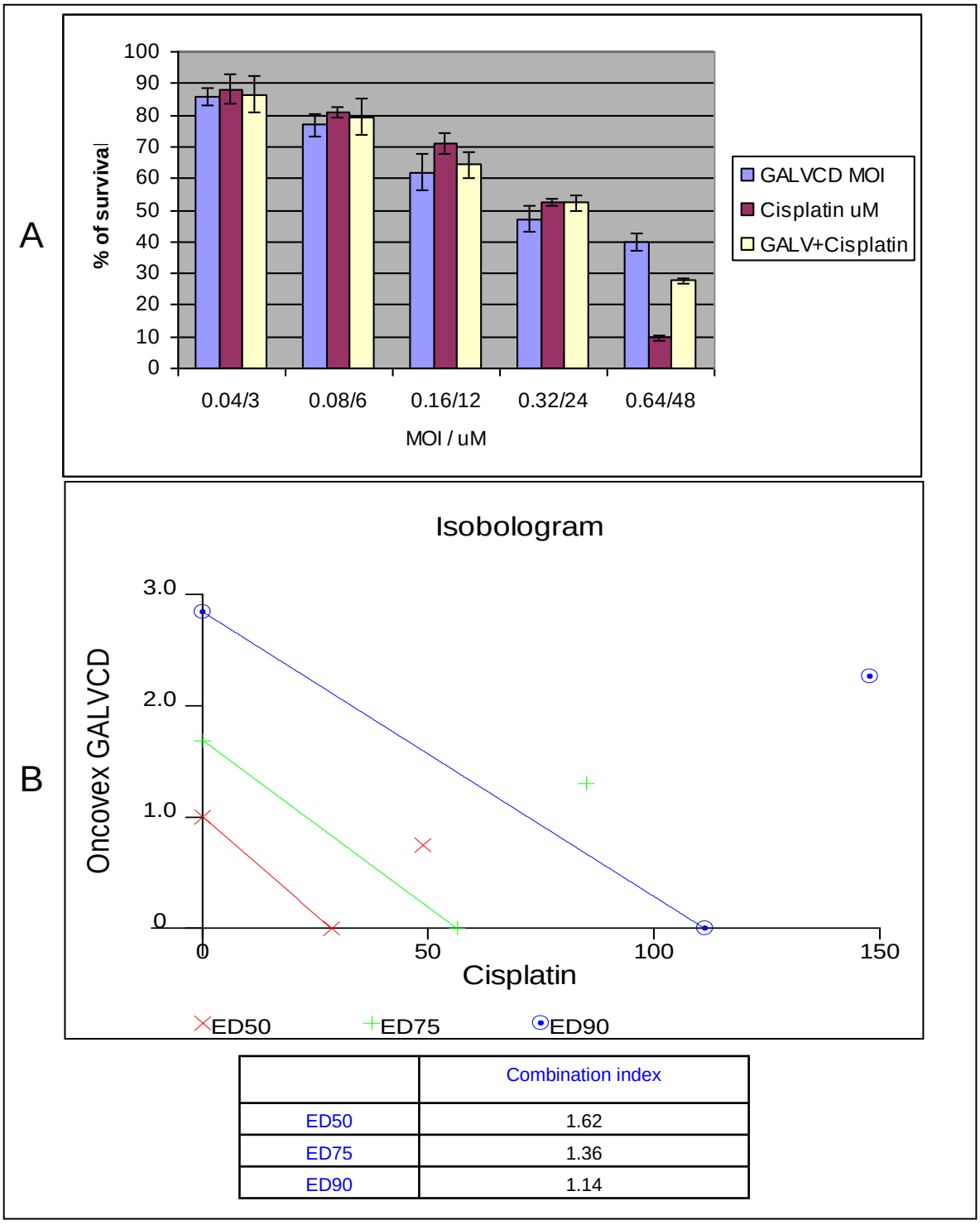

	Combination index
ED50	1.62
ED75	1.36
ED90	1.14

Figure 4.3.6 TCCSUP-G cells were plated at 1 x 10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex^{GALVCD} and cisplatin in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100µL of appropriate dilution was added to each well and incubate 48hr. After 2 days the cells were measured by the MTS assay (A). The isobologram curves (B) and the combination indexes were prepared by using CalcuSyn programme (Biosoft, Ferguson, MO). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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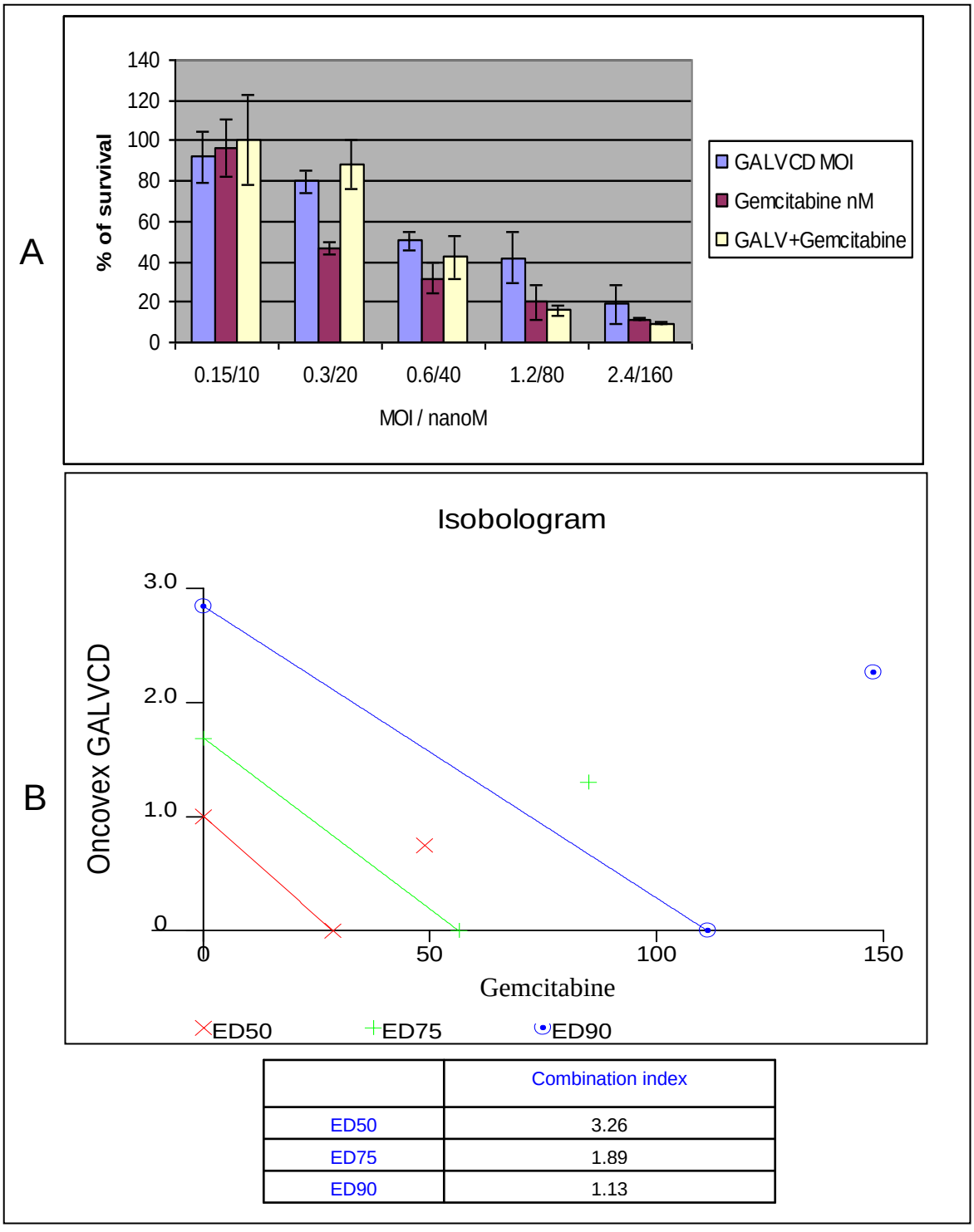

	Combination index
ED50	3.26
ED75	1.89
ED90	1.13

Figure 4.3.7 EJ cells were plated at 1 x 10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex^{GALVCD} and gemcitabine in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100µL of appropriate dilution was added to each well and incubate 48hr. After 2 days the cells were measured by the MTS assay (A). The isobologram curves (B) and the combination indexes were prepared by using CalcuSyn programme (Biosoft, Ferguson, MO). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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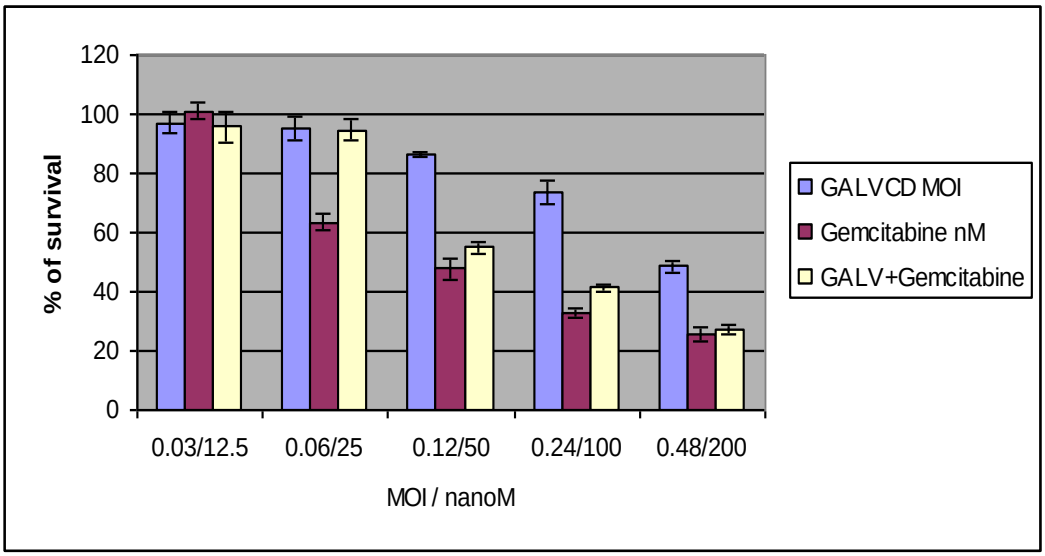

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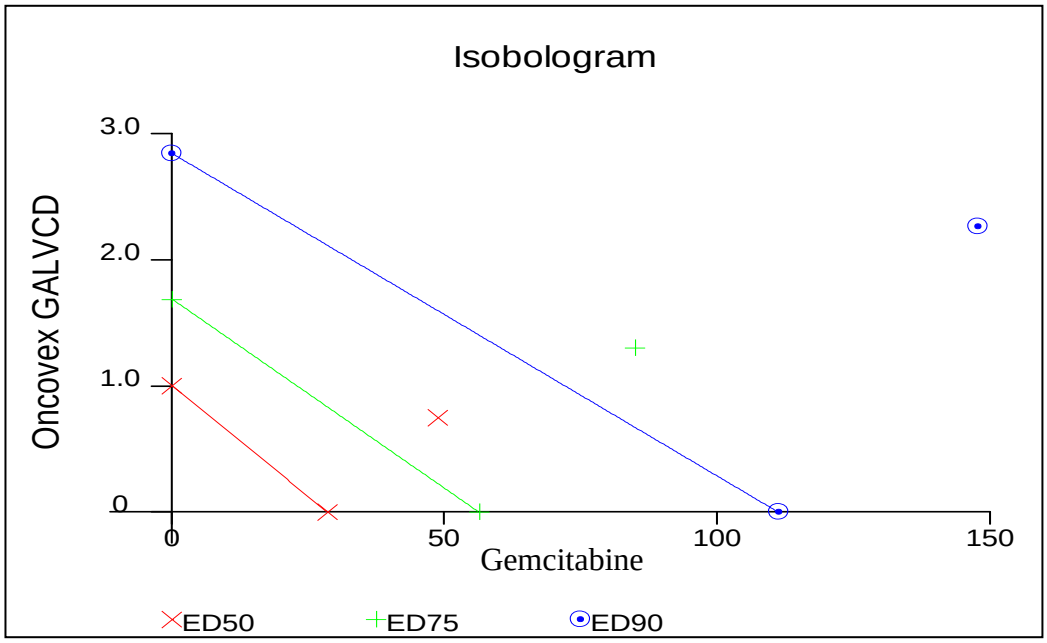

	Combination index
ED50	2.16
ED75	1.28
ED90	0.81

Figure 4.3.8 T24 cells were plated at 1 x 10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex^{GALVCD} and gemcitabine in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100µL of appropriate dilution was added to each well and incubate 48hr. After 2 days the cells were measured by the MTS assay (A). The isobolgram curves (B) and the combination indexes were prepared by using CalcuSyn programme (Biosoft, Ferguson, MO). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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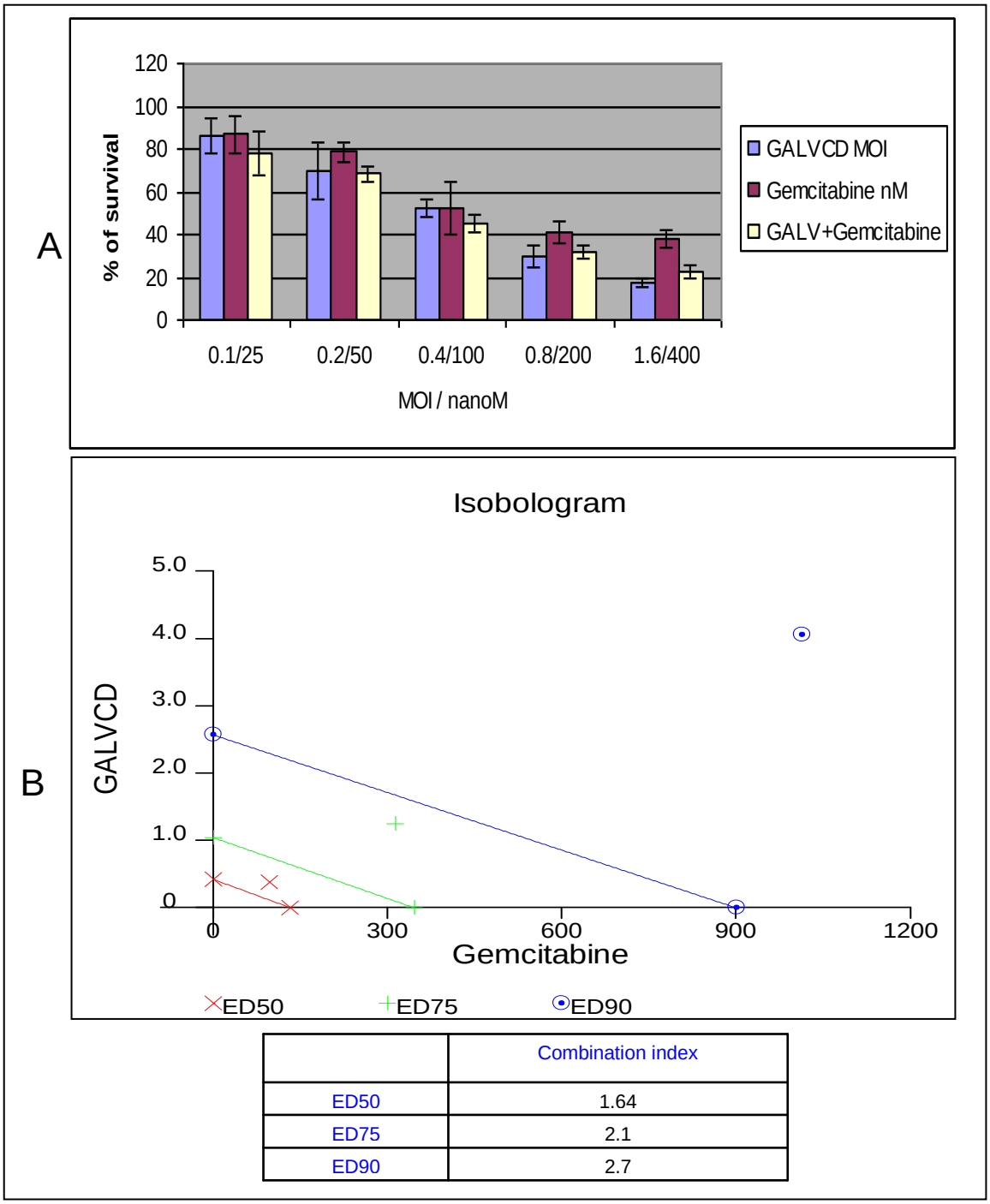

	Combination index
ED50	1.64
ED75	2.1
ED90	2.7

Figure 4.3.9 TCCSUP-G cells were plated at 1 x 10^4 per well of a 96-well tray in appropriate FGM. Doubling dilution series were prepared from Oncovex^{GALVCD} and gemcitabine in 2% FCS medium. Equipotent ED ratio of each agent was mixed for combination. 100µL of appropriate dilution was added to each well and incubate 48hr. After 2 days the cells were measured by the MTS assay (A). The isobologram curves (B) and the combination indexes were prepared by using CalcuSyn programme (Biosoft, Ferguson, MO). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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Table 4.3.1 The coadministration of Oncovex^{GALVCD} and mitomycin was synergistic whereas the coadministration of both Oncovex^{GALVCD} or Oncovex^{GFP} and cisplatin or gemcitabine was antagonistic. (NAD: no available data)

Oncovex^{GALVCD}

Origin	Cell type	Mitomycin	Cisplatin	Gemcitabine
Human	EJ	Synergistic	Antagonistic	Antagonistic
	T24	Synergistic	Antagonistic	Antagonistic
	TCCSUP-G	NAD	Antagonistic	Antagonistic
	KU19-19	Synergistic	NAD	NAD

Oncovex^{GFP}

Origin	Cell type	Mitomycin	Cisplatin	Gemcitabine
Human	EJ	NAD	Antagonistic	Antagonistic

4.4 Set up a stable rat orthotopic bladder tumour model

4.4.1 Infection screening of wild type AY-27 rat bladder tumour cells

To set up a rat orthotopic bladder tumour model first we planned to test AY-27 rat bladder tumour cells for their susceptibility for HSV entry. Oncolytic HSV viruses have been shown to infect a wide range of human and animal tumour cell types including all human bladder cancer cell lines so far tested (See more details in part 1.6.1). To assess whether rat AY-27 cells were suitable targets for HSV vectors we infected the cells with OncoVEX^{GFP} at various MOIs and incubated for 48hrs (Figure 4.4.1).

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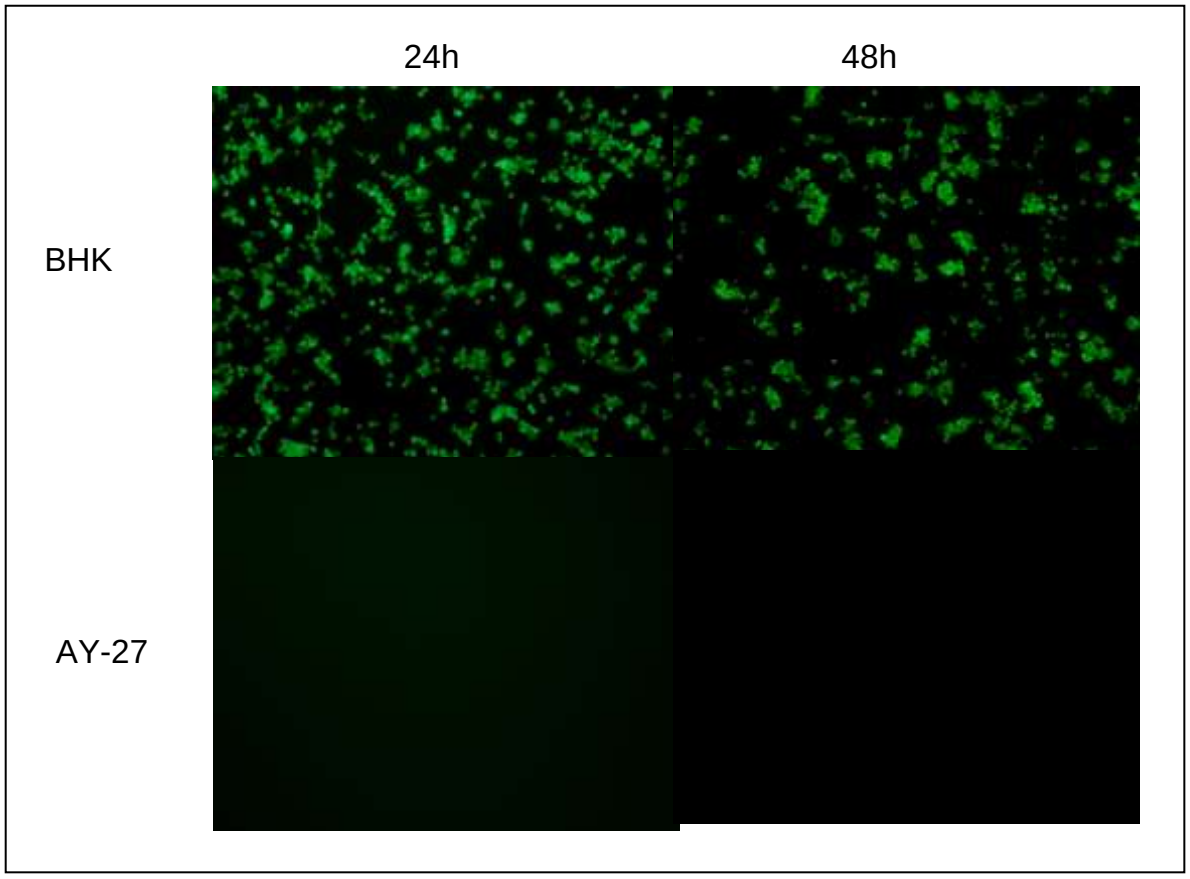

Figure 4.4.1 Infection of AY-27 and BHK control cells with OncoVex^{GFP}. AY-27 and BHK cells were plated at 1x10^{5}cells per well of a 24 well tray and incubated at 37^{o}C/5% CO_2 o/n. The media was then removed and the cells were infected with OncoVex^{GFP} virus at MOI 10 and incubated at 37^{o}C/5% CO_2 for 24/48Hr. Infection of OncoVex^{GFP} virus expressing green fluorescent protein marker gene were visualised under an inverted fluorescent microscope (*Nikon* Eclipse TE200) at wavelength 520nm. Under UV microscope control BHK cells can be seen in green colour after infection of OncoVex^{GFP}, whereas AY-27 cells cannot be seen (black field) as they fail to support OncoVex^{GFP} infection.

From the results we can conclude that AY-27 cells are a rare exception in failing to support HSV entry and/or replication. To overcome this problem AY-27 cells had to be stably transfected with the herpesvirus entry receptor. Although HSV does not appear to infect the particular rat cell line we used (AY-27), this is not a potential problem for human cells, because all human bladder cells we have tested are able to be infected by HSV. Therefore this problem should not affect the effectiveness of OncoVEX$^{GALV/CD}$ in the treatment of bladder cancer in human.

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4.4.2 Transfection of AY-27 cells with the herpesvirus entry receptor (HVEM) and infection screening

Wild type AY-27 cells were transfected using a linearised plasmid of pcDNA3 CMV HVEM with lipofectin (Introvitrogen). The cells were selected under G418 media for 5-20 days. 72 individual clones were isolated and grow up under G418 selection. The 72 clones were tested for infection by HSV where the cells were infected with OncoVEX^{GFP} at MOI 10 and incubated for 48hrs. From these results the 3 most suitable new cell clones (E6, E5, B9) were chosen which support HSV entry on the largest scale (Figure 4.4.2).

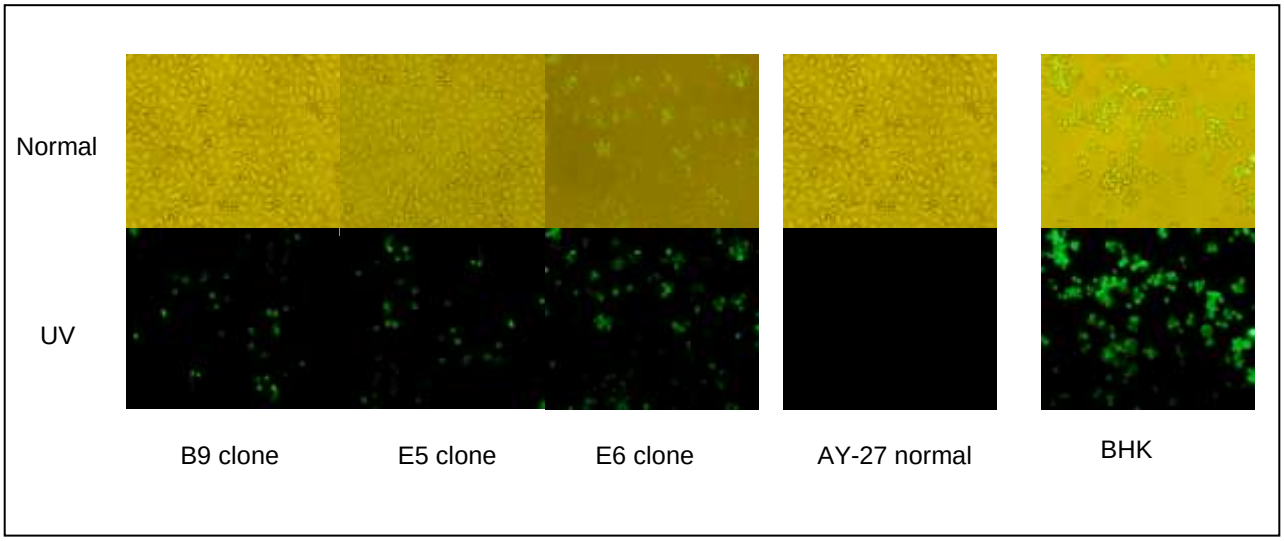

Figure 4.4.2 Infection of wt AY-27, AY27 HVEM clones and BHK control cells with OncoVex^{GFP} AY-27 HVEM clones and control cells were plated at 1x10^5 cells per well of a 24 well tray and incubated at 37^{o}C/5% CO_2 o/n. The cells infected with OncoVex^{GFP} virus at MOI 10 and incubated at 37^{o}C/5% CO_2 for 48Hr.. Infection of OncoVex^{GFP} virus expressing green fluorescent protein marker gene were visualised under an inverted fluorescent microscope (*Nikon* Eclipse TE200) at wavelength 520nm. After transfection of HSV entry receptor AY-27 HVEM clones have become visible under UV microscope in green colour, whereas AY-27 cells cannot be seen (black field) as they fail to support OncoVex^{GFP} infection. Under normal microscope there is no difference between AY-27 and AY-27 HVEM cells.

We have chosen AY-27 HVEM E6 clones for our further experiments as this cell clone showed the strongest infectibility by HSV. AY-27 HVEM E6 clone was scaled up and frozen stocks were produced.

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4.4.3 Testing the efficacy of Oncovex$^{GALV/CD}$ on AY-27 HVEM cell clone with Fusion and Prodrug assay

To test for improved tumour cell killing effects of GALV expression *in vitro*, AY-27 HVEM cells were infected with OncoVex$^{GALV/CD}$ or OncoVex^{GFP} under previously described conditions. These fusion assay results were confirmed by a reduction in metabolic enzyme activity of up to 30% (MTS assay) in AY-27 HVEM cells infected with OncoVex$^{GALV/CD}$ compared to the OncoVex^{GFP} control (Figure 4.4.3).

AY-27 HVEM cells were further tested in our prodrug assay, which showed that OncoVex$^{GALV/CD}$ can metabolize 5-FC within these cells, resulting in a decrease in metabolic enzyme activity of up to 81% (MTS assay) when compared to controls (Figure 4.4.4).

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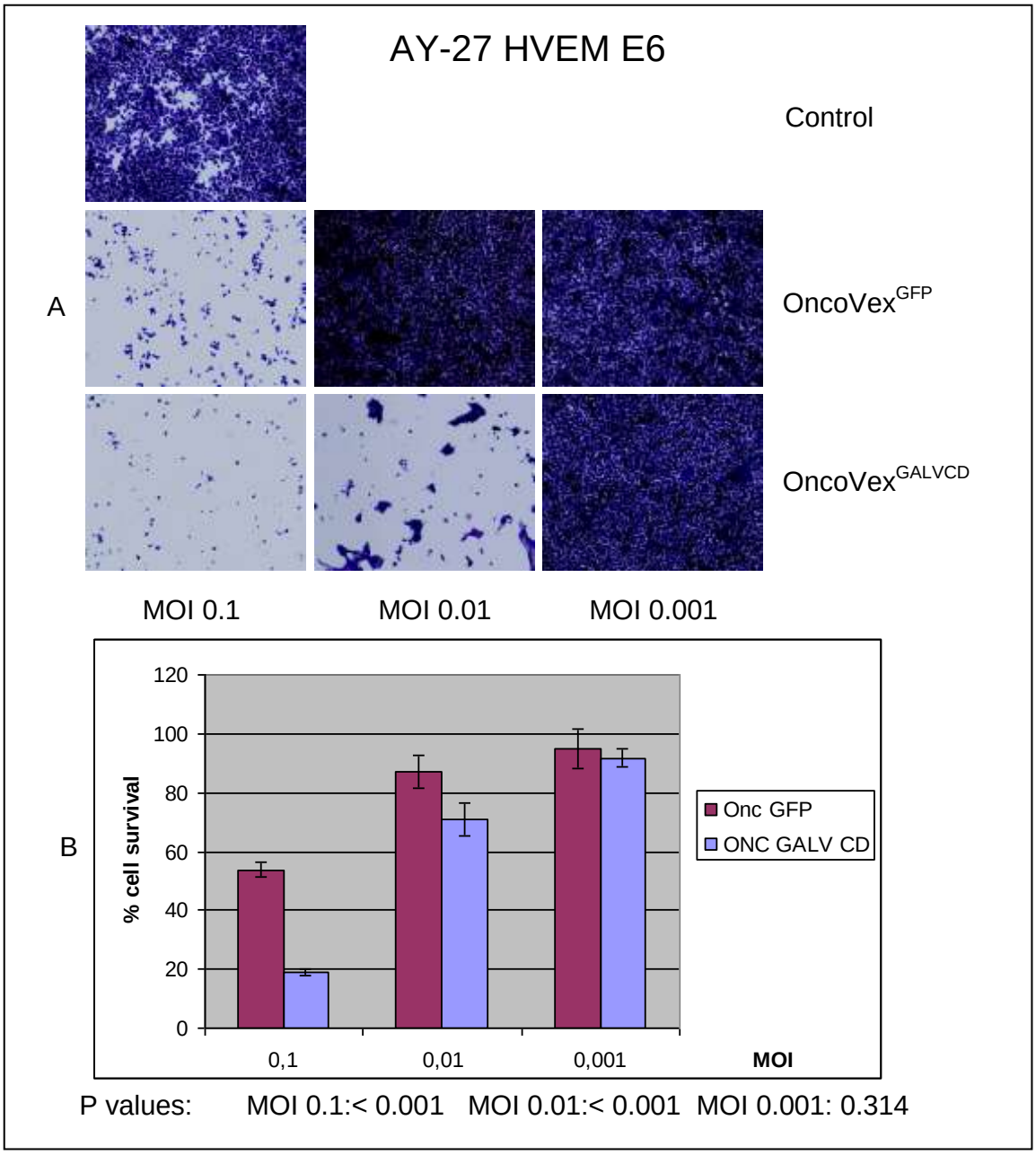

Figure 4.4.3. $2x10^4$ AY-27 HVEM E6 cells were infected with OncoVex^{GFP} or OncoVex$^{GALV/CD}$ at MOIs of 0.1, 0.01, 0.001 and incubated at 37°C/5% CO_2 for 48 hours and then assayed by Crystal Violet staining (magnification 100x)(A) or MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells (B). Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations. **A:** In the mid row cells infected with OncoVex^{GFP} show a classic HSV-1 cytopathic effect, where tumour cell killing is increasing (from right to left) by the increasing levels (MOI) of virus due to its oncolytic activity. In the lower row cells infected with OncoVEX$^{GALV/CD}$ show fusion (large multinucleated syncytia) surrounded with cells showing the classic HSV-1 cytopathic effect and higher levels of cell killing compared to the mid row.

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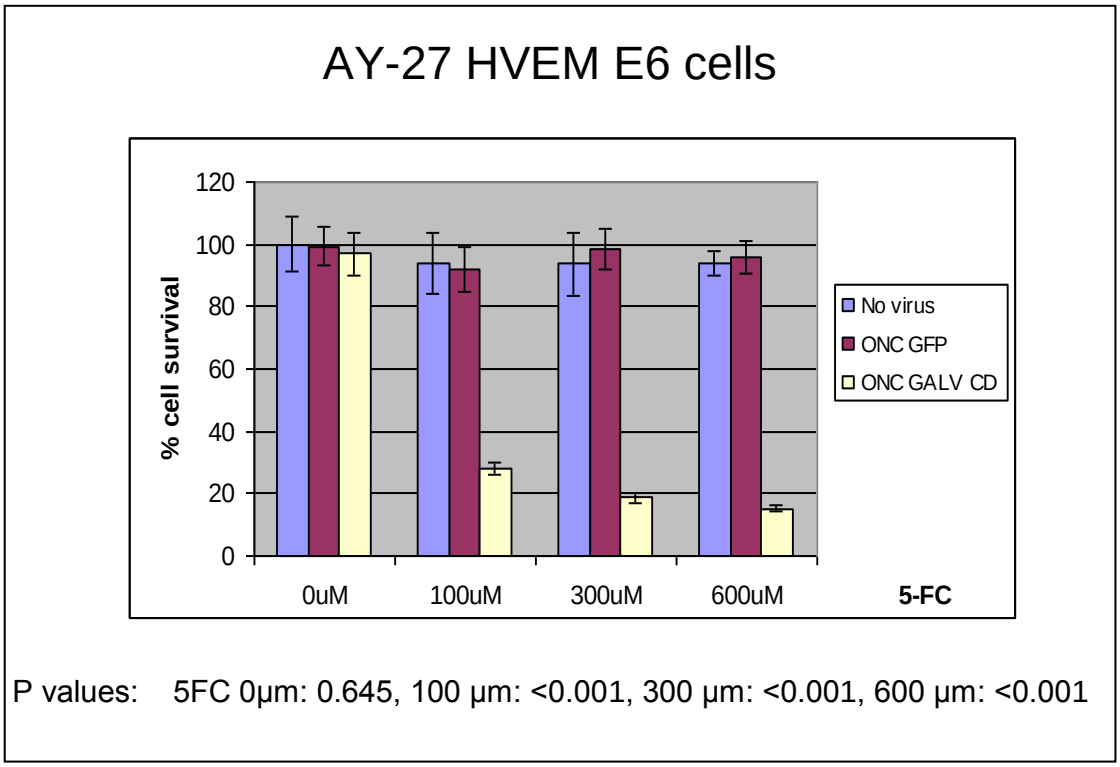

Figure 4.4.4 1 x 10^5 AY-27 HVEM E6 cells were infected with OncoVex^{GFP} and OncoVex$^{GALV/CD}$ at MOI of 0.1 according to the fusion assay results and no virus control. After 30 minutes at 37°C/5% CO_2, the virus was removed, and 1 mL of FGM containing 5-FC ($C_4H_4FN_2O$; Sigma) at different concentrations (0-100-300-600 µmol/L) was added and incubated for 48 hours at 37°C/5% CO_2. The cell supernatants were then heat inactivated and added to 1 x 10^4 fresh EJ cells and incubated at 37°C/5% CO_2 for 72 hours and then assayed by MTS assay (Promega). Average cell survival was calculated as a percentage compared to untreated cells. All results showed enchanced tumour killing in the presence of OncoVEX$^{GALV/CD}$ and 5-FC. Quantification of the prodrug studies showing that in all cases the differences were significant (P <0.05). (P values: GALV/CD+0µm Vs GFP+0µm =0.645, GALV/CD+100µm Vs GALV/CD+0µm =<0.000, GALV/CD+300µm Vs GALV/CD+0µm =<0.000, GALV/CD+600µm Vs GALV/CD+0µm =<0.000) Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

4.4.4 AY-27 and AY-27 HVEM flank tumour model

In order to assess whether AY-27 HVEM cells can grow in rats we set up a flank tumour model with the rat bladder tumour cells. It was shown that both AY-27 and AY-27 HVEM E6 rat bladder tumour cells were able to grow in rats. The rate of tumour

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growth on a flank tumour model showed slightly slower tumour development when using the new AY-27 HVEM E6 cell line compared to the original AY-27 cell line (Figure 4.4.5). Therefore slower rate of tumour growth is expected in our orthotopic bladder model as well compared to the data from the literature. The difference in xenograft growth curves might be related to differing passage numbers between the parental cell line and the derived luciferase-expressing clone (AY-27 HVEM E6).

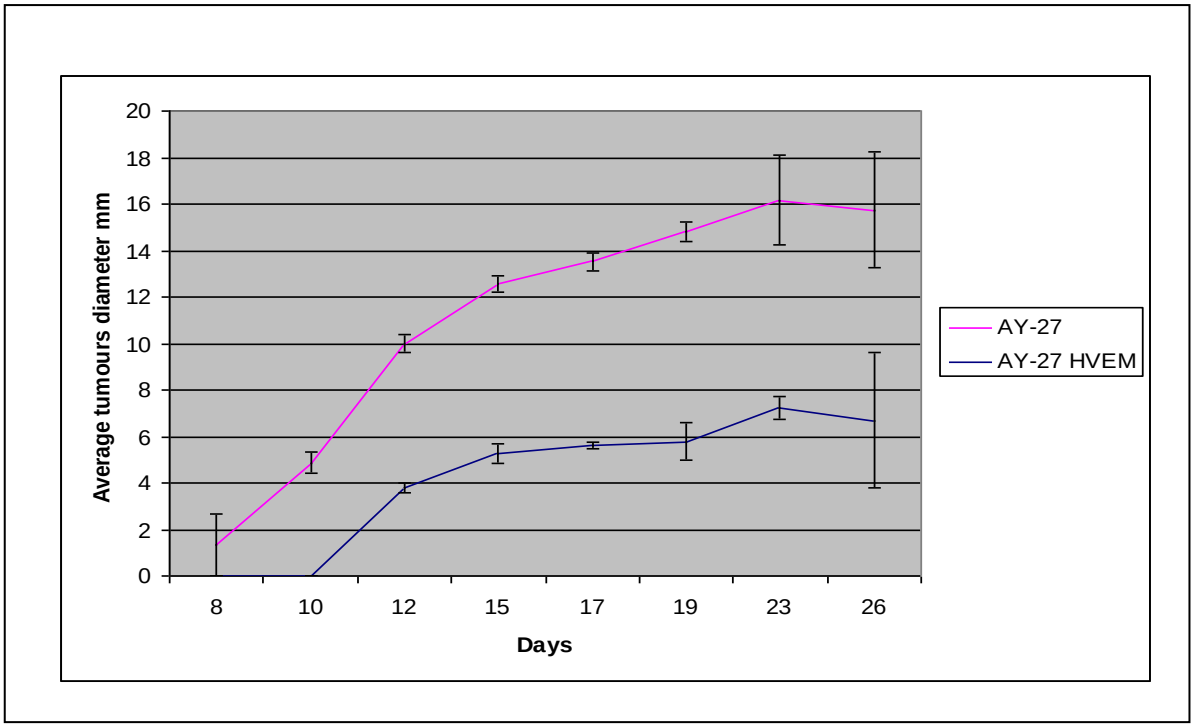

Figure 4.4.5 *In vivo* growth AY-27 HVEM cells. Eight rats randomly assigned into two groups. $1x10^6$ AY-27 HVEM or WT AY-27 cells in 100ul RPMI (without FCS) were injected subcutaneously into the flank of the rats (both side). The tumours were measured every 2-3 days. Experiments were repeated three times with two animals in each group. The figure shows a representative experiment, where the error bars are standard deviations.

4.4.5 Rat orthotopic bladder tumour model

The anaesthesia of the female Fischer F344 rats was carried out with two different protocols. In our early experiments rats were anesthetised with intraperitoneal injections of Domitor (Pfizer Animal Health) and Ketaset (Pfizer Animal Health). The doses of Domitor and Ketaset used is shown earlier in chapter 2 (3.5.1). After the procedure the same amount of antidote as Domitor (Pfizer Animal Health) was given to each animal to terminate the anaesthesia. In our later experiments we used balanced inhalation of

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Isoflurane (Novartis **Animal** Health **UK** Ltd) for anaesthesia. Comparing the two methods we found that the balanced inhalation of Isoflurane anaesthesia seemed much safer, reproducible and showed less side effects. Using this inhalation anaesthesia also showed the recovery period was much quicker and this method caused less harm to the rats. The percentage of anaesthetic related deaths was about 19.2% when using intraperitioneal injections of Domitor and Ketaset and this was elevated up to 30% when we repeated the anaesthesia weekly 3 times during the treating phase of our studies. The percentage of anaesthetic related deaths were about 6.6% when using balanced Isoflurane inhalation anaesthesia.

The tumour cell implantation was performed via a transurethral catheter. To facilitate the tumour seeding the bladder mucosa was damaged by instillation of hydrochloric acid followed by a rinse with sodium hydroxide for neutralization. A suspension of freshly harvested AY-27 cells was then instilled and maintained in the bladder for 1 hour (Figure 4.4.6).

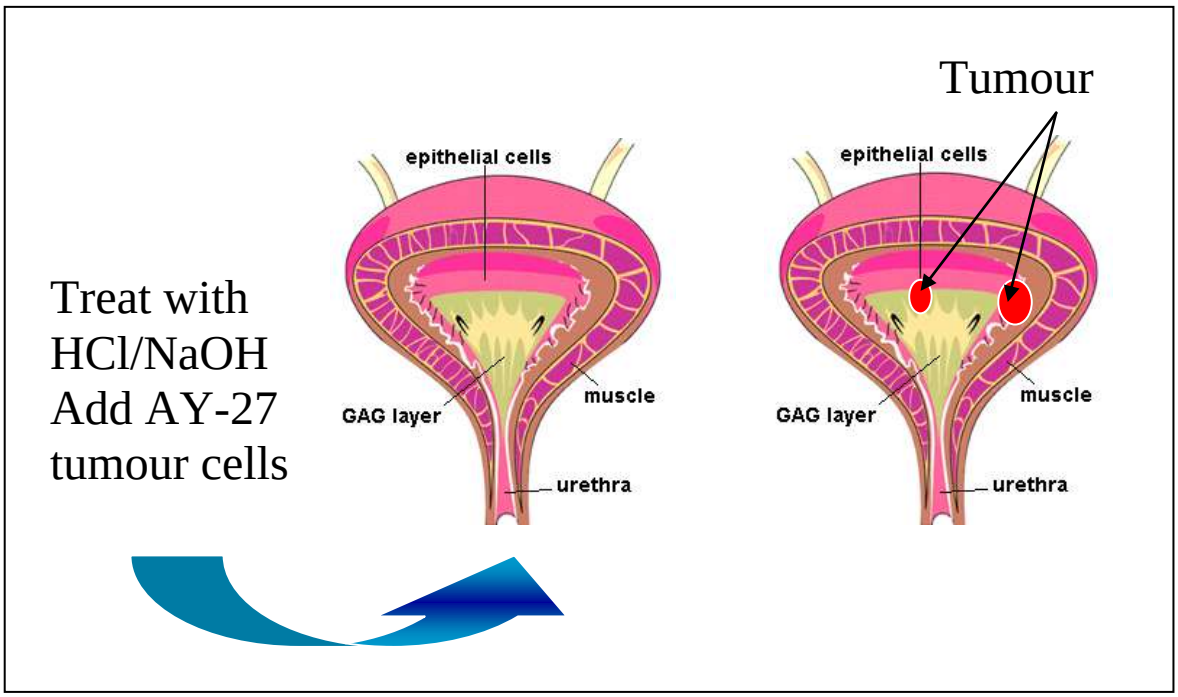

Figure 4.4.6 Fischer F344 rats were anesthetised and placed in a supine position (on a homeothermic blanket). An 18-gauge plastic (Braun) intravenous cannula was used as a catheter. The catheter was inserted into the bladder via the urethra. To facilitate the tumour seeding the bladder mucosa was damaged by instillation of 0.4 ml 0.1 N hydrochloric acid for 15 seconds followed by a 15 seconds rinse with 0.4ml of 0.1 N sodium hydroxide for neutralization. The bladder was then drained and washed five

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times with 0.6ml PBS (pH 7.4). A suspension of freshly harvested AY-27 cells (1.5-2.5x10^6 cells in 0.4ml of medium) was then instilled and maintained in the bladder for 1 hour on homeothermic blanket. The rats were turned 90° laterally every 15 minutes to ensure exposure of the entire bladder wall to the tumour cells. After 1 hour the catheters were removed, and the rats were allowed to void spontaneously.

After the tumour implantation the rats were monitored daily for general health status and for signs and symptoms of advanced bladder cancer. The animals were sacrificed in different time periods (10,14,18 days). The extent of tumour burden at necropsy was evaluated by macroscopic examination of the bladder. Our necropsy results showed 1-2 mm average tumour diameter at day 10 and 14 and 2-5mm average tumour diameter at day 18. All rats developed tumour in their bladder, resulting in a 100% success rate of tumour imlpantation. After 35-50 days all rats showed 1.5-2 cm average diameter tumour masses in their bladder (Figure 4.4.7).

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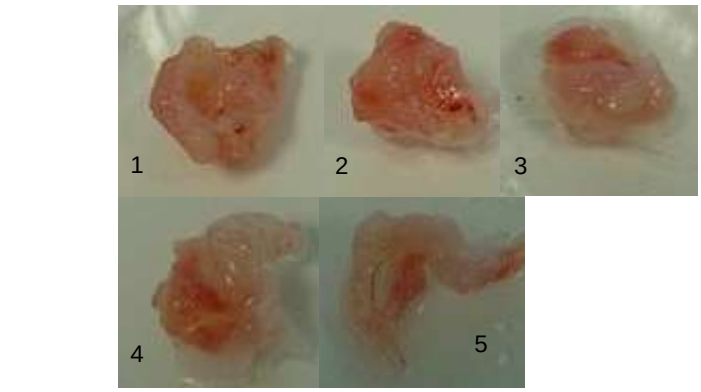
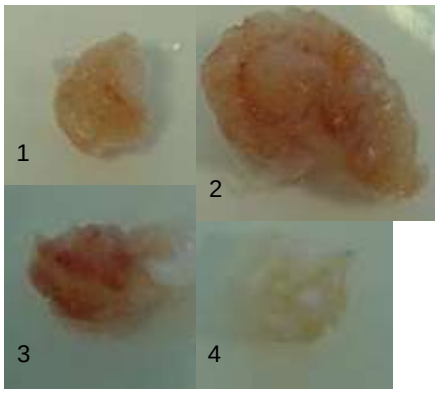
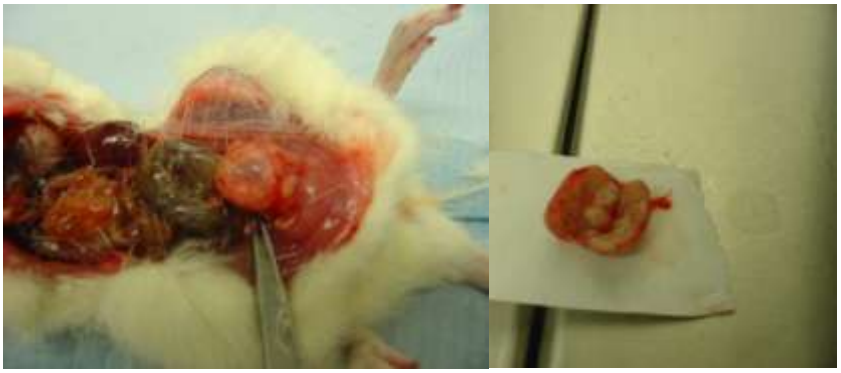


After 14 days



After 18 days
(Rat 4 did not get tumour cells)



After 34 days

Figure 4.4.7 After the tumour implantation (6.3.5) the animals were sacrificed in different time periods, at 10 (n:4),14 (n:5),18(n:4) and 34 (n:8) days of implantation. The bladders were removed and the size of the tumours were measured. (n: number of rats)

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4.4.6 Detection of HVEM RNA by QRT-PCR as a marker for tumour cells

In rat tissue and urine samples we aimed to demonstrate the presence of the HVEM receptor on AY-27 HVEM rat bladder tumour cells by QRT-PCR as a marker of tumour load. We collected urine samples by holding the rats in a metabolization cage for 1 hour on day 0, 4, 7, 11 and 14 after tumour implantation. Quantitative reverse transcription polymerase chain reaction was carried using primers to HVEM (and control primers to rat β-actin) and cDNA were produced by reverse transcription (Invitrogen) (Figure 4.4.8). QRT-PCR measures how much target cDNA was present compared to control rat β-actin (x1000). After necropsy QRT-PCR was performed on the removed bladder tissue and showed significant signals in the tumour bearing animals compared to the control animals (Figure 4.4.9).

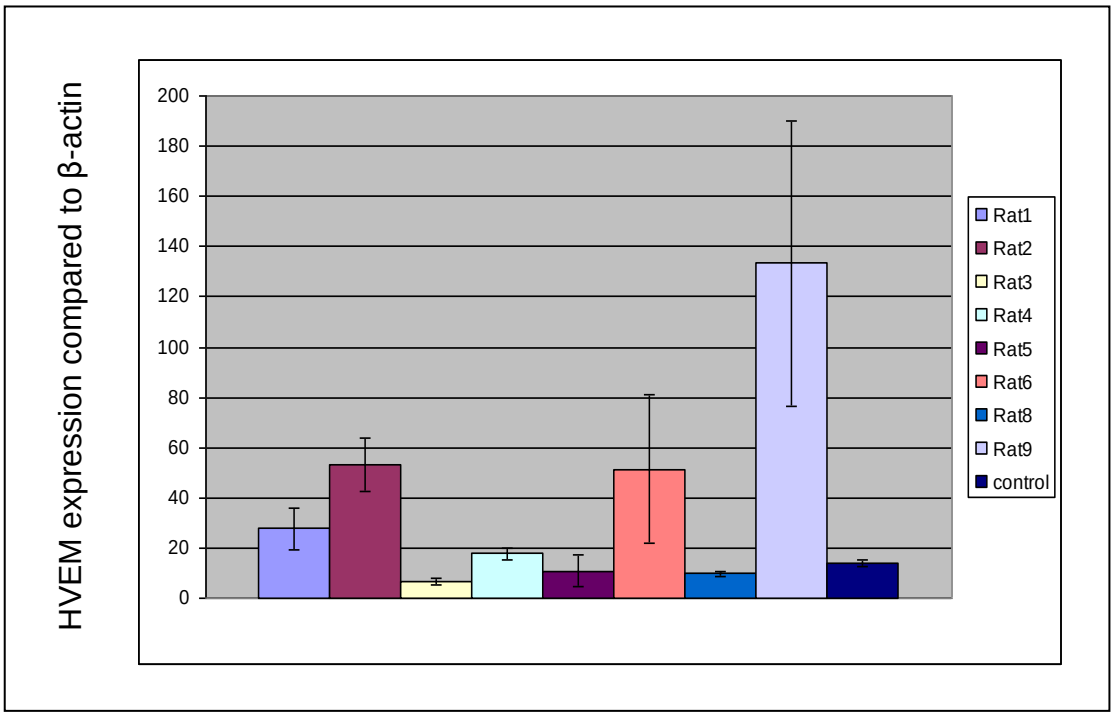

Figure 4.4.8 QRT-PCR for HVEM on rat urine. 14 days after tumour implantation quantitative reverse transcription polymerase chain reaction was performed on the collected urine samples. The method of QRT-PCR was discussed in more detail earlier in chapter 2.3. The figure shows a representative experiment, where the error bars are standard deviations.

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In collected urine samples no significant difference in the tumour bearing animals was observed compared to the controls, therefore tumour growth was not detectable by QRT-PCR on the collected urine samples (Figure 4.4.8). In contrast after necropsy QRT-PCR was performed on the removed bladder tissue and showed significant signals in the tumour bearing animals compared to the control animals (Figure 4.4.9). Our results show that we can detect HVEM in rat bladder tumour tissue but not in urine from the same animals. Therefore we can conclude that measuring HVEM in urine by QRT-PCR is not an effective method for quantitating tumour load in this model.

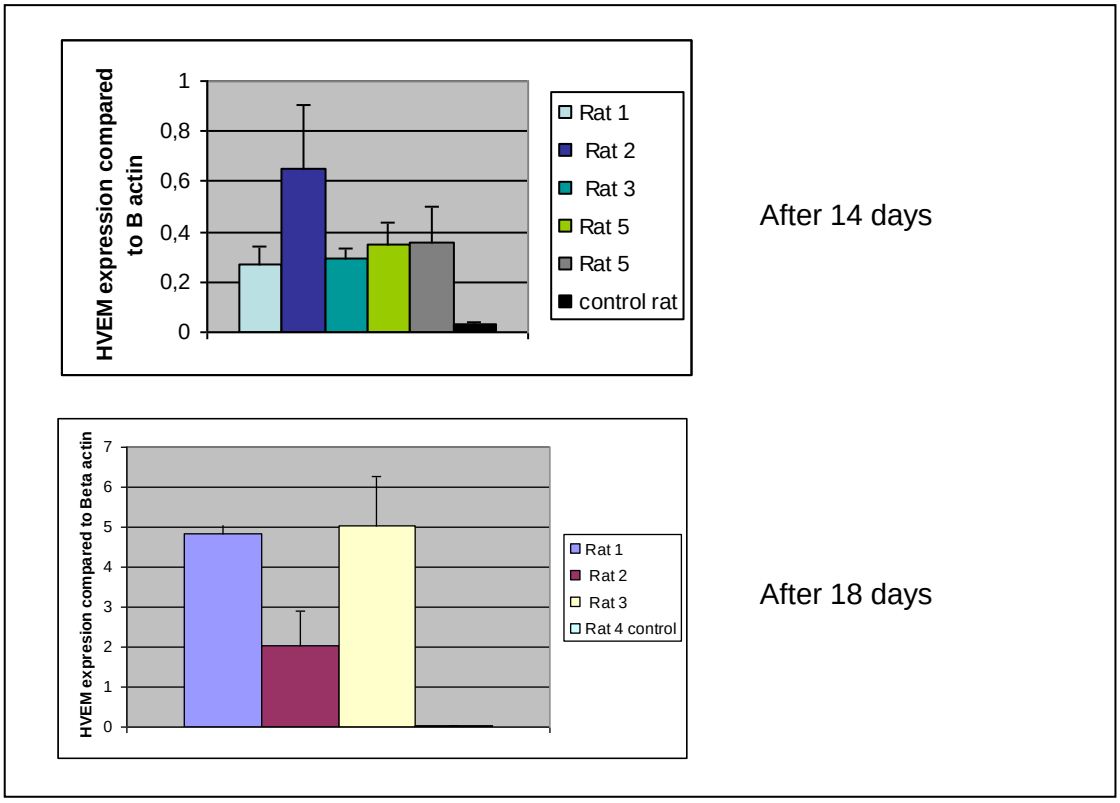

Figure 4.4.9 QRT-PCR for HVEM on rat tumour bladder tissue. 14-18 days after tumour implantation the rats were sacrificed and QRT-PCR was performed on the removed bladder tissues. The method of QRT-PCR was discussed in more detail earlier in chapter 2.3. The figure shows a representative experiment, where the error bars are standard deviations.

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4.4.7 Transfection of AY-27 HVEM cells with a luciferase encoding plasmid for bioluminescence imaging

AY-27 HVEM cells were transfected using linearised plasmid of pGAL CMV Luc Hygro (Promega) with lipofectin (Invitrogen). The cells were then selected under G418 + Hygromycin. We could observe approximately 35 individual cell colonies which were isolated and grown up under G418/Hygromycin selection. The resulting 35 clones were tested for luciferase activation (Sigma). From these results the most suitable new cell clone (HL-S) was chosen that showed the highest luciferase activity (Figure 4.4.10). Therefore, AY-27 HL S clone was chosen for further investigation *in vivo* bioluminescence imaging experiments.

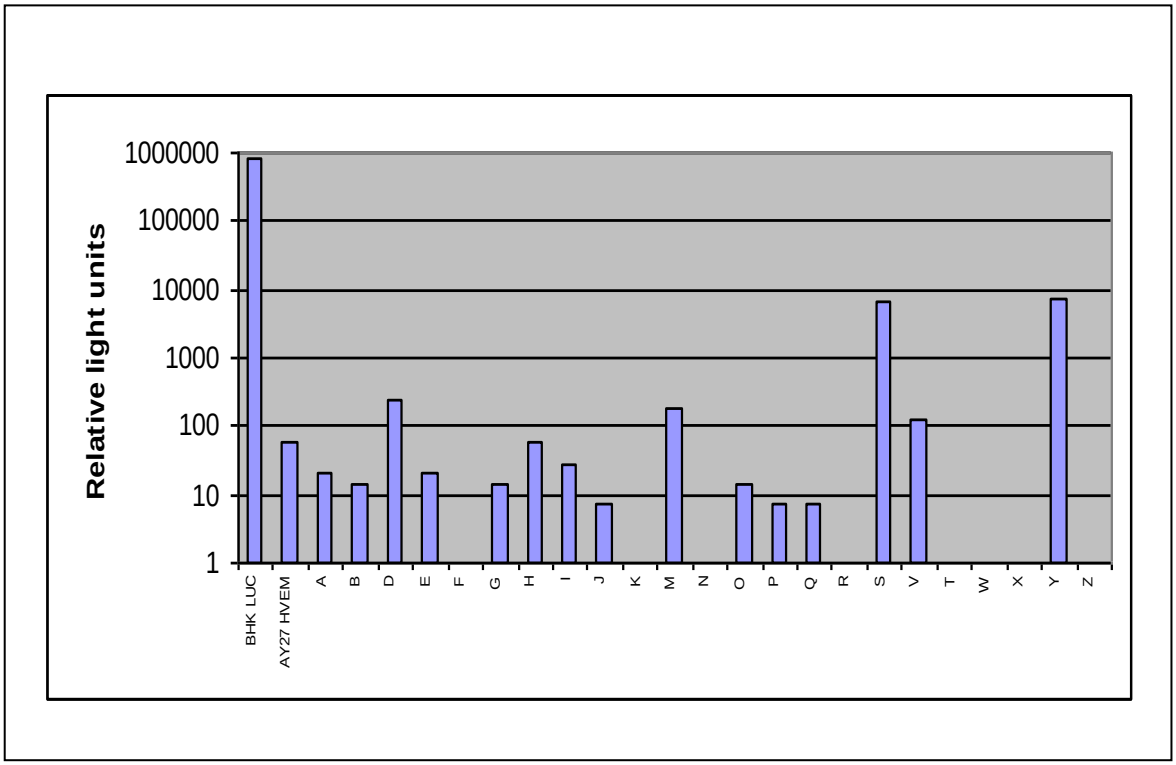

Figure 4.4.10 Luciferase assay (Sigma). AY-27 HVEM+Luciferase cells were plated at $5x10^5$ cells and incubated at 37^0C o/n. The cells were washed (3x) with ice cold PBS. 1x cell culture lysis reagent was added and the lysate was incubated at RT for 15 minutes after which the cells were scraped off and spun at 12,000g at 4^0C for 1 minute. The supernatant was removed and stored on ice. The luciferase substrate and the tested cell lysate were allowed to equilibrate to room temperature before use. 20µl of the cell lysate was added to 100µl of the luciferase substrate assay buffer. Light emission was read after 10 seconds incubation at room temperature using Beckman counter DTX 880**.**

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4.4.8 Bioluminescence imaging rat flank tumour model

(This work was carried out in Dr Kevin Harrington Laboratory at Institute of Cancer Research London)

In order to assess whether AY-27 HVEM+Luciferase cells were able to grow in rats we set up a flank tumour model. It was shown that until day 7 after tumour implantation tumours grew in the flank of the rats and they were detectable by the non-invasive IVIS bioluminescence imaging camera. After 11 days the tumours disappeared from the flank and activity was not detectable by IVIS camera (Figure 4.4.11).

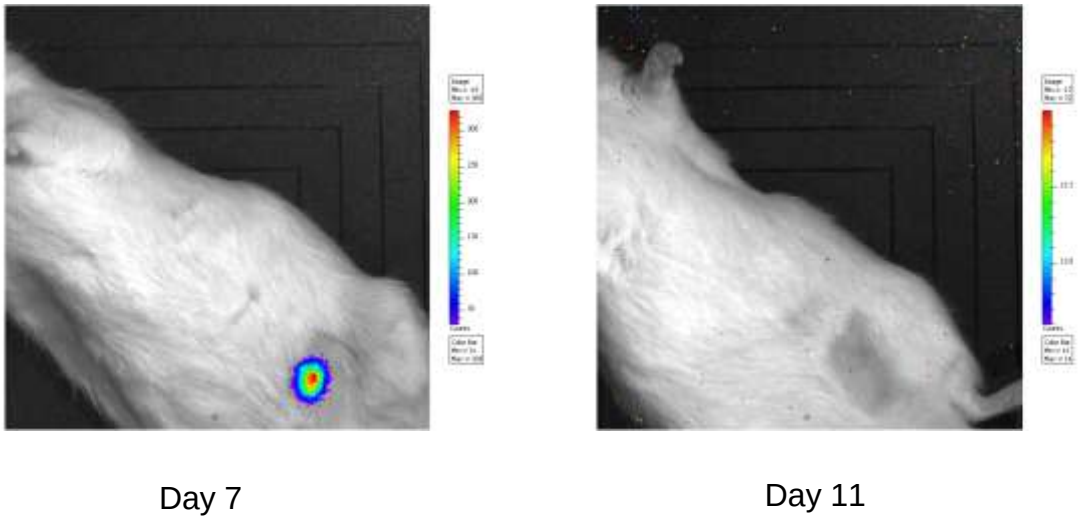

Figure 4.4.11 Fischer F344 rats were anaesthetised and 1x10^6 AY-27 HL-S cells in 100ul RPMI (without FCS) were injected subcutaneously. Luc expression was quantitated by real-time bioluminescent imaging using the IVIS System 200 series (Xenogen Biosciences) D-Luciferin Firefly Potassium salt (15 mg/mL; Xenogen Biosciences) was administered at 150 mg/kg and imaging was performed after 5 min. 30s exposures were obtained at high sensitivity using the machine default settings (f/stop, 1; emission filter, open; field of view d, 12 cm; and subject height, 1.5 cm). The Xenogen software provides visual images of bioluminescence detection using a coloured overlay on a photographic image taken immediately before luminescence measurements. The areas shown in colour represent the regions of bioluminescence detected. Results were analyzed using Living Image 2.60.1 software (Xenogen Biosciences). Areas of Luc expression were defined using the autocontouring tool and the photon flux and average radiance (photons/cm^2/s) were estimated over the defined area.

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These results suggest that the tumours may be destroyed by an immune response which may relate to the expression of luciferase protein within the tumours. We have not found any data in the literature about using similar bioluminescence imaging in immuno-competent rats. We can conclude that the non-invasive IVIS bioluminescence imaging camera is not suitable to detect tumour growth in our rat orthotopic bladder tumour model.

4.5 Evaluation of the efficacy of Oncovex$^{GALV/CD}$ on our previously developed rat orthotopic bladder tumour model *in vivo*

Here we carried out a previously described method to implant bladder tumours within the bladders of catheterized Fischer F344 rats, which involves chemical abrasion of bladder mucosa followed by installing of freshly harvested AY-27 HVEM cells (Day 0). The tumour bearing animals were assigned into three treated groups either OncoVex$^{GALV/CD}$+5-FC (n=10), OncoVex$^{GALV/CD}$ +PBS (n=10) or PBS+5-FC (control group) (n=8). Intravesical treatment of implanted tumours was carried out with virus (OncoVex$^{GALV/CD}$, 9e7 pfu) or Control PBS on Days 7, 14 and 21. Prodrug 5-FC (12mg) or PBS was installed in the same manner on Days 8, 9, 15, 16, 22 and 24 (Figure 4.5.1).

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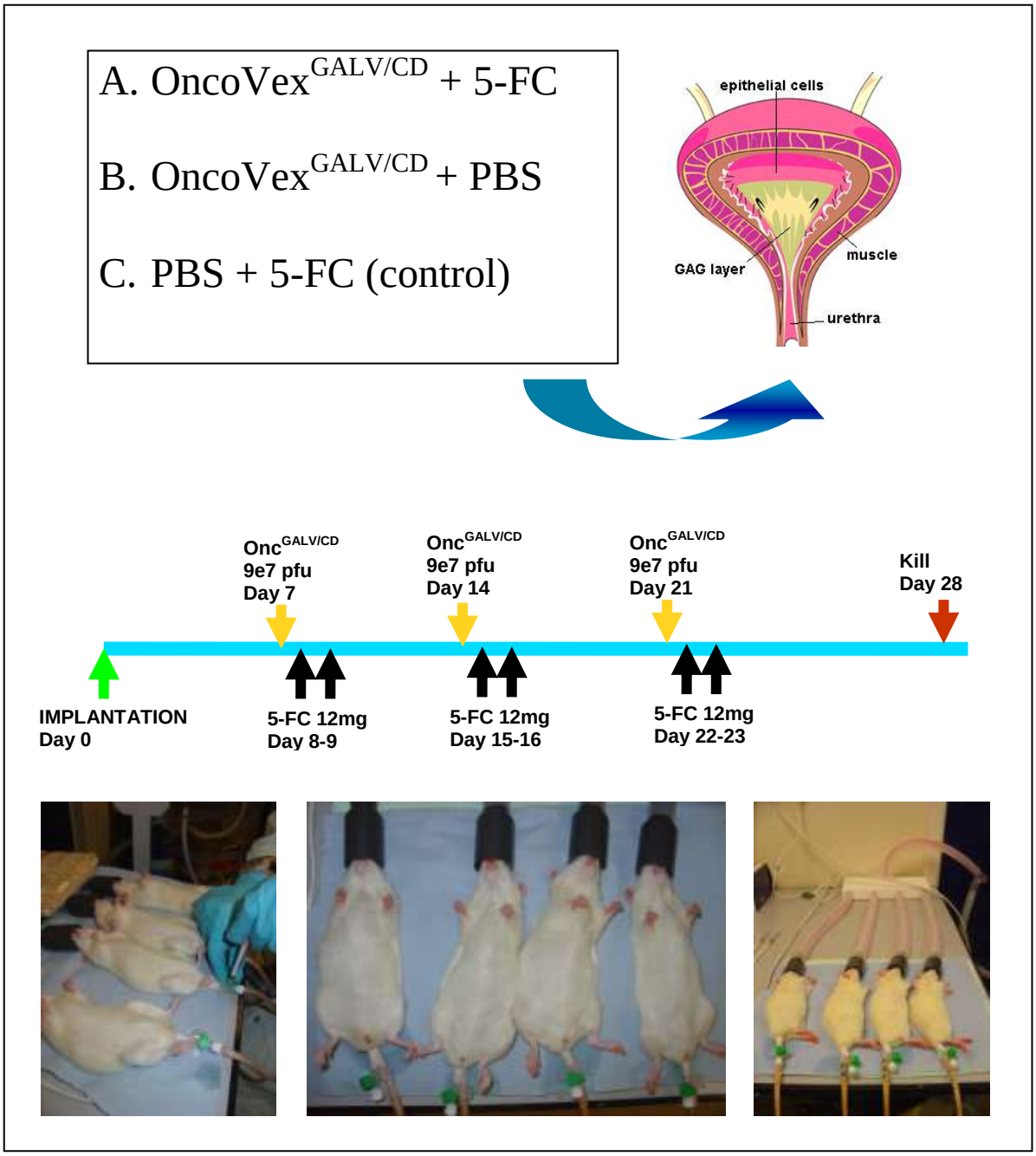

Figure 4.5.1 Treatment of a rat orthotopic bladder tumour model. Female Fischer F344 rats were anesthetised and a catheter was inserted into the bladder via the urethra with the use of an 18-gauge plastic intravenous cannula. 3 treatment groups. Group A Oncovex GALV/CD + Prodrug, Group B Oncovex GALV/CD + PBS, Group C PBS + Prodrug. On day 7,14 and 21 rats in Group A and B were treated with Oncovex GALV/CD, in Group C with PBS. On day 8-9, 15-16 and 22-23 rats in Group A and C were treated with prodrug, in Group B with PBS. All kind of treatments were instilled transurethrally through the catheter and were maintained in the bladder for 1 hour. The rats were turned 90° laterally every 15 minutes to ensure exposure of the entire bladder wall. $9x10^7$ pfu Oncovex GALV/CD virus was administered in 600µl PBS (Stock: $1.8x10^5$pfu/µl). 600µl of 5-Fluorocytosine (5-FC) in RPMI was then instilled

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(Concentration of stock: 15.0mg/ml). As control 600μl PBS (pH 7.4) was added. After 1 hour the catheters were removed, and the rats were allowed to void spontaneously.

After the treatment the animals were observed for signs and symptoms of advanced bladder cancer. There was no detectable abdominal mass during the 28 days period. The weight of the animals was measured in every 3 days (Figure 4.5.2). The data showed a trend that the animals treated with OncoVex$^{GALV/CD}$ + 5-FC are 11.5g heavier than controls, suggesting that they are in a healthier condition compared to controls. Statistical analysis of the data shows no significant difference detected between the three treated groups. Mild haematuria subsequent to treatment days was detected in some of the rats, but this was not related to each treatment group.

The animals were sacrificed on day 28 and their bladders were removed and assessed for tumour abundance. The harvested bladders were weighed, then opened up and the bladder surface which macroscopically contained tumour was measured with a caliper. From these tumour measurements we could calculate tumour volume. Results showed enhanced local tumour control in the presence of both OncoVex$^{GALV/CD}$ and prodrug when compared to control (No virus +prodrug, ***P*=0.001**) or virus alone (***P*=0.034**) (Figure 4.5.3, 4.5.4). A smaller amount of tumour shrinkage seen with virus alone was not statistically significant when compared to control animals (No virus +prodrug, ***P*=0.13**). The results strongly suggest that a combination of oncolysis, prodrug activation and fusogenic glycoprotein therapy offers an opportunity for improved tumour control within the bladder.

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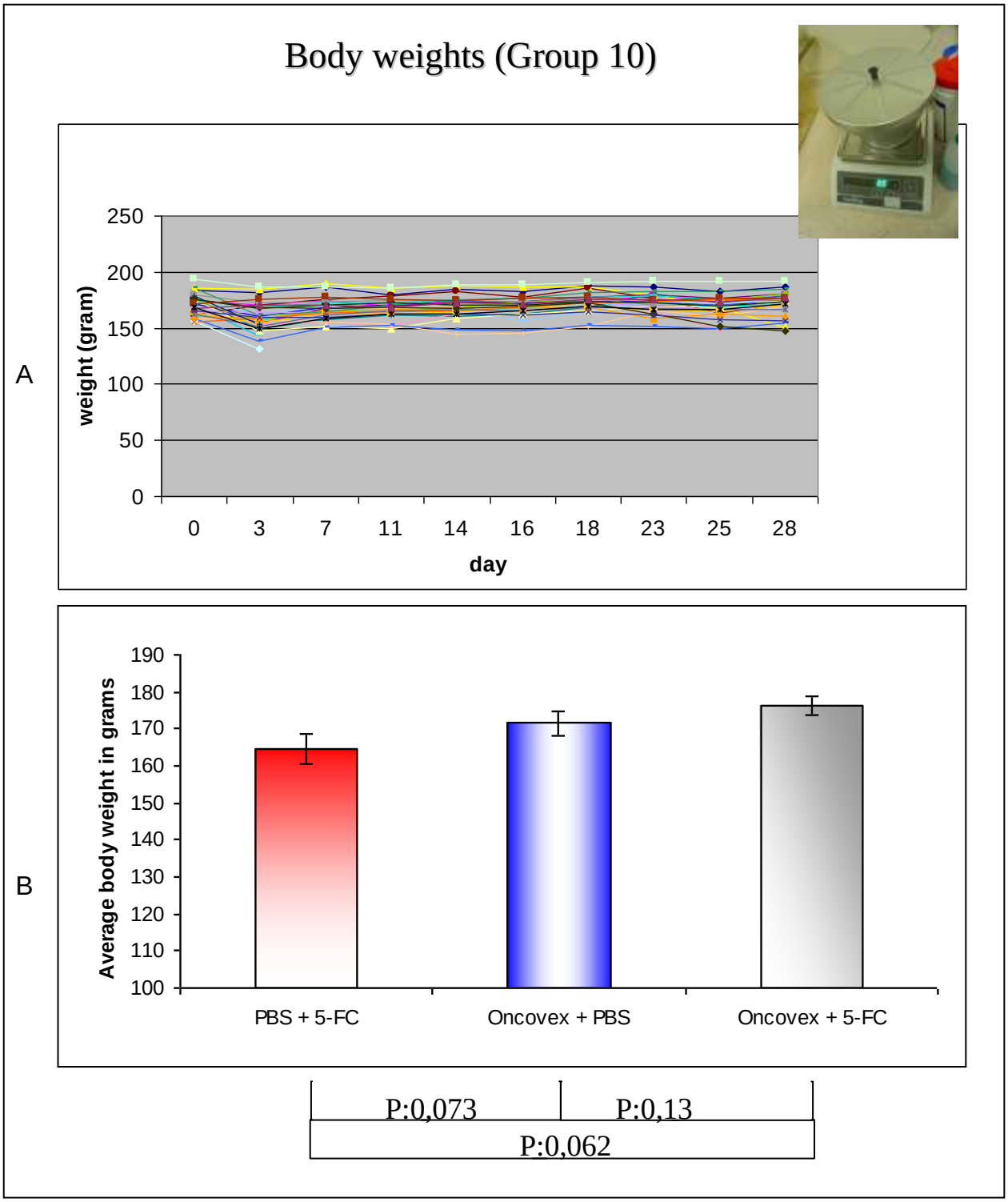

Figure 4.5.2 Animals body weight after treatment with Oncovex GALV/CD. The weight of the animals was measured in every 3 days. Body weights of all animals at various time points in the study (A): where we show that independently from treatment group there wasn't any significant difference between animals and there also wasn't any major weight loss during the experiment. Average body weights comparing each treatment group (B) did not show significant difference. (OncoVex$^{GALV/CD}$+5-FC

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(n=10), OncoVex$^{GALV/CD}$+PBS (n=10), PBS+5-FC (control group) (n=8)) Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

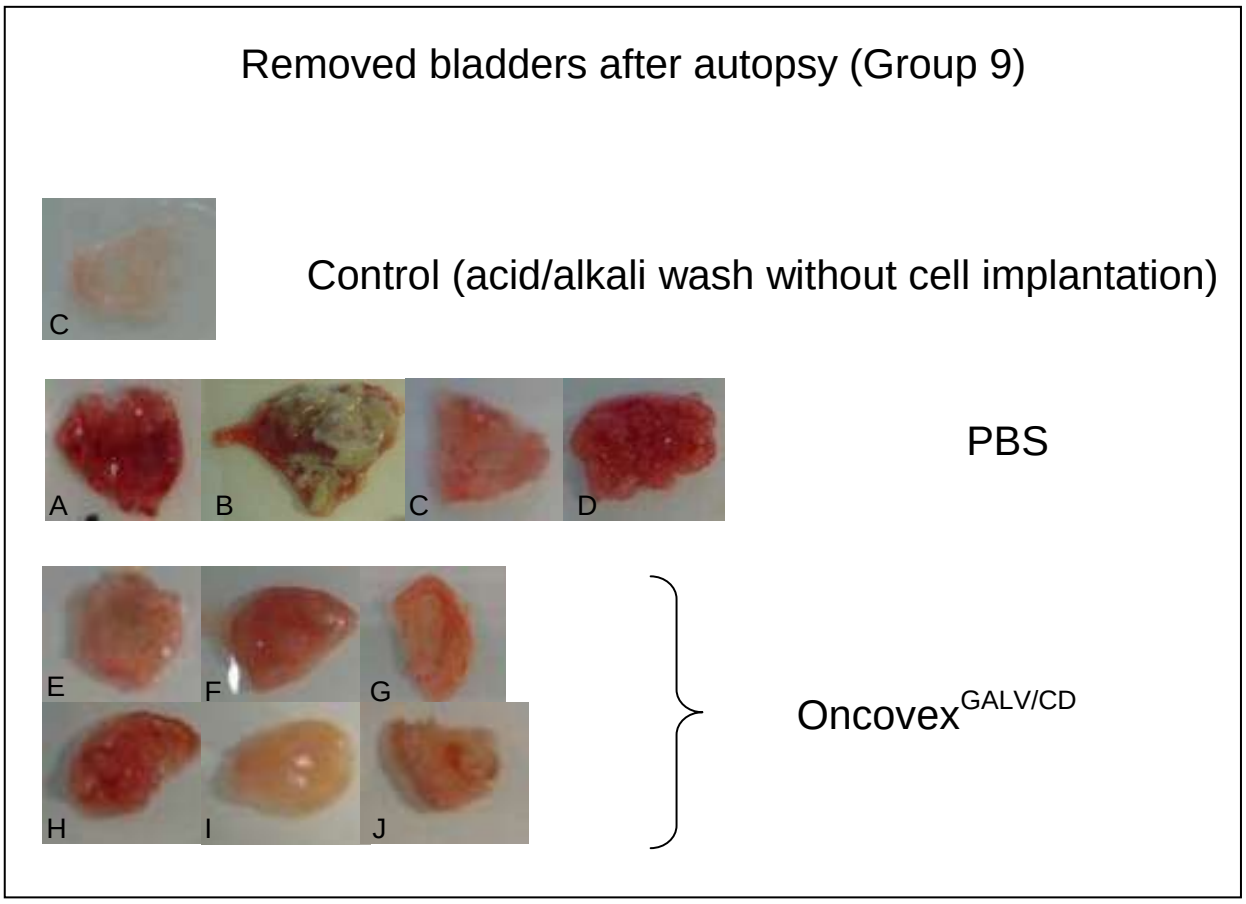

Figure 4.5.3 Bladders removed after autopsy from treatment groups Oncovex$^{GALV/CD}$ +5-FC and PBS + 5-FC.

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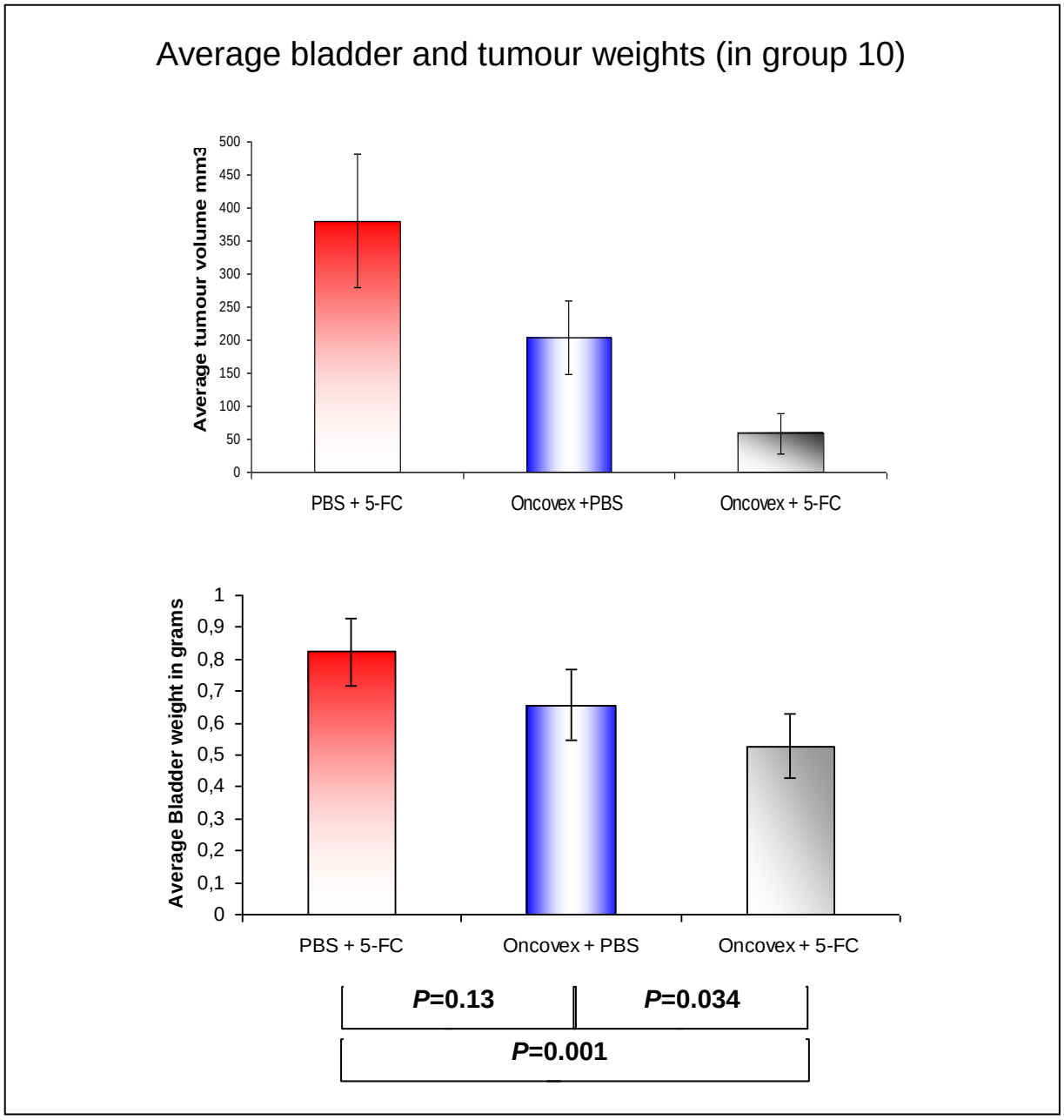

Figure 4.5.4 Average tumour volume and average bladder weight of treatment groups Oncovex GALV/CD +5-FC, Oncovex GALV/CD + PBS and PBS + 5-FC. The rats were sacrificed after 28 days. There bladders were removed and assessed for tumour abundance. The harvested bladders were weighed, then opened up and the bladder surface which macroscopically contained tumour was measured with a caliper. Average Tumour volume was calculate by measure length x width x width. This experiment was repeated and similar results were obtained. (OncoVex$^{GALV/CD}$+5-FC (n=10), OncoVex$^{GALV/CD}$+PBS (n=10), PBS+5-FC (control group) (n=8)) Experiments were repeated at least three times. The figure shows a representative experiment, where the error bars are standard deviations.

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5 Discussion

Our *in vitro* studies using different bladder tumour cell lines showed that OncoVEX$^{GALV/CD}$ increases tumour killing in over half of the bladder cancer cell lines compared to backbone virus alone. We can therefore conclude from this study that expression of the truncated retroviral envelope GALV *env* R- greatly improves the dose response of the OncoVex^{GFP} backbone in bladder tumour cell lines. It is important to remember that the aim of this study is to improve the efficacy of the oncolytic HSV backbone virus. To allow expression of the truncated retroviral glycoprotein the backbone needs to enter the target cells and viral transcription needs to take place. The MTS assay data showed that all bladder cells tested demonstrated some efficacy to HSV alone (Figure 3.1-5), with T24, VMCUB-I, 5637 cells requiring lower levels of virus to see viral infection (MOI 0.1 and 0.01) whereas other cell lines, such as EJ cells, needed higher levels (MOI 10 and 1). RT112, TCCSUP-G and KU19-19 cells did not show increase in cell survival at any MOIs (MOI 10, 1, 0.1, 0.01, 0.001).

An important point to note is that infection by HSV is needed for fusion but it is not the only control factor needed. Cellular factors are likely to play a role in fusion. Pit-1 is the GALV receptor, mandatory for fusion and entry for wild type Gibbon Ape Leukaemia Virus into their target cells.

In 1990 O'Hara and coworkers cloned, sequenced, and characterized the human cDNA encoding the receptor for GALV (O'Hara et al. 1990). The normal function of this multiple membrane-spanning receptor, designated Pit-1 (Kavanaugh et al. 1994; Olah et al. 1994), is that of a type III sodium-dependent phosphate transporter. Pit1 is ubiquitously expressed in mammalian normal tissues (Johann et al. 1992; Kavanaugh et al. 1994) and it plays a fundamental housekeeping role in Pi transport, (such as absorbing inorganic phosphate from interstitial fluid for normal cellular functions (cellular metabolism, signal transduction, and nucleic acid and lipid synthesis).

Limited studies have been carried out on the expression of Pit-1 on tumour tissue. What is known is that in human osteosarcoma, melanoma and renal carcinoma cells expression of Pit-1, at the level mRNA, occurs to different degrees (Grabarczyk et al. 2001; Palmer et al. 1997). No direct relationship was found between the Pit-1 mRNA level and transduction efficiency on melanoma and renal carcinoma cells (Grabarczyk et

al. 2001). Stimulation of retroviral receptor expression (Pit-1 and Pit-2) by phosphate depletion induced a limited increase of receptor mRNA levels, but did not improve the gene transfer efficiency (Grabarczyk et al. 2002). We can conclude that mRNA levels of Pit-1 seem to show little correlation with viral fusion.

The presence of Pit-1 receptor has not been described yet in the literature in bladder cancer cell lines. Although all cell lines tested by our group were transitional cell type of bladder cancer it is very likely that there is a difference between them in the presence of Pit-1 receptor and this may account for which bladder tumour cell types fuse and which do not fuse.

We found effective fusogenic activity by the OncoVEX$^{GALV/CD}$ at different MOI levels when testing the bladder cancer cell lines. The cause of the different effective MOI levels might be the different expression level of Pit-1 receptor in each bladder cancer cell line. In the future it would be an interesting question to evaluate the protein levels for Pit-1 receptor in bladder tumour cell lines as well.

We have detected fusogenic activity by both MTS and fixing/staining of infected cells. Each has shown a clear difference between backbone and Onc$^{GALV/CD}$ on EJ, T24, VMCUB-1 and 5637 cells. Athough we have seen some differences between the results of the MTS assay and fixing and staining method. The MTS results showed effective fusogenic activity at slightly higher MOI levels compared to the fixing and staining method in each cell line. This is related to the technique of fixing and staining method where we wash the cells at least four times with PBS which leads to a slightly lower concentration of cells on the slides.

Prodrug activation therapy strives to deliver genes to cancer cells, which convert non-toxic prodrugs into active chemotherapeutic agents. Using this method a systemically administered prodrug can be converted into high local concentrations of an active anticancer drug in the tumour. We evaluated the prodrug activating effect of the OncoVEX$^{GALV/CD}$ virus *in vitro* on seven bladder tumour cell lines.

We can conclude from these *in vitro* experiments that expression of the Fcy:Fur gene within the HSV-1 backbone vector promotes active metabolism of 5-FC into 5-FU and further into its metabolites, resulting in tumour cell killing in five out of seven bladder tumour cell lines (EJ, RT112, TCCSUP-G, 5637, KU19-19). The percentage cell

survival is based on the uninfected cells as control (100%). Most of the virus controls appear to be over 100% (120-130%). We feel that this may be due to the fact that virus infected cells will not use up as many nutrients in the media compared to uninfected cells. Therefore after heat activation the viral media offers a greater growth potential then the uninfected control. Two out of seven tested bladder tumour cell lines (T24, VMCUB-I) failed to improve tumour cell killing when infected with OncoVEX$^{GALV/CD}$ in the presence of 5-FC. This failure to improve tumour cell killing by prodrug activation, may be due to a number of factors, that include either the cells are not sensitive to 5-FU metabolites or that the cells did not allow the active metabolism of 5-FC into 5-FU metabolites.

A recent proteomic study on colorectal adenocarcinoma cells transfected with cytosine deaminase in the presence of 5-FC, has shown an upregulation of a wide range of proteins involved in resistance to anticancer drugs and resistance to apoptosis (Negroni et al. 2007). Examples of some of the genes that are upregulated include the antioxidative gene thioredoxin-like 2 protein, aldehyde dehydrogenase, dihydropyrimidine–related protein 2 and Ezrin. The antioxidative gene thioredoxin-like 2 protein controls redox balance, cell growth and apoptosis, and has also been shown to increase tumour cell growth and chemotherapy resistance (Powis et al. 2000). Aldehyde dehydrogenase is a mitochondrial enzyme, genetic variability in this gene has been linked to resistance to the anti agent cyclophosphamide (Di Paolo et al. 2004). The dihydropyrimidine–related protein 2 is involved in the pyrimidine metabolism and in the catabolism of 5-FU (Nyhan 2005). Patients with a partial dihydropyrimidine deficiency proved to be at risk of developing severe toxicity after 5-FU administration (van Kuilenburg et al. 2004). Ezrin is a cytoskeletal protein which has a major role in cell polarization through actin binding and the role of ezrin in metastasis behaviour has been well documented (Fais 2004). Also ezrin is connected to P-glycoprotein, which regulates the efflux pumps that are responsible for some of the multidrug resistance mechanisms of tumours (Luciani et al. 2002). From this study we can hypothesize that some of the drug resistance genes up-regulated in colorectal adenocarcinoma cells expressing CD/5-FC, may be also up-regulated in bladder TCC carcinoma cell lines, although the expression of UPRT will alter the down stream effects dramatically. Further to this, genetic variation in such drug resistance genes within each TCC cell line

may well be a controlling factor in the different level of susceptibility to prodrug seen in these cells. In the future a proteomic study on different TCC cell lines infected with our virus in the presence of prodrug could answer some of these questions.

The use of combined drug treatments is becoming commonplace in the treatment of cancer. We have tested the efficacy of Oncovex$^{GALV/CD}$ in combination with conventional chemotherapies such as mitomycin, cisplatin, gemcitabine on human bladder tumour cell lines.

Why is oncolytic HSV synergistic with mitomycin C but not cisplatin/gemcitabine on bladder transitional cell carcinoma? During the past decade gemcitabine gained widespread use for the treatment of bladder cancer (Fechner et al. 2003; Moore et al. 1997; Muramaki et al. 2004). Gemcitabine, a cytotoxic pyrimidine deoxynucleoside analogue, is transported into the cell mostly by human nucleoside transporters (hENT and hCNT, respectively. As with other analogues of pyrimidines, the triphosphate analogue of gemcitabine replaces one of the nuclosides to be incorporated into growing DNA strands and therefore acts as a chain terminator to stop DNA polymerase (Blackstock et al. 2001). The process arrests tumour growth, resulting in apoptosis (Blackstock et al. 2001). The majority of drugs used in the clinical treatment of HSV-1 related diseases target the viral DNA polymerase activity by also acting as a nucleoside analog inhibitor (Ganciclovir, Acyclovir and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracyl). The virus thymidine kinase gene catalyses the phosphorylation of the purine nucleosides analogues converting them to their corresponding nucleoside monophosphates, which are catalysed to nucleoside diphosphates by mammalian nucleoside monophosphate kinases and are subsequently converted to the tri phosphate form by nucleoside diphosphate kinase (Miller and Miller 1980). The triphosphate form is able to stall DNA synthesis by inhibiting DNA polymerase and by incorporation into DNA causing chain termination (Davidson et al. 1981; Elion 1980; Mar et al. 1985), thus killing infected cells (Elion 1980). It is possible that gemcitabine causes chain termination of both viral and host DNA which results in the failure of replication of the virus and synergy between this chemo agent and oncolytic HSV-1.

Cisplatin is commonly used in combination regimes for the treatment of bladder cancer. Its cytotoxicity is mediated through platinum-DNA adducts, resulting in apoptosis and cell cycle arrest (Boulikas and Vougiouka 2003). However, this apoptosis is responsible for the characteristic nephrotoxicity, ototoxicity, and neurotoxicity (Boulikas and Vougiouka 2003). Adusumilli et al (Adusumilli et al. 2006) showed that low-dose cisplatin was used to induce the cellular stress response, with minimal activation of apoptotic pathways against malignant pleural mesothelioma. High-dose cisplatin caused not only toxicity but also a high apoptotic cell fraction, which hindered HSV-1 oncolysis by limiting viral replication (Stanziale et al. 2004). Other investigators have shown that cisplatin did not inhibit the efficacy of replication-competent HSV in the treatment of head and neck squamous cell carcinoma and in non-small cell lung cancer (Chahlavi et al. 1999; Toyoizumi et al. 1999).

Mitomycin C is an antibiotic that is a potent cross-linker of DNA and has widely been used in cancer chemotherapy (Tomasz 1995). A single crosslink per genome has shown to be effective in killing bacteria (Szybalski and Iyer 1964). A general mechanism of action for MMC has emerged that is activated regardless of the source of reducing equivalents, comprising three competing pathways that give rise to unique reactive intermediates and different DNA adducts. Partitioning into the pathways is dictated by chemical considerations such as pH and drug concentration. DT-diaphorase stands out in this mechanism, since it is much less effective at metabolizing MMC at neutral pH. At least five different enzymes can catalyse MMC bioreduction in vitro, and as many activities may be present in solid tumours, including a series of novel mitochondrial reductases such as a cytochrome P450 reductase. Competition between reductases for MMC appears to be based solely on protein levels rather than enzyme kinetics. Consequently, DT-diaphorase can occupy a central role in MMC metabolic activation since it is often highly overexpressed in cancer cells (Cummings et al. 1998). It would be interesting to study the expression of DT-diaphorase within infected cells. Oncolytic HSV-1 viruses (NV1066, G207) has previous been shown to be synergistic with mitomycin C on one bladder transitional cell carcinoma cell line (Ru19-19)(Mullerad et al. 2005) and a number of gastric cancer cell lines (Bennett et al. 2004). Mitomycin C cross-links in a specific DNA sequence manner, its target being CpG sites. CpG sites are regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in

the linear sequence of bases along its length. Why does oncolytic HSV act synergistically with mitomycin C? A hypothesis could be that the HSV genome lacks the target CpG and therefore does not inhibit the cross-linker effects of mitomycin C. It has been shown that the genome of HSV resembles bacterial DNA in having a high content of "CpG motifs" (Klinman et al. 1996; Krieg et al. 1995) suggesting it would be a better target for cross-linking than the host genome. Mitomycin C is one of a range of DNA-damaging agents which induces the expression of GADD34 (growth arrest and DNA damage –inducible protein) (Chou and Roizman 1994; Hollander et al. 1997). There is great homology between the carboxyl terminus of the mammalian GADD34 gene and the corresponding carboxyl domain of the viral ICP34.5 (Chou and Roizman 1994). ICP34.5 functions as a virulence factor by preventing the shutoff of protein synthesis in virus-infected cells (Chou and Roizman 1992), deletion of which allows tumour selective viral replication (Rampling et al. 2000). Bennett et al (2004) (Bennett et al. 2004) shows that mitomycin C and HSV were synergistic on gastric cancer cell lines. Their Northern blot analysis confirmed that expression of GADD34 mRNA was increased by mitomycin C treatment. RNAi targeting GADD34 decreased mitomycin C -associated enhancement of HSV replication and resulted in decreased viral synergy with the chemotherapy on gastric cancer cell lines (Bennett et al. 2004). This would suggest in bladder tumour cells, that synergy seen between oncolytic HSV and mitomycin C maybe due to the up regulation of GADD34, which aids viral tumour replication. On this basis we would expect cisplatin to show synergy with HSV in bladder transitional cell carcinoma because cisplatin has been shown to up-regulate GADD34 in malignant mesothelioma and head / neck squamous carcinoma cell lines (Adusumilli et al. 2006; Fishel 2006). Our results do not show synergy between cisplatin and oncolytic HSV on bladder transitional cell carcinoma cells. Therefore, it suggests that the cellular components needed for up regulation of GADD34 by cisplatin are present in malignant mesothelioma and head/neck squamous carcinoma cell lines but may not be present in bladder TCC cells.

These mechanisms describe viral exploitation of the host cellular stress response following exposure to chemotherapeutic agents or ionizing radiation. Eisenberg et al (Eisenberg et al. 2005) demonstrated that the production of viral progeny is significantly enhanced in the presence of either 5-FU or gemcitabine. Their data also suggest that this

potentiation of viral replication is responsible for the synergism observed. The differential improvement in cell killing in the combination therapy part of the experiments did not appear to be an initial cytotoxic effect. Rather, it became evident five to six days following treatment after several viral life cycles had been completed and after the differential increase in viral progeny production was evident by plaque assay. This amplification is limited only to the extent that viable cancer cells are present to support viral replication.

Our results showed synergistic effect when OncoVEX$^{GALV/CD}$ was combined with mitomycin C in EJ, T24 and KU19-19 cells. The synergistic interaction between OncoVEX$^{GALV/CD}$ and MMC may provide an opportunity to eradicate bladder cancer microscopic deposits that each agent alone at acceptable dosage cannot reach. The combined effect of OncoVEX$^{GALV/CD}$ with cisplatin or gemcitabine was antagonistic in EJ, T24 and TCCSUP-G cells. This antagonistic effect was not related to the implanted GALV and CD genes as the backbone OncoVEX^{GFP} virus showed antagonistic effect in combination with cisplatin or gemcitabine.

In order to evaluate the mechanism of interaction between OncoVEX$^{GALV/CD}$ and cisplatin or gemcitabine further experiments are needed. According to previous studies we plan to examine simultaneous and subsequent adiminstration of these agents, where primary administration of chemotherapeutical drug followed by a subsequent administration of the OncoVEX$^{GALV/CD}$ virus may lead to promising results.

An animal tumour model is essential for preclinical evaluation of new treatment modalities. We have set up a useful rat orthothopic bladder tumour model to test the effectiveness of the Oncovex$^{GALV/CD}$ virus. Entry of herpes simplex virus type 1 (HSV-1) into cells requires the envelope glycoprotein D (gD), gB, gH, and gL. After virion adsorption to cell surface proteoglycans, gD binds to one of its receptors, herpesvirus entry mediator (HVEM), nectin-1 (HveC), or 3-*O*-sulfated heparan sulfate (3-*O*-S HS) (Geraghty et al. 1998; Montgomery et al. 1996; Shukla et al. 1999). Receptor binding results in a conformational change in gD, which is believed to activate either or both gB and gH as mediators of fusion between the viral envelope and the plasma membrane or an endosomal membrane. Fusion leads to nucleocapsid release into the cytoplasm for subsequent viral genome delivery to the nucleus. Since

productive HSV-1 infection is set in motion by the gD-receptor interaction, cellular susceptibility to HSV-1 is initially determined by the presence of one or more functional gD receptors on the cell surface.

In addition to supporting HSV-1 entry, gD receptors also support HSV-1 cell-to-cell spread (Cocchi et al. 2000; Roller and Rauch 1998). However, it is not clear whether they act by the same mechanism in entry and spread. Two additional glycoproteins, gE and gI, are important for HSV-1 spread but are dispensable for entry (Johnson and Huber 2002). The individual contributions of HVEM and nectin-1 to infection in vitro and in vivo are difficult to assess since cells often express both receptors (Krummenacher et al. 2004).

Oncolytic HSV viruses have been shown to infect a wide range of human and animal tumour cells. The following studies have shown that oncolytic HSV infects and replicates *in vitro* and *in vivo* in a wide range of human tumour cell types: glioma (Andreansky et al. 1997; Andreansky et al. 1996; Chambers et al. 1995; Detta et al. 2003; Samoto et al. 2002), colon carcinoma (Carroll et al. 1996), retinoblastoma (Nicolo and Chiocca 1998), epithelial ovarian cancer (Coukos et al. 1999), colorectal cancer (Kooby et al. 1999; Reinblatt et al. 2004), prostate cancer (Cozzi et al. 2002; Walker et al. 1999), non-small cell lung cancer (Toyoizumi et al. 1999), gallbladder carcinoma (Nakano et al. 2001), head and neck squamous cell carcinoma (Wong et al. 2001), esophageal adenocarcinoma (Stiles et al. 2003), breast cancer (Liu and Rabkin 2005; Pin et al. 2004a; Teshigahara et al. 2004), thyroid cancers (Yu et al. 2004), hepatocellular carcinoma (Pin et al. 2004b) and rhabdomyosracoma (Currier et al. 2005).

Although HSV viruses have been shown to infect a wide range of human and animal tumour cells, but AY-27 rat bladder tumour cells are rare exceptions in failing to support HSV entry and/or replication. To overcome this problem AY-27 cells were stably transfected with the herpesvirus entry receptor (HVEM).

After the transfection our in vitro fusion assay results showed increased tumour cell killing with OncoVex$^{GALV/CD}$ compared to the OncoVex^{GFP} control on AY-27 HVEM E6 cells. OncoVex^{GFP} gave a classic HSV-1 cytopathic effect, in contrast, cells infected with OncoVEX$^{GALV/CD}$ showed fusion in the form of large multinucleated syncytia,

which were then surrounded with cells showing the more classic HSV-1-mediated effect.

AY-27 HVEM E6 cells were further tested in our prodrug assay. We can conclude from these *in vitro* experiments that expression of the Fcy:Fur gene within the OncoVex$^{GALV/CD}$ promotes active metabolism of 5-FC into 5-FU and further into its metabolites, resulting in tumour cell killing in AY-27HVEM E6 cells.

In our in vivo experiments we used a technique to develop a rat ortothopic bladder tumour model that was described by Xiao et al in 1999. To facilitate tumour seeding, the bladder mucosa was conditioned with an acid rinse followed by neutralization with alkali. After tumour implantation all rats developed tumour in their bladder, resulting in a high success rate of tumour imlpantation. The tumour implantation procedure was well tolerated by the animals and resulted in minimal morbidity associated with occasional mild haematuria subsequent to tumour cell instillation.

We planed to use QRT-PCR to demonstrate the presence of the HVEM receptor of the AY-27 rat bladder tumour cells as a marker for tumour load. However while we obtained positive signals on bladder tissue QRT-PCR, signals using urine samples were insignificant, therefore tumour growth was detectable by QRT-PCR only on bladder tissue samples.

As another option we planed to use a bioluminescence imaging technique that has become a very popular tool for noninvasive monitoring of fundamental biological and molecular processes in small living subjects. The non-invasive IVIS camera can detect the luciferase activity of the implanted tumours. We have cloned a new AY-27 bladder tumour cell line that contains both HVEM receptor and luciferase activating gene. Unfortunately our experiments showed that the implanted AY-27 tumours expressing luciferase regress after a period growth. We think this tumour regress may be due to immune responses to the luciferase (insect) proteins. This view is support by the fact that the majority of IVIS bioluminescence imaging studies are carried out on nude mice which have a deletion of thymus function resulting greatly reduced number of T cells. Therefore nude mice are unable to mount most types of immune responses, which results them fail to reject xenograft tissue. Fischer F344 rats (inbred) on the other hand have a fully competent immune system, which do not reject wild type AY-27 cells due to the fact that the tumour cells are derived from the same strained of mice. But Fischer

F344 rats may make specific immune responses to the insect luciferase protein leading to the regress of the tumours. Therefore the non-invasive IVIS bioluminescence imaging camera is not suitable to detect tumour growth in this rat orthotopic bladder tumour model. In the future we plan to use a non-invasive MRI scanner to detect the tumour development and the effect of the different treatment options.

With an immune-competent rat orthotopic bladder model it was necessary to use a non-human cell line, which required transfection of a viral receptor-encoding construct, and raises questions regarding immune response against luciferase transgene expression. The use of an immune-deficient model would lose the component of treatment response which may be attributable to immune recognition of tumour antigens following induced death of tumour cells, but would avoid all the above problems.

The reasons for choosing an immune-competent rat orthotopic bladder model are complex and are as follows. Advantages of a rat versus a mouse model include its size and evidence that the rat model may represent the human disease processes better than the mouse equivalent (Oyasu 1995). It was also important for us to match our in vivo model with the anti cancer agent used. We are testing Onco$^{GALV/CD}$ which expresses the gibbon ape leukaemia virus envelope, which needs the pit1 receptor for fusion. The effects of this could be shown in both rat immune-competent model and a xenograft mouse (immune-deficient model). But any side effects of GALV control fusion would not be evident on normal mouse tissue because a pit1 receptor homolog is not present mouse cells (Sommerfelt and Weiss 1990). This could be overcome by using a costly immune-deficient rat model. Oncolytic viruses work by replicating in and therefore destroying tumours, but can also show efficacy by stimulating an immune response to viral and tumour antigen's by cross priming (Pesonen et al. 2011). Onco$^{GALV/CD}$ has a deletion in ICP47 which increases the antitumor immune response in the presence of HSV and this efficacy would not be see in an immune- deficient model (Liu et al. 2003). Therefore we felt to see full efficacy and possible side effects of Onco$^{GALV/CD}$ our model needed to based on an immune-competent rat.

OncoVex$^{GALV/CD}$ has been found to be effective in treating various experimental cancers while maintaining an excellent safety profile. Bladder cancer is an ideal target for novel therapies because the ease of intravesical delivery allows the tumor to be exposed to

large titres of vector. In addition, replication-competent oncolytic viruses, which selectively infect and replicate within rapidly dividing cells, are potentially very useful for bladder cancer because the umbrella cell layer (i.e., the luminal surface of the urothelium) of the bladder is not rapidly dividing and should therefore be resistant to infection and lysis. HSV is inactivated at low pH, and the relative acidity of the urine is of concern with this route of administration. These experiments demonstrate that these viruses maintain their infectivity and efficacy in this animal model when administered by intravesical instillation in PBS. It is likely that these agents will exert their therapeutic effect prior to inactivation by a low pH when administered with PBS as a buffer.

Data from our current experiments in a well-accepted, orthotopic rat bladder model confirm safety, efficacy, and ease of delivery of oncolytic viral therapy for experimental treatment of bladder cancer. OncoVex$^{GALV/CD}$ virus, when administered with prodrug weekly for 3 weeks by intravesical instillation, was clearly more effective than control or virus alone at reducing tumor burden. In these experiments, treated animals continued to groom themselves and behave normally, no adverse events were noted, and there were no treatment-related deaths. However, these oncolytic viruses will likely be more efficacious *in vivo* in humans because HSV are human viruses and human tumors are significantly more susceptible to their effects than murine tumors (Cozzi et al. 2001). Although OncoVex$^{GALV/CD}$ is highly attenuated, therapeutic use of replicating viruses still raises concerns about viral proliferation and dissemination. Unlike many other viruses, effective antiviral agents currently available for the treatment of HSV infection. Antiviral agents such as acyclovir can be used effectively for therapy if dissemination of virus and severe systemic toxicities are encountered. It is also worth noting that a version of this virus expressing GM-CSF has shown promising results in Phase I and II (Hu et al. 2006; Senzer et al. 2009) with limited toxicity to patients and is currently in III clinical trials. Our results suggested that OncoVex$^{GALV/CD}$ intravesical therapy in the presence of 5-FC prodrug is suitable for further human phase I studies.

In conclusion our results on human bladder carcinoma cell lines and rat orthotopic bladder tumour model indicate that OncoVex$^{GALV/CD}$ may improve local tumour control within the bladder and it can be used in combination with mitomycin. It is hoped that

this will lead to new therapeutic option for bladder cancer.

The current work has raised questions that would be the basis of our future work. In the in *vitro* part (fusion and prodrug assay) we plan to extend the number of tested cell lines and also test other histological type of bladder cancer than TCC. We plan to repeat our prodrug assay with more concentrations of administered 5-FC to be able to accurately evaluate the IC50 levels for each cell line. Further combination therapy studies are needed with other chemotherapeutic agents (epirubicin, doxorubicin) and with immunotherapy (BCG) as we would plan to test OncoVex$^{GALV/CD}$ as a potential treatment for NMIBC. We plan to further study the mechanism of the OncoVex$^{GALV/CD}$ virus (presence of Pit1 receptor in bladder cancer, proteomic study). Using the rat orthotopic bladder model we plan to use a non-invasive MRI or CT imaging technique in order to monitor tumour growth and prove therapeutic effect of OncoVex$^{GALV/CD}$ during the treatment. With an immune-competent rat orthotopic bladder model it was necessary to use a non-human cell line, which required transfection of a viral receptor-encoding construct, and raises questions regarding immune response against luciferase transgene expression. In chapter 6 we discussed more in details why had chosen a rat immune-competent model (absecnce of pit1 receptor in mice). Therefore we plan to study OncoVex$^{GALV/CD}$ on an immune-deficient rat model as well to overcome these problems.

According to the literature gene therapy using viral vectors seems to be a promising tool in the field of oncotherapy. Several phase I-III ongoing studies shows that this is one of the burning issues in cancer therapy. In bladder cancer there are many published *in vitro* and *in vivo* studies using different vectors and different therapeutic genes, and all have pros and cons as we discussed in the introduction part of the thesis. Unfortunately, to date there have been few clinical trials of gene therapy for bladder cancer. It is also worth noting that a version of this virus expressing GM-CSF has shown promising results in phase I and II (Hu et al. 2006; Senzer et al. 2009) with limited toxicity to patients and is currently in phase III clinical trials. Once we finish our *in vitro* and *in vivo* studies we plan to set up a phase I trial with OncoVex$^{GALV/CD}$. The planned treated group would be medium risk NMIBC patients in combination with currently used chemotherapies and probably high risk patients in combination with BCG.

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6 Conclusion

- The transduction of bladder tumour cells with viral fusogenic membrane glycoprotein (GALV) is a new and effective approach for bladder cancer gene therapy *in vitro* using the OncoVEX$^{GALV/CD}$ virus.
- The transduction (by an oncolytic HSV) of bladder tumour cells with prodrug activating (CD) gene in the presence of 5-FC led to enhanced tumour control *in vitro*.
- All human bladder tumour cell lines tested are susceptible to HSV oncolysis and showed enhanced tumour cell killing in at least one type (fusion or prodrug) of the assays when infected with OncoVEX$^{GALV/CD}$ virus.
- The combination of oncolytic transduction of bladder tumour cells with viral fusogenic membrane glycoprotein (GALV) and a prodrug activating system (CD) can further increase tumour control *in vitro.*
- The coadministration of OncoVex$^{GALV/CD}$ and mitomycin showed synergistic effect *in vitro.*
- The coadministration of OncoVex$^{GALV/CD}$ with cisplatin or gemcitabine showed antagonistic effect *in vitro.*
- AY-27 rat bladder tumor cells are rare exception in failing to support HSV entry and/or replication.
- The transfected AY-27 HVEM cell line that contains the herpesvirus entry receptor supports HSV entry and replication.
- The transduction of AY-27 HVEM cell line with viral fusogenic membrane glycoprotein (GALV) was effective treatment *in vitro* using the OncoVEX$^{GALV/CD}$ virus.
- The transduction of AY-27 HVEM cell line with prodrug activating (CD) gene in the presence of 5-FC led to enhanced tumour control *in vitro* using the OncoVEX$^{GALV/CD}$ virus.

- The rat orthotopic bladder tumour model (described by Xiao) using AY-27 HVEM cell line was suitable for further *in vivo* testing. The percentage of tumour implantation using the orthotopic tumour model was strong (almost 95% after necropsy).
- QRT-PCR on the removed bladder tissue samples (after necropsy) showed effective detection of tumour growth, but failed to detect a signal in urine from the same animals.
- Non-invasive IVIS bioluminescence imaging camera was also not suitable to detect tumour growth in this rat orthotopic bladder tumour model, due to host immune response to the luciferase expression.
- OncoVex$^{GALV/CD}$ intravesical therapy with the combined transduction of viral fusogenic membrane glycoprotein (GALV) and a prodrug activating system (CD) in the presence of 5-FC prodrug led to enchanced local tumour control within the bladder *in vivo* in the rat orthotopic bladder tumour model.

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7 Summary

In Hungary approximately 2,600 new cases of bladder cancer are diagnosed every year. Approximately 80% of these cases are non muscle invasive bladder cancer (NMIBC). Despite current operative and postoperative treatment options there is recurrence rate of 15-61% and significant risk of progression (1-17%).

Gene therapy strategies have been developed as a therapeutic approach for a number of malignances. OncoVex$^{GALV/CD}$ is a modified oncolytic herpes simplex virus, where the virus is deleted for ICP34,5 region to provide tumour selective replication and also deleted for the ICP47 region to increase antigen presentation and the antitumor immune response. The OncoVex$^{GALV/CD}$ virus carries a highly potent prodrug activating gene for suicide gene therapy (CD) and a fusogenic glycoprotein gene from gibbon ape leukaemia virus (GALV). Effective use (enhanced cell killing and tumour shrinkage) of OncoVex$^{GALV/CD}$ vector has been previously described *in vitro* and *in vivo* other tumour sites but not the bladder.

The present thesis aimed to test *in vitro* and *in vivo* the OncoVex$^{GALV/CD}$ as an intravesical therapy for NMIBC. In vitro tumour cell killing by OncoVex$^{GALV/CD}$ was assessed by Fusion/Prodrug MTS assays and staining by Crystal Violet. Treatment of seven human bladder carcinoma cell lines with the virus resulted in higher tumour cell killing through oncolysis, pro-drug activation and glycoprotein fusion. The effect of OncoVex$^{GALV/CD}$ treatment in combination with chemotherapies was assessed by calculating combination index. The coadministration of OncoVex$^{GALV/CD}$ and mitomycin showed synergistic, whereas the coadministration with cisplatin or gemcitabine showed antagonistic effect *in vitro*. For the *in vivo* tests we have developed a rat orthotopic bladder tumour model using AY-27 HVEM cells for implantation. *In vivo* efficacy of the OncoVex$^{GALV/CD}$ treatment was studied on the orthotopic bladder tumour model, where the results showed enhanced local tumour control and higher body weights in the presence of both OncoVex$^{GALV/CD}$ and prodrug when compared to control (No virus + prodrug) or virus alone.

In conclusion our results on human bladder carcinoma cell lines and rat ortotopic bladder tumour model indicate that OncoVex$^{GALV/CD}$ may improve local tumour control within the bladder and it can be used in combination with mitomycin.

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Összefoglalás:

Magyarországon mintegy 2600 új hólyagtumoros eset kerül felismerésre évente. A diagnosztizált hólyagdaganatok kb. 80%-a felületes hólyagtumor. A jelenleg alkalmazott transurethralis rezekció és a posztoperatív hólyaginstillációs kezelések ellenére a kiújulás aránya 15-61%, a progresszió aránya pedig 1-17%.

Alternativ terápiás lehetőségként számos daganat típusnál kutatják a gén terápia alkalmazhatóságát. Az OncoVex$^{GALV/CD}$ egy módosított herpes simplex vírus, mely kombinálja a vírus saját onkolítikus aktivitását egy prodrug ("előanyag") aktíváló gén (citozin deamináz (CD)/uracil foszforibozil transzferáz enzim) és a gibbon ape leukémia vírus (GALV) egy a sejtek fúzióját elősegítő membrán glükoproteinjének expressziójával. Emellett az Oncovex$^{GALV/CD}$ vírus tartalmaz más mutációkat is, melyek tovább fokozzák annak hatékonyságát. Így az ICP34.5 vég törlődése tumor szelektív osztódáshoz vezet, az ICP47 vég törlése pedig a tumor elleni immunválaszt fokozza. Korábbi az Oncovex$^{GALV/CD}$ vírussal végzett *in vitro* és *in vivo* vizsgálatok fokozott tumorsejt pusztulást mutattak fej-nyaki, vastagbél, hasnyálmirigy, tüdő és glioma eredetű daganatokban.

Kutatásunk során az OncoVex$^{GALV/CD}$ vírus hatékonyságát vizsgáltuk *in vitro* és *in vivo* intravesicalis kezelés során. Az *in vitro* kísérleteket fúziós és prodrug MTS kísérletekkel valamint kristályibolya festéssel végeztük. 7 különböző human hólyagtumor sejttípuson *in vitro* végzett kísérletek során jelentős mértékű tumoros sejthalált észleltünk a HSV onkolítikus hatása, a prodrug aktíváció és a glycoprotein sejtfúzió révén. Az OncoVex$^{GALV/CD}$ kezelés kemoterápiával kombinációban történő alkalmazását *in vitro* vizsgáltuk, a kombinációs index meghatározásával (CalcuSyn szoftver segítségével). Mitomycin C együttadása esetén szinergista hatást észleltünk, míg a cisplatinnal vagy gemcitabinnal történő kombinációs kezelés antagonista volt. Az *in vivo* kísérletekhez kifejlesztettünk egy orthotopikus patkány hólyagtumor modelt AY-27 HVEM patkány hólyagtumor sejtek alkalmazásával. Az orthotopikus patkány hólyagtumor model segítségével végezett *in vivo* vizsgálatok szignifikánsan kisebb tumor volument és nagyobb testtömeget igazoltak az OncoVex$^{GALV/CD}$ vírussal kezelt csoportban, szemben a kontrol csoportokkal.

Vizsgálataink során hólyagtumorok esetében igazoltuk az OncoVex$^{GALV/CD}$ vírus hatékonyságát *in vitro, in vivo* és Mitomycinnel történő kombinált kezelés esetén.

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9 List of publications

Publications related to the thesis:

1. **Horváth A**, Mostafid AH. (2009) Therapeutic options in the management of intermediate risk non muscle invasive bladder cancer. British Journal of Urology International, 103(6): 726-729. **Impact Factor: 2.865**
2. Simpson GR[£], **Horvath A[£]**, Annels NE, Pencavel T, Metcalf S, Seth R, Peschard P, Price T, Coffin RS, Mostafid H, Melcher AA, Harrington KJ, Pandha HS, ***[£]These authors have contributed equally to this work.*** (2012) Combination of a fusogenic glycoprotein, pro-drug activation and oncolytic HSV as an intravesical therapy for superficial bladder cancer. British Journal of Cancer, 106: 496-507 doi:10.1038/bjc.2011.577. **Impact Factor: 4.831**
3. **Horváth A**, ChanawaniM, Mostafid AH. (2008) Immediate post operative administration of intravesical Mitomycin in theatre for non-muscle invasive bladder cancer. British Journal of Urology International, Website: Atlas of Surgery and Surgical Devices 2008.08

Other publications:

1. **Horváth A.** (2005) A kőképződés, mint anyagcsere betegség -a recidív köves betegek kivizsgálása. Családorvosi Fórum, 11: 9-12.
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3. **Horváth A**, Majoros A, Mavrogenis S, Istók R, Romics I. (2007) Lymphoepithelioma-szerű hólyag carcinoma ritka esete. Uroonkológia, 4(2): 59-61.
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9. **Horváth A.** (2010) Kommentár - J.R. Brill: Férfiak húgycsőgyulladásának felismerése és kezelése - című cikkére. Orvostovábbképző Szemle, XVII (12)
10. Blázovics A, Nyirády P, Bekő G, Székely E, Szilvás Á, Kovács-Nagy E, **Horváth A**, Szűcs M, Romics I, Sárdi É. (2011) Changes in Erythrocyte Transmethylation Ability are Predictive Factors for Tumor Prognosis in Prostate Cancer. Croatica Chemica Acta, 84(2): 127-131. **Impact Factor: 0.713**
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