The Impact of Chemoprevention on Treatment Regimens for Non-Muscle Invasive Bladder Cancer

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Abstract

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Chemoprevention is becoming a highly promising approach to lowering the incidence of cancers. There is, however, insufficient data to determine whether these agents are safe to be used alongside established treatment regimens such as chemotherapy.

Non-muscle invasive bladder cancer is readily treatable with surgery (TURBT), yet there is a high rate of recurrence (~60%), 20-30% of patients recurring with the muscle invasive form of the disease, making it a good candidate for chemopreventive intervention. Anthocyanins are one example of dietary compounds currently under investigation as chemopreventive agents. They have been found excreted in the urine of mice at levels capable of causing 50% growth inhibition in cancer cell lines, making them potential bladder cancer chemopreventive agents.

Mirtoselect, a standardised bilberry extract containing a mixture of 15 different anthocyanins, was investigated *in vitro* as a potential chemopreventive agent for bladder cancer alongside chemotherapeutic agent mitomycin C (MMC). Mirtoselect itself was found to inhibit cell survival and growth, causing significant a decrease in clonogenic cell survival, as well as causing an increase in apoptosis in two of the bladder cancer cell lines investigated.

In combined studies mirtoselect pre-treatment did not inhibit the effects of MMC in measures of growth, cell survival, apoptosis, cell cycle distribution or DNA damage. In fact, there was a mirtoselect dependent increase in MMC-induced crosslinks, an enhancement of MMCs anti-proliferative effects at low concentrations of mirtoselect and in some instances apoptosis was greater than additive. Furthermore mirtoselect was shown to enhance the DNA damaging effects of radiation.

Mirtoselect itself appears to be a good chemopreventive candidate for non-muscle invasive bladder cancer. In combination it does not appear to interfere with the cytotoxic actions of MMC, potentially enhancing its effects, and could also provide a therapeutic advantage in radiotherapy, therefore warranting further investigation for use in clinic.

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Table of Contents

Tables, Figures and Equations

Abbreviations

%D%TD	% Decrease in % tail DNA
%TD	Percentage tail DNA
ALS	Alkaline labile site
ARE	Anthocyanin rich extract
ATM	Mutated in ataxia telangiectasia
ATR	AT- and Rad3-related
BBN	*N*-butyl-*N*-94-hydroxybutyl) nitrosamine
BCG	Bacillus Calmette-Guérin
C3G	Cyanidin-3-glucoside
Cdks	Cyclin-dependant kinases
CFDASE	carboxyfluorescein diacetate succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
COX2	Cyclo-oxygenase 2
CRUK	Cancer Research UK
ddH_20	Double distilled water
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DSB	Double strand break
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EMEM	Eagle's minimal essential medium
EGFR	Epidermal growth factor receptor
EtBr	Ethidium bromide
FA	Fanconi's anemia
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein-5-isothiocyanate
FL2-A	Area of the fluorescence peak
FL2-W	Width of the fluorescence peak
FSC	Forward scatter

GSE	Grape seed extract
HDAC	Histone deacetylase
HPV	Human papilloma virus
ICL	Interstrand crosslink
LMP	Low melting point
MMC	Mitomycin C
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NDNS	National diet and nutrition survey
NEAA	Non-essential amino acid
NER	Nucleotide excision repair
NMSC	Non melanoma skin cancer
OTM	Olive Tail Moment
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PE	Plating efficiency
PI	Propidium iodide
ProI	Proliferation index
RB	Retinoblastoma
RDA	Recommended daily allowance
RFU	Relative fluorescent uni
RLU	Relative luminescent unit
RNase A	Ribonuclease A
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
R-point	Restriction point
RT	Radiotherapy
SCE	Sister chromatid exchange
SCGE	Single-cell gel electrophoresis
SD	Standard deviation
SEM	Standard error of the mean
SF	Surviving fraction
SSB	Single strand break
SSC	Side scatter

TCC	Transitional cell carcinoma
TD	Tail DNA
TM	Tail moment
TNM	Tumour, nodes and metastasis
TMRE	Tetramethylrhodamine ethyl ester
TrypE	Trypsin/EDTA
Thiotepa	Triethylenethiophosphoramide
TURBT	Transurethral resection of the bladder tumour
WT	Wild type

Chapter One

Introduction

1.1 Cancer – a 21st Century Global Disease

1.1.1 The Impact of Cancer on Society

The human race is becoming an aging population. Between 1983 and 2008 the number of people aged 65 and over in the UK increased by 1 per cent, an increase of 1.5 million people (Office for national statistics). Age is a fundamental factor in the development of cancer, with incidences increasing dramatically with age. Carcinogenesis is a multi-step process taking time for the accumulation of genetic errors to occur, it therefore follows that the longer we live the more likely we are to develop cancer. Amongst 50-64 year olds cancer is the most common cause of death (Table 1.1-1).

Cause of Death in England and Wales	Women (%)			Men (%)		
	50-64	65-84	85+	50-64	65-84	85+
Cancers	39	32	18	53	29	12
Circulatory	36	42	42	22	40	44
Digestive system	7	4	3	6	5	4
Respiratory system	7	13	19	8	13	17
Injury and poisoning	4	1	2	3	1	2
Nervous system	2	3	3	3	3	3
Mental and behavioural	1	1	3	-	2	6
Other	4	5	9	5	7	14
All deaths (=100%)(thousands)	35.6	144.6	53.6	23.0	129.6	116.3

Table 1.1-1 Major Causes of Death: by Sex and Age, 2002
Causes of death (%) in the over 50's in 2002 in England and Wales (Office for national statistics.).

Cancer is prominent in both developed and developing countries and in 2004 it accounted for 7.4 million deaths worldwide, around 13% of all deaths (World health organisation. 2009). Deaths from cancer are estimated to continue to rise worldwide, with an estimated 12 million deaths by 2030 being accountable for by cancer (World health organisation, 2009). In Great Britain cancer mortality rates, for all types of cancer excluding non melanoma skin cancer (NMSC), have been steadily falling since 1989 but the incidence of cancer is still rising (Figure 1.1-1). In 2006 there were just

under 294,000 people diagnosed with cancer in the UK, of these the five most common cancers, excluding NMSC were; prostate, lung, colorectal, bladder and non-Hodgkin lymphoma for men and breast, colorectal, lung, uterine and ovarian for women, these eight cancers make up over 65% of all the incidences of cancer in the UK.

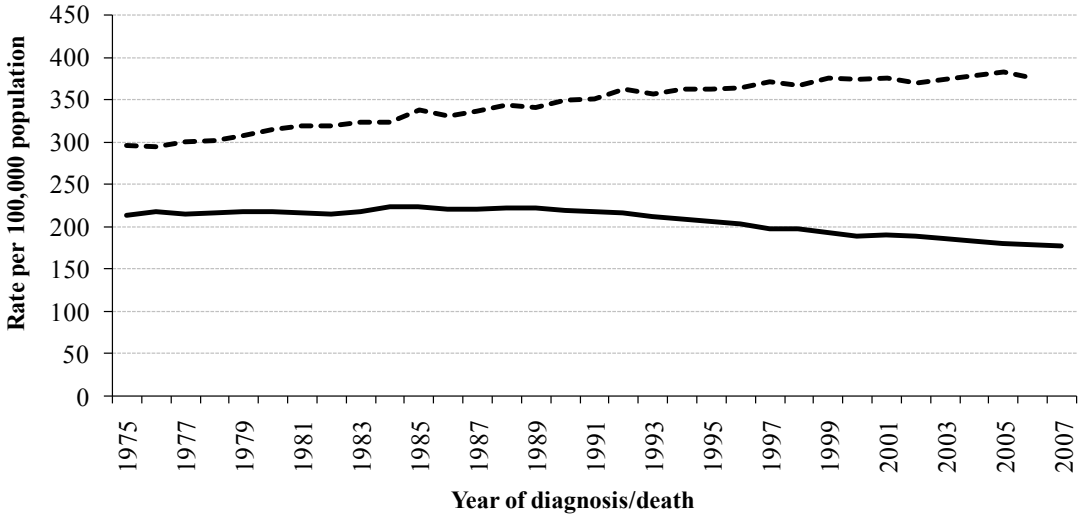


Figure 1.1-1 Age-Standardised (European) Incidence and Mortality Rates; all Cancers (excluding NMSC), GB 1975-2007.
Graph showing the trend in incidence (dashed line) and mortality (solid line) rates for all cancers, excluding non-melanoma skin cancer, in Great Britain since 1975 (Cancer research UK, http://info.cancerresearchuk.org/cancerstats/mortality/timetrends/).

1.1.2 Major Causes of Cancer

Three main causes exist for cancer; infectious agents, genetic factors and lifestyle (King, 2000). The majority of cases of cancer caused by infectious agents, such as viruses, are associated with the developing world, however, the Human Papilloma Virus (HPV) presents a worldwide risk. There is now strong molecular evidence that this sexually transmitted virus is a causative agent for cervical cancer. Although screening in women aged 25 years and over is routine in the UK, and is estimated to prevent 75% of cancer cases in women who are regularly screened, with girls becoming sexually active younger, preventative action has been taken against cervical cancer with the

introduction of a vaccination program for girls aged 12-13 years of age to vaccinate them against the HPV 16/18 (Cancer research UK). Even cancers caused by infectious agents result because of the accumulation of genetic mutations, be that single or multiple base changes, deletions or insertions, yet very few are inheritable; only 0.1% of cancers have a well defined familial genetic involvement (e.g. Retinoblastoma and Wilms'). Mutation rates differ significantly between somatic cells, for example, kidney epithelium has a mutation rate of around 250 mutations per million cells compared to blood lymphocytes, which have a frequency of around 8 mutations per million cells, giving some insight as to why the majority of cancers occur in epithelial cells (King, 2000). Cancers are clonal in their origins and require at least two 'hits' before initiation occurs, i.e. DNA damage of an oncogene (activation) followed by loss of a tumour suppressor gene (deactivation) leading to uncontrolled proliferation or in the instance of familial cancers an inheritable germline mutation, followed by a somatic mutation usually caused by an environmental factor. Environmental factors play a huge role in the accumulation of mutations. Smoking is the single largest avoidable contributing lifestyle factor leading to an increased cancer risk. Tobacco has been associated with around 14 different cancers and accounts for approximately a quarter of all deaths from cancer (29%) (Cancer research UK). Recent awareness of the risk to public health of widespread low dose exposure to carcinogens, i.e. passive smoke, has lead to the introduction of legislation, such as the smoking ban which was brought into the UK in July 2007, to minimise risk to the general public (Jamrozik, 2005). Occupational exposure to carcinogens (e.g. chemicals and radiation) and other lifestyle choices (e.g. diet, exposure to sunlight) also contribute significantly to cancer risk. Many types of cancer are believed to be avoidable by reducing your exposure to the causative carcinogens. Cancer incidences vary quite significantly between different geographical

regions, for instance, cancers such as breast, prostate and colon are mainly associated with western countries including the UK and America. In oriental countries, cancers of the stomach and oesophagus are much more prominent. The incidence of breast cancer in Japanese and Chinese women born in Asia is relatively low and compared to women born in the USA, the occurrence is 50% lower in women of Asian descent born in Asia compared to USA-born white women (Stanford *et al*. 1995). Japanese women under the age of 50 born in the USA, however, actually have similar breast cancer incidence to USA-born white women, revealing that within 3-4 generations of a person immigrating to a new country they will have adopted the cancer incidence of that country (Ziegler *et al*. 1993). This suggests environmental and lifestyle factors play a major role in cancer risk. It is therefore feasible that cancer is, in part, preventable by removing the harmful environmental influences and adopting the lifestyles of countries where incidences are lower.

1.1.3 Hallmarks of Cancer – Uncontrolled Growth

Cancers are often thought of being made up of cells that proliferate at much faster rates than normal cells. In well differentiated tumours, however, proliferation rates are not dissimilar to the progenitor normal cells. The fundamental difference between cancer and normal cells, therefore, is that cancer cells are able to evade the stop signals that ensure normal tissue stasis (King, 2000). In 2000, Hanahan and Weinberg published a paper detailing the six hallmarks of cancer (Hanahan and Weinberg, 2000); growth in the absence of growth factors, evasion of growth inhibitory signals, evasion of apoptosis, unlimited replicative potential, angiogenesis and invasion and metastasis (Figure 1.1-2). At the recent (2010) National Cancer Research Institute (NCRI) Cancer Conference, however, Doug Hanahan announced an updated list, including a number of

new hallmarks; generation of alternative energy supplies, evading detection by the immune system, genetic instability and inflammation (Hanahan, 2010). The ability of cancer cells to grow in an environment with a low supply of growth factors and in disregard of other negative environmental stimuli, such as cell to cell contact, is all part of the evasion of cancer cells to the checkpoints which make up the cell cycle. In order for all cells to divide, including cancer cells, the DNA must be replicated and distributed equally between the progeny cells. The cell cycle depicts the order in which these events must take place, along with the regulatory checkpoints that ensure that only cells that have progressed through the cell cycle in the correct manner are able to proceed.

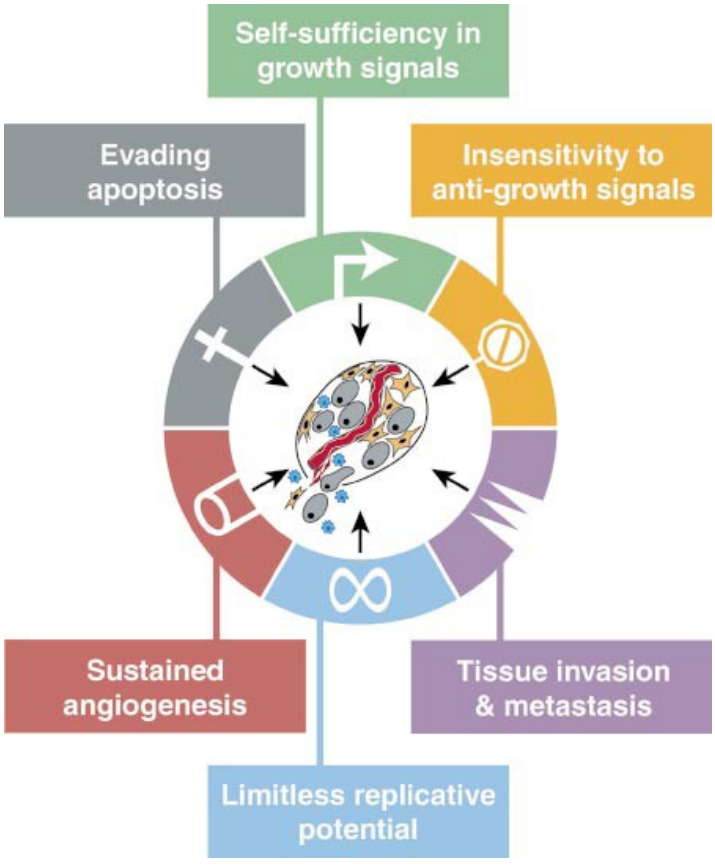


Figure 1.1-2 Hallmarks of cancer
The six essential alterations acquired by cells during the development of cancer. (Picture with permission of Elsevier and published in Hanahan and Weinberg, 2000).

1.1.3.1 Cell Cycle

Tight control is needed in order that cells undergoing cellular division distribute their cellular contents evenly between the daughter cells and to ensure division only occurs as and when necessary. In some instances it is loss of, or errors in, control of the cell cycle which leads to cancers forming. Normal cells have a finite number of rounds of cellular division after which they undergo programmed cell death (apoptosis). It is this balance between cellular division and programmed cell death which ensures tissues maintain a certain size and loss of this equilibrium which can result in cancer (King, 2000).

The cell cycle is made up of four phases; G_1 (first gap) in which amplification of the synthetic machinery occurs and the cells prepare for synthesis, S (DNA synthesis) during which synthesis of DNA and other macromolecules occurs, G_2 (second gap) where the cell duplicates its contents and reorganises to prepare for mitosis and finally, M (mitosis) where the cell divides in two. Regulation of the cell cycle is complex and involves many different molecular mechanisms. Advancements in methodologies and a better understanding of the consequences of errors in the process have progressed knowledge into the molecular controls involved in cell cycle progression (Nurse, 2000). As the processes involved in the cell cycle are too complex to be fully described here, a summary of the major events and processes involved in each phase will be given.

1.1.3.1.a Quiescence

G_0 distinguishes quiescent cells from those preparing to enter the next round cell division and actually depicts the majority of cells in a normal tissue (Murray, 1993). Quiescent cells are not actively growing, lacking the necessary molecules for cell growth, providing a way of cells to exit the cell cycle to perform the specialised

functions needed to support their tissue. In cancer cells this molecular mechanism is lacking, meaning that cancer cells never exit the cell cycle into a quiescent state but instead continue growing (Pardee, 2002).

1.1.3.1.b G_1 Phase

The first gap phase (G_1) is where the cell prepares for DNA synthesis and is the phase in which the cell commits to division, differentiation or death. G_1 is sub-divided to include G_0. The first Growth/Gap phase is the most variable phase amongst different cells. In general it is the longest of all the phases and it is this that ultimately governs the length of the cell cycle and the doubling time of cells (ranging from 6-24 hours in culture). In order for a normal cell to enter the cell cycle from G_0, nutrients (sugars, salts, vitamins and essential amino acids) and externally supplied growth factors have to be present that signal to the cell to start cellular growth (Pardee, 2002). During this growth phase there is a cascade of signals which ultimately leads to gene activation and the production of messenger RNA (mRNA) and proteins which start DNA synthesis in S phase.

One of the main protein families involved in the cell cycle are the cyclins. Cyclins complex with cyclin-dependent kinases (cdks) and together they catalyse stages of the cell cycle progression. During the cell cycle four main cyclins are produced; D, E, A and B, their ordered appearance (and disappearance) throughout the cell cycle governing whether a cell is able to proceed through the checkpoints. The first cyclin to increase in early G_1 is cyclin D which complexes with cdk4 and cdk6, triggering the synthesis of cyclin E in late G_1 (Pardee, 2002) (Figure 1.1-3). Cyclin E then activates cdk2, triggers the production of cyclin A and initiation of DNA synthesis (Pardee, 2002).

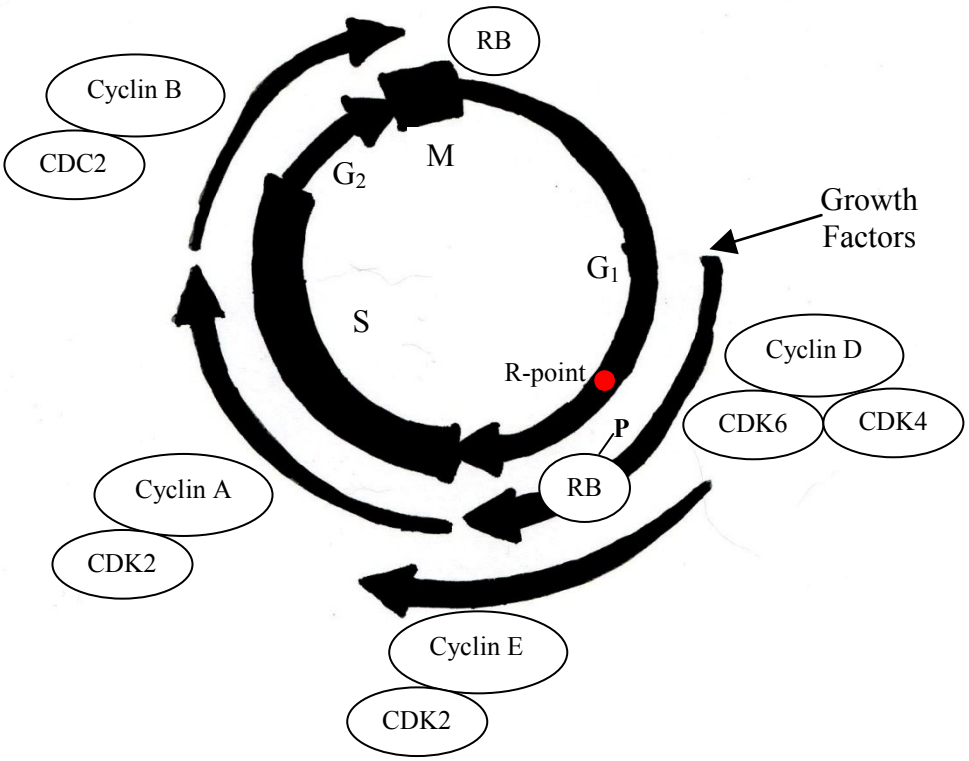


Figure 1.1-3 The Cell Cycle
Diagram depicting the order of events which make up the cell cycle and the proteins which control entry into the subsequent phase. A primary growth phase (G_1) is followed by duplication of the cells chromosomes during S phase. A second growth phase (G_2) prepares for mitosis in which the cell separates its chromosomes into two sets and finally divides into two separate cells (cytokinesis).

1.1.3.1.c S Phase

S phase is the period in the cell cycle in which the cell duplicates its DNA. This phase usually takes between 6-8 hours and by the end the cell has doubled its DNA content in preparation of mitosis (Pardee, 2002).

After a certain point during late G_1, called the restriction point (R-point), the passage of cells through the cell cycle is no longer controlled by external growth factor signals but is instead driven forward by the production of internal controls (Pardee, 2002). In order to traverse the R-point, gene activating protein E2F must be present, and active, in order to synthesis cyclin E which in turn brings about the initiation of enzymes involved in

DNA synthesis such as DNA polymerase (Murray, 1993). As well as the activation of proteins it is also vital that inhibitory proteins are removed if the checkpoints are to be traversed. Progression through S phase is dependent on the production of cyclin A (Figure 1.1-3) and also the removal of cyclins D and E by proteasomes (Pardee, 2002).

1.1.3.1.d G_2 Phase

Before a cell can enter mitosis it is first necessary that enzymatic machinery is produced and cellular contents are duplicated in order that each daughter cell receives the appropriate cell organelles, a process which takes several hours (3-4 hours). Cyclin B accumulates during S phase and increases during G_2 complexing with cdc2 (Figure 1.1-3), a cdk kinase, resulting in its phosphorylation (Murray, 1993, Pardee, 2002). It is only at the G_2/M boundary that the complex finally becomes active as another cdk kinase, cdk25, becomes active and results in further phosphorylation and activation of cyclin B/cdc2 (Murray, 1993). In addition to its activation, the complex is also relocated from the cytoplasm to the nucleus at the G_2/M boundary where it breaks down the nuclear membrane allowing mitosis to proceed (Pardee, 2002).

1.1.3.1.e M Phase

The actual process of mitosis takes less than 1 hour to complete. Mitosis is separated into sections which depict the different stages the cell goes through in this relatively short space of time. After leaving G_2, cells first enter prophase where the chromosomes condense, in late prophase, sometimes referred to as prometaphase, spindles are formed. During the second stage of mitosis, metaphase, the nuclear membrane breaks down and the chromosomes attach and align along the spindles. After aligning along the equator of the cell the chromosomes are pulled apart and each sister chromatid is pulled to

opposite poles of the cell. The final stage of mitosis is telophase in which a new nuclear membrane forms around each of the groups of separated chromatids and the chromosomes de-condense. After mitosis is complete the cells undergo cytokinesis to form two completely separate daughter cells (Simpkins and Williams, 1989).

1.1.3.2 Cell Cycle Control

During each of the phases of the cell cycle there are proteins whose role it is to block the activities of cyclin-cdk complexes by binding to the complexes and inhibiting the activating effects of the cyclins. These proteins are known as inhibitors of kinases. There are two families of inhibitors; the INK4 family specifically inhibits cdk4 and cdk6 and has four members (p16, p15, p18 and p19) and the Kip family which inhibit G_1 and S cdk enzymes and has three members (p21, p27 and p57) (Lewin, 2000) (Figure 1.1-4). Of the inhibitors, p21, is the most universal, acting on cdk2, 4 and 6 to inhibit progression through all stages of the cell cycle. When p21 expression is high, growth arrest ensues and progression through the cell cycle does not continue until its levels drop again. These inhibitors therefore play an important role in the cell cycle checkpoints providing a system of pathways and feedback mechanisms so that each phase does not begin before the previous has not only been completed, but has done so with a high level of fidelity.

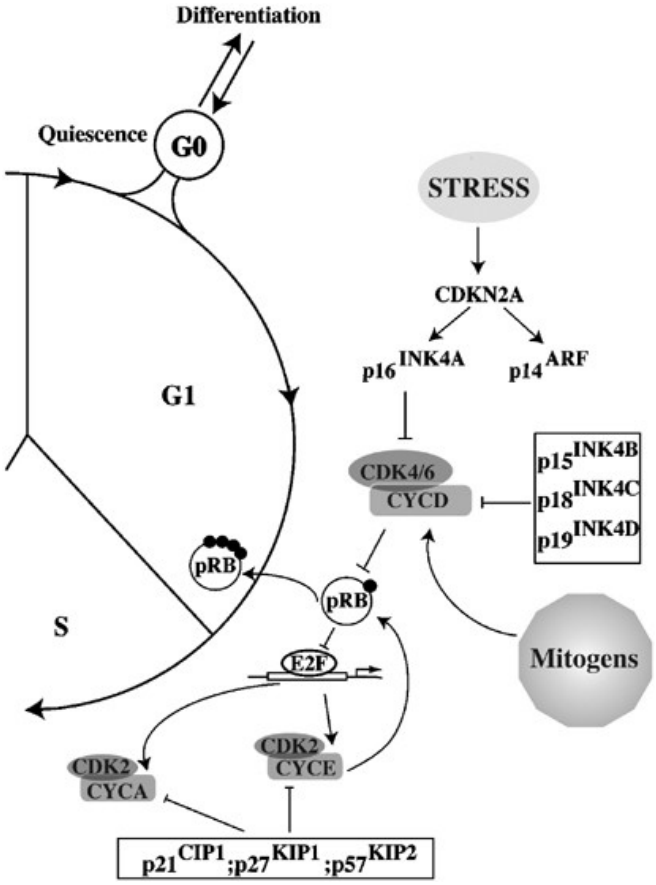


Figure 1.1-4 G_1/S Cell Cycle Control
Diagram showing the inhibitory actions of the INK4 (p15, p18 and p19) and Kip (p21, p27 and p57) inhibitors of kinases acting at the G1/S boundary (Picture reproduced with permission of Nature Publishing Group and published in Deshpande *et al*. 2005).

In order to ensure that DNA synthesis only occurs when the cell is ready, that it only occurs once and that it occurs prior to mitosis, there are a number of checkpoints at the entry and exit of S and M phases. The proteins involved in controlling these checkpoints are the same as those that are involved in damage recognition, so rather than there being a unique DNA damage pathway, it is a system of feedback mechanisms so ensuring high genetic fidelity upon replication. The first of these regulatory points is the R-point.

1.1.3.2.a Restriction Point

The R-point occurs within G_1 and defines the cells commitment to replicating its chromosomes. Exit from G_0 is dependent of the availability of nutrients and growth factors and once in G_1 in order to pass the R-point, synthesis of the unstable protein, cyclin E, must outweigh its degradation in order to be in excess of the cdk inhibitor p21. Only under optimal conditions does this occur, causing an accumulation of the protein and allowing for a cell to pass into S phase. Once past the R-point there is no longer any requirement for extracellular growth factors. In many cancers the R-point is defective providing cancer cells the freedom to enter S phase and progress through the cell cycle in the absence of growth factors, bestowing their growth advantage over normal cells, which are signalled to return to a quiescent state (Pardee, 2002).

1.1.3.2.b DNA damage-induced checkpoints

In order that normal cells can repair any mutagenic DNA damage, and avoid progressing along the path to becoming a cancerous cell, delays in the cell cycle are necessary. The term checkpoint refers to the controls that are activated in the instance of any DNA damage being detected. The checkpoints delay entry into the next phase of the cell cycle, so limiting the opportunity for any damaged cells to progress through to mitosis so passing on a deleterious mutation to daughter cells.

The first of the DNA damage checkpoints occurs at the G_1/S boundary, preventing any damaged cells from beginning DNA synthesis, the second occurs during S phase and the third and final checkpoint occurs at the G_2/M boundary and provides time for repair before cells enter mitosis (Pardee, 2004).

Several proteins are associated with checkpoint control, in particular, p53, a tumour suppressor that has been given the title 'the guardian of the genome' and is mutated in more than 50% of cancers (Pardee, 2004) and potentially non functional in the majority of others due to indirect inactivation by mutations in other proteins (Bode and Dong, 2004). The fundamental function of p53 is to act as a transcription factor activating a number of different target genes. One such gene is that of the cdk inhibitor, p21 (Zamamiri-Davis and Zambetti, 2004). As previously mentioned p21 blocks proliferation by inhibiting G_1 and S phase cdks. The G_2/M checkpoint is also controlled by similar proteins (e.g. p53) resulting in arrest due to the activation of the Cyclin B/ cdc2 complex being inhibited (Pardee, 2004, Zamamiri-Davis and Zambetti, 2004).

DNA damage is detected in cells through two main proteins; ATM (mutated in ataxia telangiectasia) and ATR (AT- and Rad3-related). ATM is thought to mainly be involved in the detection of double strand breaks (DSBs) whereas ATR detects all other types of DNA damage (e.g. stalled replication forks, bulky adducts, single strand breaks [SSBs]) (Braastad *et al*. 2004). ATM and ATR phosphorylate many different proteins that are important in cell cycle inhibition and DNA repair, one such target is p53 (Braastad *et al*. 2004).

1.1.3.2.c Mitotic checkpoints

Checkpoints also exist within mitosis to ensure that correct alignment of the chromosomes along the spindle microtubules occurs and that each daughter cell gets only one copy of each of the chromosomes. The checkpoints can be triggered by a number of factors, including unattached chromosomes and spindle damage (Braastad *et al*. 2004).

1.2 Bladder Cancer

1.2.1 Demographics and Causes of Bladder Cancer

In the UK bladder cancer is a relatively common malignancy with over 10,000 new cases diagnosed in 2007, making it the 4th commonest cancer in men and 12th in women (Cancer research UK). The main cause of bladder cancer is the presence of high concentrations of carcinogens present in the urine, e.g. specific aromatic amines and nitrosamines (Wallace, 2005). Aromatic amines can be absorbed through the skin and appear in urine as a result of metabolic processing whilst nitrosamines can be produced in the bladder by bacteria acting on urinary amines and nitrates (Wallace, 2005). As is true for many cancers, cigarette smoking is the main avoidable cause of bladder cancer, with an estimated 40% of cases in the developed world being accountable for through cigarette smoke. Cigarette smoke produces known urothelial carcinogens (Figure 1.2-1). Although the precise mechanism of how cigarette smoke causes bladder cancer is not fully elucidated, it is thought that metabolites of these carcinogens accumulate in the urine of smokers and cause direct damage to the cells of the urothelium (Leppert *et al*. 2006). Studies have also shown a link between exposure of foetuses to tobacco carcinogens *in utero* and infants through breastfeeding and the development of bladder cancer later in life (Hemminki and Chen, 2006).

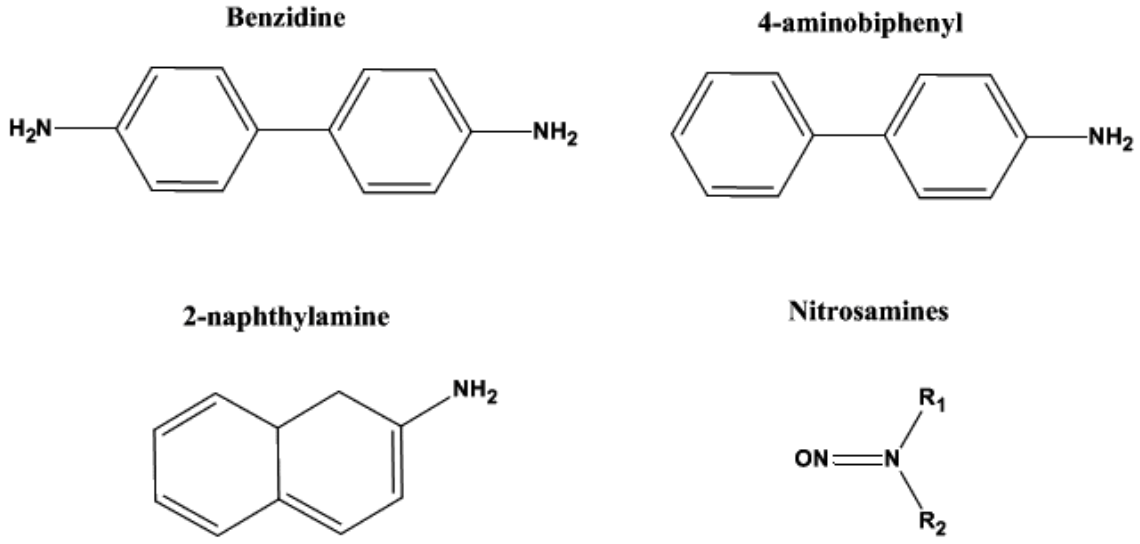


Figure 1.2-1 Urothelial Carcinogens
The structures of some of the harmful components of cigarette smoke that have been linked to bladder cancer (Wallace, 2005).

The second commonest cause of bladder cancer is occupational exposure to urothelial carcinogens. Aromatic amines were traditionally used in the production of dyes and pigments associated with the textile industry, an industry which forms a major part of Leicester's industrial heritage. Other occupations linked to increased risk of bladder cancer include truck and bus drivers, metal workers, paper and rubber manufacturers and those jobs which involve organic chemicals such as beauticians, barbers and dry cleaners (Golijanin *et al*. 2006). A 1.5 fold increase in the risk of bladder cancer has been associated with textile and rubber workers and although the association between aromatic amines and DNA damage is now apparent, due to the long latency period of 20-50 years between toxic exposure and development of bladder cancer, industrial exposure is still a risk factor. This long latency period is thought to be due to the specialised cells of the urothelium, which normally grow very slowly, allowing a substantial amount of time for the carcinogenesis process to occur. Once physically

damaged/injured, however, cells are capable of rapid proliferation which could account for the high rate of recurrence post surgery.

As well as occupational exposure to carcinogens and cigarette smoking, other risk factors for bladder cancer include age, sex and race. Bladder cancer is more common in men than women, with a sex ratio of 5:2 (male: female) (Table 1.2-1). This can, in part, be explained through higher occupational exposure to carcinogens and larger numbers of men smoking, yet in studies adjusting for these factors male risk is still higher, suggesting a genetic link between disease pathogenesis and sex (Pashos *et al*. 2002).

	England	**Wales**	**Scotland**	**N. Ireland**	**UK**
Males	5886	637	499	167	**7189**
Females	2393	249	249	49	**2940**
Persons	**8279**	**886**	**748**	**216**	**10129**

Table 1.2-1 Numbers of New Cases of Bladder Cancer During 2003
Number of new cases of bladder cancer, UK 2003 (Cancer research UK. http://info.cancerresearchuk.org/cancerstats/types/bladder/incidence/)

Incidence of bladder cancer increases exponentially with age with the majority of cases presenting in men over the age of 50, with the most at-risk age group being the 75-79 year olds (Cancer research UK). Risk is also thought to be associated with the ability of certain individuals to metabolise carcinogens (Wallace, 2005). Smokers are threefold more likely to develop bladder cancer than non-smokers with long term smokers being at highest risk (Brennan *et al*. 2000). Even 25 years after quitting, an ex-smoker is still at higher risk than the general public (Brennan *et al*. 2000). This is thought to be due to differences in the body's natural detoxifying systems that convert carcinogens into non-toxic chemicals for excretion. Genetic predisposition, determining if a person is a rapid acetylator or carries polymorphisms in genes such as CYP1A and GSTM1 making them

better metabolisers of carcinogens, may play a role in establishing if someone is more likely to develop bladder cancer or not (Cohen *et al*. 2000).

1.2.2 Bladder Cancer, Progression and Recurrence

Bladder cancer is rarely discovered incidentally at autopsy suggesting a short pre-clinical period (Foresman and Messing, 1997). Patients suffering from bladder cancer become aware of a problem mainly due to the presence of blood in the urine and an increased frequency and difficulty, due to pain, in urination. Diagnosis is then confirmed via direct visualisation of the urothelium by endoscopy. Over 90% of bladder cancers diagnosed in the UK are transitional cell carcinomas (TCC). Approximately 70% are diagnosed as non-muscle invasive bladder cancers, where the disease is confined to the mucosa (stages Ta or CIS) or submucosa (stage T1) (Figure 1.2-2).

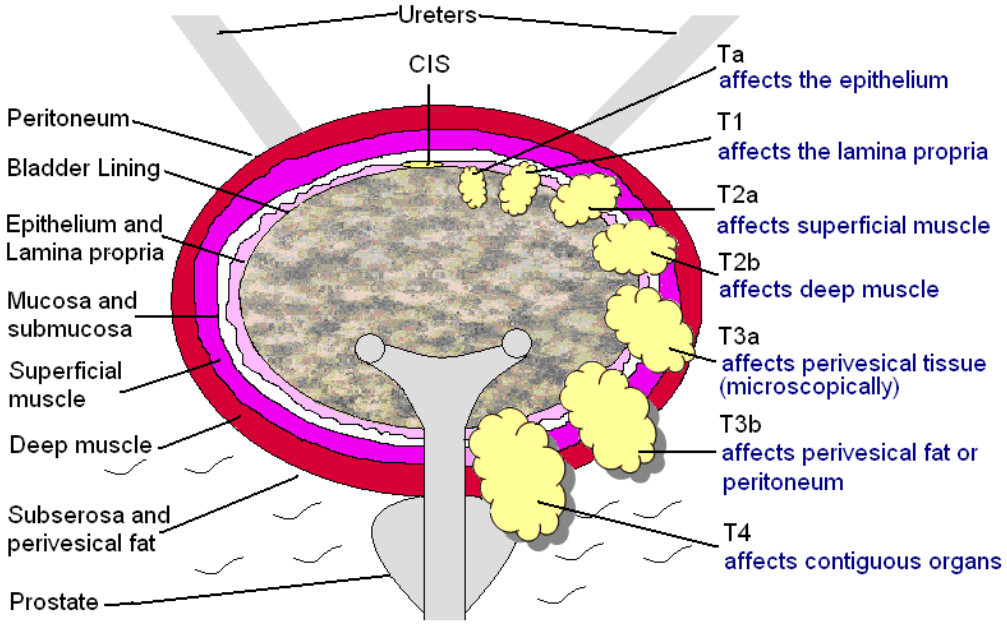


Figure 1.2-2 Pathological Tumour Staging of Bladder Cancer
The pathological staging of bladder cancer using the tumour, nodes and metastasis (TNM) system. The staging of a cancer indicates how far the tumour has spread and helps determine the best treatment regime. CIS denotes carcinoma *in situ*.

Non-muscle invasive bladder cancer, formally known as superficial bladder cancer, is readily treatable via surgical resection of the bladder (transurethral resection of the bladder tumour [TURBT]). Unfortunately non-muscle invasive bladder cancer has a high rate of recurrence (60%) with 20-30% of reoccurrences occurring as the muscle-invasive form of the disease (stages T2a-T4) a condition which is much harder to treat, often involves the lymph nodes and is linked with a higher risk of metastasis. If a patient with non-muscle invasive bladder cancer is considered at high risk of recurrence or progression then further treatment in the form of an intravesical instillations of a chemotherapeutic or immunological agents are administered post TURBT. One such class of high risk non-muscle invasive bladder cancers are the pT1G3 tumours (low stage [T1] but high grade [G3] tumours). These tumours are known to be high risk in terms of recurrence, as for most bladder cancers, but also in progression (40%). For these patients treatment options also include intravesical bacillus Calmette-Guérin (BCG), radiotherapy or cystectomy.

Upon diagnosis of bladder cancer, patients are almost always found to have multiple tumours (multifocal disease). The multifocal nature of bladder cancer and high recurrence rate has resulted in many different theories to explain the mechanisms behind this disease, these include; incomplete resection of the original tumour leading to re-growth, a new occurrence at a remote site and/or lesions that were microscopic at presentation but have subsequently become macroscopic, or implantation and subsequent growth of cancer cells shed from the original tumour during growth or as a consequence of TURBT (Brausi *et al*. 2002, Fadl-Elmula *et al*. 1999). Studies using molecular genetic analysis have been carried out to determine if secondary tumours are really monoclonal, derived from the original tumour, or polyclonal, new carcinomas.

Work by Fadl-Elmula *et al.* (1999) used chromosomal banding in samples obtained by resection of the bladder from six patients to determine the mechanism of recurrence. Amongst the six patients, primary tumours all had distinct karyotypes yet upon comparison of original vs. recurrent tumours for each patient, tumours were found to be cytogenetically identical or at very least clonally related differing only due to progression of the tumour to a higher stage (Fadl-Elmula *et al*. 1999). This, along with other studies (Chern *et al*. 1996, Habuchi *et al*. 1993, Sidransky *et al*. 1992, Xu *et al*. 1996), supports the theory of monoclonal origin and gives weight to the hypothesis that cells shed from the original tumour, either during its growth or during TURBT, are able to re-imbed into the bladder wall. In light of this evidence every patient, whether at high risk of recurrence or not, is given a single dose of intravesical agent post resection in Leicester NHS hospitals in order to reduce the rate of recurrence of bladder cancer.

1.2.3 Management of Bladder Cancer

1.2.3.1 Non-Muscle Invasive Bladder Cancer

Agents given as part of non-muscle invasive bladder cancer treatment are given intravesically, i.e. directly into bladder. A basic understanding of the pharmacokinetic principles associated with the bladder help to understand why this strategy is employed. In order for intravesical agents to work they have to penetrate through the urothelium and underlying tissue. The urothelium does not contain blood capillaries and is around 5-10 cells thick. As the agent diffuses across this barrier there is a decrease in the concentration of the drug, in a linear relationship to depth (Malmstrom, 2003, Badalament and Farah, 1997). Intravesical agents will therefore work best for non-invasive tumours (Ta, T1 and CIS) whereas systemic chemotherapy is best reserved for invasive tumours which have penetrated the urothelium into tissue containing blood

vessels and are at risk of metastasis (Wallace, 2005). Systemic chemotherapy is considered less effective in treating non-muscle invasive disease as the wider toxicity associated with systemic chemotherapy is deemed unnecessary and in order to overcome this, would have to be administered at lower doses which are further diluted in passing through the lamina propria and urothelium into the urine potentially becoming less effective than direct intravesical administration (Malmstrom, 2003). Although intravesical instillations are not free from side effects (patients can experience irritation, burning, diarrhoea and inflammation) (Pashos *et al*. 2002) toxicity is much lower as the drugs used have a high molecular weight and due to the structure of the urothelium, as already explained, very little drug gets absorbed systemically. Studies investigating single instillations of chemotherapeutic agents immediately after resection of the bladder have shown a 15-30% reduction in recurrences in low grade bladder cancers compared to surgery alone (Pashos *et al*. 2002).

The role of adjuvant intravesical therapies is to aid in the eradication of disease, minimize recurrence and prevent progression. There are two different types of agents used in intravesical therapy; immunological and chemotherapeutic.

1.2.3.1.a Immunotherapy

First line treatment for bladder cancer, in terms of immunotherapy, is BCG. BCG was originally developed as a tuberculosis vaccine and it is generally thought that the agent's ability to raise a local immune response against the tumour is responsible for its anti-tumour potential. BCG's exact mechanism of action is, at present, not completely understood, however, there is evidence pointing towards three mechanisms by which BCG may achieve its therapeutic action; 1. Chemical signals, called cytokines, are released in response to BCG which direct killer cells to destroy the tumour, 2. BCG

promotes anti-angiogenesis removing the tumours blood supply, thereby halting progression of the tumour and 3. It stimulates synthesis of nitric oxide, a chemical known to kill cells (Meyer *et al*. 2002). BCG has been used in the treatment of non-muscle invasive bladder cancer since the late 1980's and although effective it does cause some side effects and around 20% of treatments have to be halted due to these side effects (Pashos *et al*. 2002, Lodillinsky *et al*. 2010). Other immunological agents used in the treatment of non-muscle invasive bladder cancer include the interferons, interferon alpha in particular, which also work by raising a natural immune response and so directing killer T cells to destroy the tumour.

1.2.3.1.b Chemotherapy

Triethylenethiophosphoramide (thiotepa) is the oldest chemotherapeutic agent successfully used in the treatment of non-muscle invasive bladder cancer (Malmstrom, 2003). Thiotepa is an alkylating agent which forms crosslinks between nucleic acids and proteins resulting in the inhibition of DNA synthesis. Due to its low molecular weight (Table 1.2-2), however, systemic toxicity is a problem and treatment can result in serious toxicities such as myelosupression, leukopenia and thrombocytopenia (Badalament and Farah, 1997). Mitomycin C (MMC) is also an alkylating agent but is associated with far lower systemic toxicity due to its larger molecular weight, which limits absorption, as a result, many of the side effects of MMC treatment only occur locally.

The other types of compounds used for intravesical chemotherapy are anthracyclines. These are antibiotic-related agents and for the treatment of bladder cancer include doxorubicin (Dox), epirubicin (4'-epidoxorubicin) and valrubicin. Anthracyclines exert their cytotoxicity through the disruption of DNA and RNA synthesis, by binding to the

DNA helix causing conformational changes inhibiting topoisomerase II, and via the production of free radicals (Muller *et al*. 1998). Topoisomerases are enzymes involved

	Dose (mg)	Efficacy	Molecular weight (Da)	Toxicities
Thiotepa	30-60		189	**Systemic**
Mitomycin C	20-60	43% -papillary 58% carcinoma in situ	329	**Chemical cystitis**
Doxorubicin	30-100	34% - carcinoma in situ Lower response Ta or T1	580	**Chemical cystitis**
Epirubicin	30-80	60-70% carcinoma in situ	580	**Chemical cystitis**

Table 1.2-2 Intravesical Chemotherapy Agents used in Bladder Cancer
Commonly used intravesical chemotherapeutic agents used in the treatment of non-muscle invasive bladder cancer (Pashos *et al*. 2002, Malmstrom, 2003)

in the unwinding of supercoiled DNA, through the transient cutting of the DNA, in order for transcription and replication to occur. Topoisomerase II enzymes cut both strands of the DNA relaxing the DNA, allowing helicase and other enzymes access to the DNA after which the strand breaks are repaired (Hortobágyi, 1997). Anthracyclines inhibit the activity of topoisomerase II by blocking the binding of replication enzymes and inhibiting the repair of the DSBs ultimately resulting in cell death (Hortobágyi, 1997). Anthracyclines can also generate hydroxyl radicals, through electron reduction, in the presence of oxygen and metals which can interact with DNA resulting in DNA damage and as a consequence of increasing the total burden upon the cell, can induce apoptosis (Muller *et al*. 1998).

MMC is a potent anti-cancer drug, which has been used in the clinic for over 35 years in the treatment of various cancers, including bladder, gastric, lung, oesophageal and breast (Doll *et al*. 1985, Cummings *et al*. 1993, Coia, 1993, Kelsen, 1994, Schnall and Macdonald, 1993). MMC is currently the first line intravesical chemotherapeutic

treatment for carcinoma *in situ* and low grade non-muscle invasive bladder cancer (Pashos *et al*. 2002, Whelan, 2007). As mentioned above, MMC is an alkylating agent, whose main mode of action is by inhibiting DNA synthesis through the production of crosslinks, however, these agents also produce free radicals which damage DNA (Doll *et al*. 1985, Crooke and Bradner, 1976, Bachur *et al*. 1978, Begleiter, 2000). Crosslinks can either be interstrand, formed on opposite complimentary DNA strands, or intrastrand, formed on the same DNA strand; with regard to MMC the majority of crosslinks formed are interstrand. The crosslinks distort the DNA helix (Figure 1.2-3) making DNA replication and transcription impossible unless the crosslinks are removed. MMC forms crosslinks at specific sequences in DNA, preferentially forming at CG-CG sequences, via bifunctional alkylation at carbons C1 and C10 (Bizanek *et al*. 1992, Borowy-Borowski *et al*. 1990b, Borowy-Borowski *et al*. 1990a, Sastry *et al*. 1995, Tomasz *et al*. 1986). In order to react with DNA MMC needs to be reductively activated, which opens the aziridine ring, allowing interaction with the N^2 positions on guanine residues. CpG islands are areas of the genome where the frequency of the dinucleotide is relatively high and therefore these areas have a greater potential of MMC adduct formation (Borowy-Borowski *et al*. 1990b). CpG islands are normally associated with the 5' regions of housekeeping genes and ubiquitously expressed genes (Larsen *et al*. 1992), covalent modification within these regulatory regions probably accounts for MMC's potent cytotoxicity and antitumour activity.

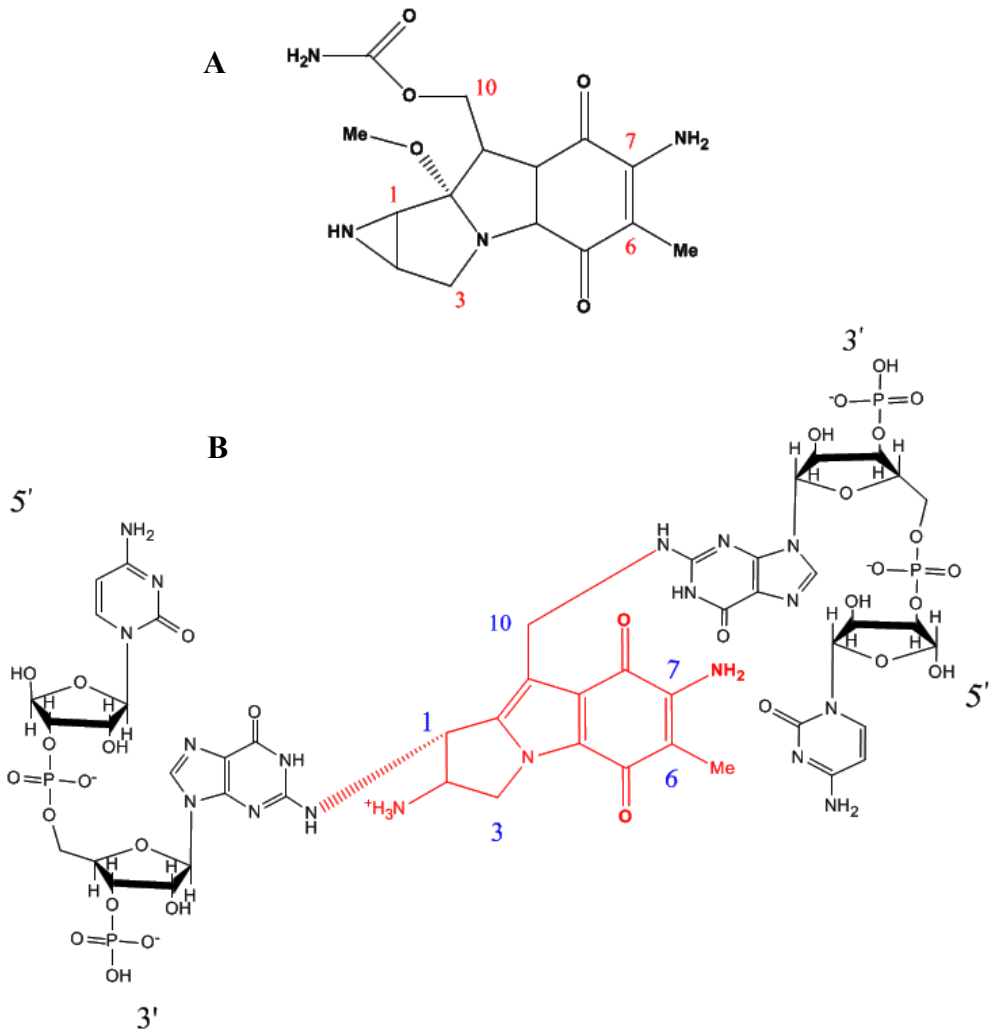


Figure 1.2-3 Configuration of DNA Binding by Mitomycin C
The formation of DNA adducts in the form of crosslinks in DNA by MMC. (A) Chemical structure of MMC and (B) Schematic showing the binding of MMC to two guanines on opposite strands of the helix (Borowy-Borowski *et al*. 1990b). MMC binds to the two guanines in a sequence specific manner (CG – GC) forming covalent bonds with the N^2 positions of the guanines through its arzidine, at C1, and carbamate, at C10, alkylating functions.

Interstrand crosslinks are repaired through a combination of nucleotide excision repair (NER) and recombination mechanisms. DNA damage recognition occurs throughout the cell cycle to ensure the integrity of the DNA is maintained at all times. MMC crosslink formation is not cell cycle specific (Badalament and Farah, 1997) but predominantly results in S phase arrest (Mladenov *et al*. 2007), which allows for their repair(Mladenova and Russev, 2006). It is thought that interstrand crosslinks distort the DNA to such an extent that during transcription the replication forks are stalled as the

replication machinery is unable to pass the crosslink. Stalled and/or collapsed replication forks instigate DNA damage repair responses resulting in an arrest of cell cycle progression and in the case of MMC, the formation of a DSB. The formation of a DSB leaves an unpaired 3' end available for XPF•ERCC1 and RPA, repair factors involved in NER, to bind to and degrade one strand of the DNA in a 3'-5' direction past the crosslink. Removal of the surrounding nucleotides releases the crosslink and generates a gap, this is recognised by recombination proteins which create a Holliday junction intermediate which is then resolved by MUS81•MMS4 complex and the crosslink removed via NER (Sancar *et al*. 2004) (Figure 1.2-4).

If MMC-induced crosslinks are not removed then ultimately the cell dies. Direct induction of apoptosis in residual malignant tumour cells is thought to be the main mode of action by which MMC reduces recurrence rates in bladder cancer. It is also possible that MMC could prevent the reseeding of floating tumour cells, present after TURBT, through its cytotoxic effects (Zincke *et al*. 1983). Due to its high molecular mass and therefore low systemic absorption rate, MMC can be administered as a single instillation relatively early after resection (~6 hours). At present there is no standard treatment regime for early instillations of MMC but in general it is administered in 20-60mg doses and is retained in the bladder for 1-2 hours.

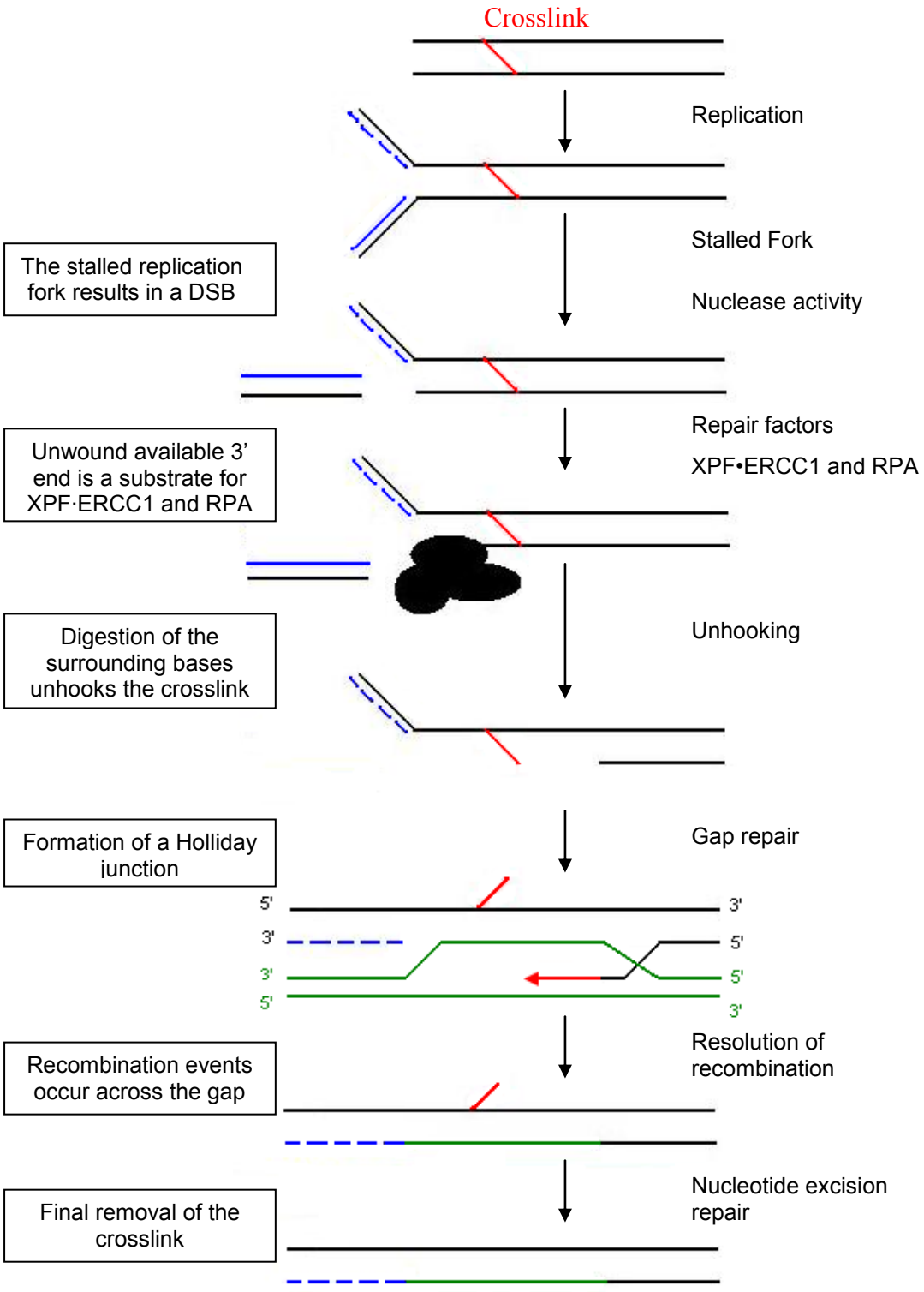


Figure 1.2-4 Interstrand Crosslink Repair
Repair of interstrand crosslinks via recombination mechanisms and NER (adapted from (Sancar *et al*. 2004, McHugh *et al*. 2001).

1.2.3.2 Muscle Invasive Bladder Cancer

In America radical cystectomy remains the gold standard therapeutic option for patients with muscle invasive bladder cancer as it provides local control (for T2-T4 tumours) (Hassan *et al*. 2005) with disease-free survival at 5 years being 40-60% (Efstathiou *et al*. 2006). However, removal of the bladder and neighbouring organs does present complications and some patients are too ill to undergo such an invasive operation. It is also argued that in some cases cystectomy presents too big an impact on the patient's quality of life and that bladder preservation techniques, such as radiotherapy and/or chemotherapy, should be utilised (Hassan *et al*. 2005).

1.2.3.2.a Radiotherapy as a Bladder Preservation Technique

Radiotherapy (RT), as a single modality therapy, applied with curative intent, has been used in invasive bladder cancer treatment since the 1950's and was used extensively until the 1980's (Troiano *et al*. 2009). Comparisons between RT and radical cystectomy outcomes are difficult as patients are often selected for treatment based on the stage of their tumours, age and general health. A retrospective study of 459 patients who underwent RT at an Italian institution between 1980 and 1998 found that 5-year survival for RT alone (dose of ≥60 Gy) was 36%. However, patient age, stage and grade of tumour at presentation had a significant impact on overall survival (Tonoli *et al*. 2006). Although RT alone has been shown to give equivalent 5-year survival rates to that of pre-operative RT with radical cystectomy (Bloom *et al*. 1982, Sell *et al*. 1991), patients undergoing RT alone do suffer recurrence (~50%) and ultimately have to undergo salvage cystectomy (Jenkins *et al*. 1989, Quilty *et al*. 1986). Standard treatment schedules for RT involve 2-3Gy fractions of radiation, ultimately exposing the cancer to a total of 60-66Gy over 6.5 weeks (Petrovich *et al*. 2001). Bladder

radiotherapy dose, in bladder sparing therapy, is limited due to the bowel and bladder both being included in the radiation field. One potential solution is radiosensitizers. Radiosensitizers are agents that are designed to enhance radiation-induced tumour cell killing whilst having little or no effect on normal tissue, therefore, giving a therapeutic differential effect. To date there are only two types of classical sensitizers (that enhance DNA damage) that give a differential effect and have been investigated clinically, halogenated pyrimidines and hypoxic-cell sensitizers, however the vast majority of these have failed clinically due to increased normal tissue toxicity (Hall and Giaccia, 2006). More recent advances into radiosensitizing tumours has involved using regular chemotherapeutic drugs (Candelaria *et al*. 2006). Although advantages have been documented using these (Seiwert *et al*. 2007), their use is limited, mainly due to the associated side effects and toxicities of the drugs themselves on normal tissues.

1.2.4 Models of Non-Muscle Invasive Bladder Cancer

It is now commonly considered that two pathways exist to give rise to the two distinct biological phenotypes of bladder cancer each with their own fingerprint of genetic mutations; non-muscle invasive disease, which is low grade, has a high level of recurrence (60%) but low progression (20-30%), and can be classed as the less aggressive, compared to muscle invasive disease which is thought to develop from CIS and have a higher malignant potential (high progression, metastasis and poor response and survival rates). A number of genetic alterations associated with bladder cancer have been identified, notably p53 and retinoblastoma (RB) (Cote and Datar, 2003). Many of the genes identified have been linked to cell cycle control in particular the G_1/S boundary. Although loss of function of both p53 and pRb have been associated with poor prognosis, i.e. muscle invasion and metastasis, no distinct pattern of alterations has

been able to model the two different phenotypes and so provide a combination of prognostic markers that could predict clinical outcome.

A number of established cell lines have been developed from human bladder cancer tumours representing different grades and stages of the disease, however, the success rates for developing cell lines from primary tumours are very low and depend greatly on the ability of the original tumour to adapt and survive in the cell culture environment (Crallan *et al*. 2006). Characterisation of a number of human urothelial cell lines was carried out by Masters *et al*. (1986). Of the twenty two cell lines studied, three were derived from 'normal' urothelium, however, the authors warned of their sole use as models for normal urothelium due to their abnormal ability to grow indefinitely in culture. Of the remaining cell lines derived from bladder tumours, only one (RT4), was unique in origin and from a patient with low grade (G1) and stage (T2) bladder cancer (Masters *et al*. 1986). In this study three cell lines have been chosen which best represent the characteristics of transitional cell carcinomas of non-muscle invasive bladder cancer.

1.2.4.1 RT4, RT112 and HT1376 Cell Lines

As previously mentioned RT4 is the only cell line, to our knowledge, that was originally established from a tumour that was well differentiated and non-invasive. RT112 and HT1376 cell lines were both established from female patients who had poorly differentiated (G2 and G3 respectively) tumours. In RT112 the clinical stage of disease was not recorded, however others have stated it to be T1 stage tumour (Gerby *et al*. 2007), HT1376 was classed as T2 (minimum). In xenograft models all three of these cell lines were all found to develop tumours which were similar in histopathology and grade to the original description from the parent tumour (Masters *et al*. 1986).

1.2.5 Future Strategies for Tackling Bladder Cancer.

Although treatments including surgery, radiotherapy and chemotherapy, have been successful in the management of some cancers, current treatments have in many cases reached their potential and advances in new cures are costly and more often targeted, so only beneficial to a limited number of individuals. Some cancers are also associated with problems such as difficulty in early diagnosis or high levels of recurrence resulting in higher incidences. Avoiding the onset of disease is preferable to dealing with the consequences of its development, it is therefore widely accepted that prevention is better than cure; nevertheless the complete elimination of carcinogens from society is neither realistic nor likely or practical and so intervention in the form of chemoprevention strategies are being considered promising approaches in tackling the incidence of cancer (Tanaka, 1997). In particular, compounds found naturally in food (e.g. phytochemicals, chemicals found in plants that are not needed for nutrition (Surh, 2003)) and therefore present in many individuals diets already, provide an attractive option, as these compounds should be suitable for long-term administration due to a good safety profile.

1.3 Chemoprevention, a Strategy for Tackling Cancer Incidence

Chemoprevention is the use of chemical agents, drugs or food supplements to prevent disease, in particular cancer, by inhibiting or retarding the progression or development of disease through a number of different mechanisms. Cancer is now known to develop through a multi-step process involving four distinct phases: initiation, promotion, progression and metastasis. Chemopreventive agents can act at any of these different stages; however, it is far more realistic that patients with premalignant disease at a high risk of cancer, or individuals at high risk due to other reasons, such as a familial

mutation, will comply with long term administration of a chemopreventive agent to prevent the promotion and/or progression of disease than a healthy disease free individual will comply in preventing its onset, which is why much of the chemopreventive research has been carried out in healthy high-risk individuals with agents that target preventing the promotion or progression of disease (Arun *et al*. 2010, Duffield-Lillico *et al*. 2009, Kim *et al*. 2010, Chow *et al*. 2010).

If an agent is to interfere with the initiation of disease then it must have some effect on the processes involved in carcinogen activation, either by interfering with the uptake or transport of the carcinogen, impeding metabolic activation or preventing interaction of the activated carcinogen with its target. Once the carcinogen is metabolised, chemopreventive agents must be targeted towards inhibiting the promotion or progression of the effects of the carcinogen i.e. inhibitors of oxidative damage resulting from genomic instability (Tanaka, 1997).

The time frame between initial exposure to a carcinogen and development of cancer can be years, therefore, any chemopreventive agent designed to act by delaying or preventing the process of carcinogen activation needs to be safe enough to be administered long term. Toxicity, as part of therapies aimed to increase life expectancy or cure disease in individuals suffering from advanced malignancy, is accepted as a trade off. However, individuals who are identified as being at high risk of developing cancer due to epidemiological data or genetic risk factors and are otherwise 'healthy', treatment regimes need to be risk free in order to ensure the benefits are worthwhile. In recent years evidence has arisen to suggest aspirin may have a role to play in reducing the risk of some kinds of cancer, alongside it's know benefits for people at high risk of heart attacks or stroke (Sanmuganathan *et al.* 2001). A recent publication by Rothwell

et al. (2010) analysed the results from eight different randomised trials involving patient treatment with daily doses of aspirin. The authors concluded that individuals who were taking daily aspirin had a reduced risk of death from cancer, with benefits being evident after 5 years of follow-up (Rothwell *et al.* 2010). In the six trials where the site of the primary cancer was available, aspirin reduced numbers of deaths was most evident in gastrointestinal solid tumours after 5 years follow-up (HR 0.46, p=0.003), with significant reductions in colorectal and pancreatic cancer. Non-gastrointestinal cancers were found to have a non-significant reduced risk of death due to aspirin allocation (HR 0.76, p=0.12), with bladder and kidney cancer having a HR of 1.28 (p=0.70) after 5 years follow-up (Rothwell *et al.* 2010). Aspirin has been hypothesised to reduce the incidence or growth of pre-cancerous lesions through the inhibition of cyclo-oxygenase (COX) enzymes. Clinical data from trials using selective cyclo-oxygenase 2 (COX2) inhibitors to prevent colorectal cancer showed that chemoprevention with rofecoxib at 25mg per day for 18 months actually increased the number of cardiovascular events (heart attack and stroke) in patients (Bresalier *et al*. 2005). Therefore, ensuring a chemopreventive agent is safe to be administered long term to healthy individuals forms one of the major issues in investigating new potential chemopreventive agents and has led to the recent interest in phytochemicals rather than synthetic compounds (Brenner and Gescher, 2005).

1.3.1 Chemoprevention using Dietary Agents

Whilst the evidence for tobacco increasing cancer risk is now overwhelming, evidence of the beneficial effect that fruits and vegetables have in lowering our risk of cancer is still under investigation. The 2003 national diet and nutrition survey (NDNS) found that British men and woman were eating on average less than three portions of fruit and

vegetables a day, compared to the recommend five (Henderson *et al*. 2003). With only 14% of the British population eating the recommended daily intake of fruit and vegetables, compared to 26% who smoke (Cancer research UK.), a national campaign to get people healthier was undertaken. This campaign has had a positive impact on the public's attitude to diet and in the most recent NDNS it was shown that a third of all men and women aged between 19-64 years old were eating the recommended '5-a-day' fruit and vegetables, with an average of 4.4 portions a day (Bates *et al*. 2008/2009). Amidst this a new term was coined for the labelling of some foods; broccoli, blueberries, olive oil, which were believed to have positive effects on health. These 'superfoods' gained popularity mainly due to their claims of possessing powerful antioxidant capabilities. New legislation, which came into effect in July 2007, now prohibits the use of such terms by food companies unless they are based on scientific evidence (FSA. 2007).

A study investigating the anti-proliferative effects of thirty four different vegetables in various different cancer cell lines found that cruciferous vegetables (cabbage, kale, brussel sprouts, broccoli) as well vegetables such as garlic and onions possessed powerful inhibitory effects (Boivin *et al*. 2009). The cancer chemopreventive properties of some dietary sources have been narrowed down to individual constituents such as epigallocatechin gallate (EGCG) in green tea, sulphoraphane in broccoli, diallyl sulphide in garlic, curcumin in turmeric, genistein in soya and anthocyanins in fruits and berries. Studies investigating the effect of pure constituents derived from dietary sources have so far been inconclusive and have lead to further investigations in order to understand the mechanisms of action of these constituents.

1.3.2 Mode of Action of Chemopreventive Agents

There are a numerous different phytotchemicals that have been identified from dietary sources that have chemopreventive properties in preclinical models (reviewed by Surh, 2003). These agents are believed to have many different mechanisms of action including antioxidant, anti-mutagenic, anti-inflammatory and anti-carcinogenic (Shureiqi *et al*. 2000) to bring about various different cellular and molecular events effecting, DNA repair, cell cycle progression, apoptosis, cell proliferation, expression and/or functional activation of oncogenes and tumour-suppressor genes, and metastasis (Surh, 2003).

Carcinogenesis is driven by damage to DNA and therefore control of that damage is of upmost importance in the chemoprevention of cancer. As well as the exogenous DNA damaging agents present in our environment, endogenous oxidative DNA damage is thought to play a significant role in the development of cancer. Reactive oxygen species (ROS) are by-products of mitochondrial respiration and can attack DNA to form oxidised bases, such as 8-oxo-guanine, which have altered base pairing properties and can result in mutagenesis (Evans *et al*. 2004, Cadet *et al*. 2003). During aerobic respiration, oxygen undergoes electron reduction resulting in free electrons as part of the electron transport system of mitochondria, to produce the ROS superoxide anion, O_2^-. Oxidation of superoxide, to form oxygen again, requires a catalyst in the form of an oxidised metal ion e.g. Iron (III) ion or Copper (II) ion. Superoxide can also be converted into hydrogen peroxide, catalysed by superoxide dismutase, which can then take part in the Fenton reaction to produce the highly reactive hydroxyl radical OH· (Collins, 1999) (Equation 1.3-1).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{+}$$

Equation 1.3-1 Fenton Reaction
Conversion of hydrogen peroxide to hydroxyl radicals catalysed by metal ions (copper and iron).

The formation of hydroxyl radicals in superoxide radical generating systems has given rise to the Haber-Weiss reaction which, if catalysed by a transition metal ion, can occur *in vivo* (Equation 1.3-2). As copper plays a role in the attachment of DNA to the nuclear matrix, OH· is likely to be produced in very close proximity to DNA, making the potential for DNA damage extremely high

$$Fe^{3+} + O_2^{-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+}$$

Net reaction: $0_2^{-} + H_2O_2 \xrightarrow{Catalyst} O_2 + OH^{\cdot} + OH^{-}$

Equation 1.3-2 Iron Catalysed Haber-Weiss reaction
The formation of the highly reactive hydroxyl radical OH· from the relatively unreactive superoxide O_2^{-} catalysed by transitions metal ions such as iron.

Protection against the damaging effects of ROS occurs in three main ways: enzymatic inactivation, prevention of the incorporation of damaged bases into DNA and repair of oxidative damage. The prevention and repair of oxidative DNA damage has been extensively reviewed elsewhere (Evans *et al*. 2004, Slupphaug *et al*. 2003). As mentioned earlier the role of dietary constituents as chemopreventive agents has frequently been associated with their antioxidant potential in preventing the formation of ROS and protecting against its DNA damaging effects (Gerster, 1993). Biomarkers of DNA damage or mutation are often used in epidemiological studies as markers of carcinogenic risk. Measurement of oxidised DNA can be achieved using a number of

different techniques, one of which is the modified enzymatic comet assay. This modified version of the comet assay allows for the detection of oxidised pyrimidines in DNA which can be used as a marker of oxidised DNA damage (Collins *et al*. 1997). In a molecular epidemiology study by Duthie *et al.* (1996) participants were given dietary supplementation with antioxidants for up to 40 weeks. During the period of supplementation the participant's antioxidant levels were measured along with markers of DNA damage using the comet assay. The investigators found that after 20 weeks of supplementation with α-tocopherol, β-carotene or ascorbate, antioxidant levels in both smokers and non-smokers were significantly higher compared to the placebo group; this correlated with measures of oxidised bases which were significantly lower, indicating these antioxidants were able to protect against endogenously produced ROS. Duthie *et al*. (1996) also showed that lymphocytes from participants in the supplementation arm of the study were able to show an increased resistance to *in vitro* oxidative DNA damaged when challenged with hydrogen peroxide compared to those isolated from volunteers in the placebo arm. Although evidence for agents such as anthocyans being used as chemopreventive agents is increasing, patients or healthy individuals are not yet advised to take these regularly for the prevention of cancer mainly due to the fact that these agents haven't been investigated thoroughly enough yet and there is insufficient clinical evidence to make any recommendations (Brown *et al*. 2001, Seifried *et al*. 2003). A stark example of this was highlighted in the recent Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study in which male smokers received daily supplementation with α-tocopherol, β-carotene, both agents or placebo for 5 to 8 years (ATBC. 1994). At follow up, it was found that there was a higher incidence of lung cancer and higher mortality for participants who had received β-carotene (Virtamo *et al*. 2003). Mayne *et al*. (2001) also found an association between β-carotene

supplementation and increased risk of lung cancer in patients who had recently been treated for stage I or II squamous cell carcinoma of the tongue, gum and mouth and deemed free of cancer at the start of the intervention. There is also a potential risk that these compounds may interfere with subsequent therapies but this has been little studied

1.3.3 Chemopreventive Agents in Bladder Cancer

Non-muscle invasive bladder cancer, due to its high rates of recurrence which are associated with frequent visits to the hospital for check-ups, is one of the most expensive cancers to treat (Botteman *et al*. 2003). In the UK during 2000-2002 the cost of management of bladder cancer per patient was on average £8349, with the total costs for non-muscle invasive disease equating to £35.25 million, a staggering £6943 per patient (Sangar *et al*. 2005). In comparison to prostate cancer another urological cancer and the most common cancer in men, bladder cancer is £1055 more expensive to treat per patient, yet in relation to the amount spent on research into the diseases, prostate cancer research receives five times the amount of money compared to bladder (Sangar *et al*. 2005). Bladder cancer is, however, a good candidate for chemopreventive research as a reduction in recurrence of non-muscle invasive disease could help save the NHS a significant amount of money as the major cost burden of this disease is associated with the regular cytoscopies which are required for adequate follow-up (Sangar *et al*. 2005).

1.3.3.1 Analysis of Agents to be used as Chemopreventive Agents in Bladder Cancer

Since the excretion and concentration of many genotoxic compounds occurs in the urine, chemoprevention of bladder cancer is a rather logical approach to lowering its incidence and recurrence. Primary and secondary chemoprevention are concerned with

the prevention of the disease; blocking or metabolising an initiating carcinogen, as well as the slowing of promotion and progression of premalignant or early stage malignancies in normal or high-risk individuals (Golijanin *et al*. 2006). Chemoprevention in patients who have already undergone successful treatment for a malignancy, but are at high risk of recurrence, is referred to as tertiary chemoprevention (Sharma *et al*. 2001). A number of agents have been evaluated clinically for their use as bladder cancer chemopreventive agents, a few agents will be highlighted here, however for a full review see Busby and Kamat (2006).

1.3.3.1.a Vitamins

Patients with bladder cancer have been shown to have lower levels of vitamins A, C and E (Yalcin *et al*. 2004). These vitamins are known antioxidants able to scavenge free radicals and prevent DNA adducts from forming (Sram *et al*. 2009). Vitamins C and E have both shown positive results in epidemiological studies in reducing bladder cancer incidence (Michaud *et al*. 2000, Bruemmer *et al*. 1996, Castelao *et al*. 2004). A clinical study using etretinate, a vitamin A analogue, showed that adjuvant treatment after TURBT could significantly increase the time taken to a second recurrence in patients with Ta-T1 bladder cancers compared to those in the control arm (Studer *et al*. 1995).

In a study by Lamm *et al*. (1994) recurrence rates were compared between patients treated with BCG with recommended daily allowance (RDA) of vitamins A, B_6, C, E and zinc or megadose vitamins (RDA plus 40,000 IU of vitamin A, 100mg vitamin B_6, 2000mg vitamin C, 400 IU of vitamin E and 90mg zinc). Five year recurrence rates showed that megadose vitamins produced a significant decrease in tumours compared to RDA and provides evidence of a secondary chemopreventive role of multivitamins (Lamm *et al*. 1994).

1.3.3.1.b Fruits and Vegetables

Flavonoids occur naturally in foods derived from the plant kingdom and have been shown to have a range of biological activities (Ren *et al*. 2003a). Studies have shown that a diet rich in fruits and vegetables, especially cruciferous vegetables, can be linked with a decreased risk of bladder cancer (Nagano *et al*. 2000, Michaud *et al*. 1999, Nomura *et al*. 1991). *In vivo,* isothiocyanates have been shown to significantly inhibit bladder cancer development in rats exposed to *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) (Munday *et al*. 2008). Similarly tomato juice has been shown to decrease the number of tumours in rats exposed to BBN (Okajima *et al*. 1998). *In vitro*, resveratrol has been shown to be able to inhibit proliferation, regulate cell cycle proteins and induce apoptosis in the T24 bladder cancer cell line and in a xenograft model using T24 cells, resveratrol also inhibited tumour growth (Bai *et al*. 2010).

One class of flavonoids currently under investigation for their potential chemopreventive effects in bladder cancer are the anthocyans, constituents of fruits and berries responsible for the bright red or blue colour, which have been found to inhibit chemical-induced carcinogenesis and interfere with the promotion and/or progression of malignancies in preclinical models (Hagiwara *et al*. 2001, Harris *et al*. 2001, Mutanen *et al*. 2008).

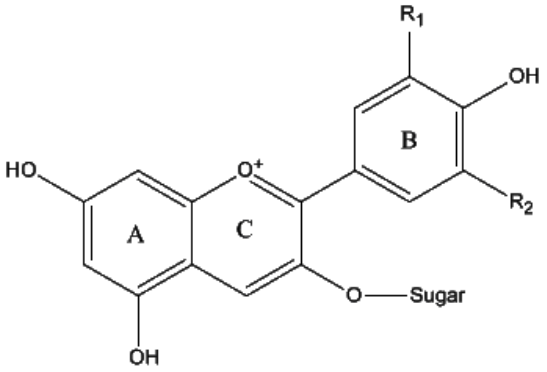
1.3.3.2 Anthocyans as Chemopreventive Agents

The majority of human tumour cells have low antioxidant enzyme levels and in particular are susceptible to oxidative damage by hydrogen peroxide due to low catalase and glutathione peroxidase levels (Oberley and Oberley, 1997), thus making cancer cells more sensitive to ROS. Anthocyans have been shown to induce certain

detoxifying enzymes *in vitro*, potentially improving an individual's ability to deal with carcinogens and increase the antioxidant potential of cells, decreasing the likelihood of ROS-induced DNA damage (Madhavi *et al*. 1998).

To date little work has been reported investigating the differential effects of anthocyans on DNA damage and cell growth in cancerous verses normal cells, but a few studies have shown that they inhibit cancer cell growth more strongly (Duthie, 2007); this could be due to cancer cells being more sensitive to ROS due to low antioxidant enzyme levels and therefore more susceptible to the antioxidant activities of anthocyans. Bladder cancer provides an excellent candidate for investigations of the chemopreventive effect of anthocyanins as just half an hour after administration they have been detected in urine of rodents (Talavéra *et al*. 2006) where they can be stored in the bladder for up to 5 hours.

Anthocyan is the collective name for anthocyanins (glycoside) and anthocyanidins (aglycon) (Figure 1.3-1). Anthocyans occur in fruits and vegetables and are also used as natural food colourings in products such as jams and squash (Bridle and Timberlake, 1997). Among the different dietary sources of anthocyanins, bilberry (*Vaccinium myrtillus* L.) has one of the highest contents of anthocyanins per Kg of fresh fruit (Prior *et al*. 1998). Berry extracts used *in vitro* in bacteria have proved to be anti-mutagens (Ames test) and in cultured cells have shown to inhibit DNA damage (sister chromatid exchange (SCE) assay), cell proliferation and malignant transformation and metastasis (Duthie, 2007) yet how they achieve this is unknown. One mechanism by which these compounds are believed to work is by reducing or inhibiting the promotion or progression of malignancy by reducing the amount of DNA damage present in cells (Ren *et al*. 2003b, Lopaczynski and Zeisel, 2001).



	R_1	R_2
Cyanidin	OH	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3
Pelargonidin	H	H

Figure 1.3-1 Structures of Anthocyanins
The structures of some of the anthocyanins found in higher plants

Anthocyanidins act as potent antioxidants by scavenging free radicals and through the inhibition of lipid peroxidation (Talavéra *et al*. 2006). Lipid peroxidation is the formation of fatty acid radicals and lipid hydroperoxides through the reaction of fatty acids with ROS resulting in DNA damage which can be mutagenic, genotoxic and cytotoxic (Talavéra *et al*. 2006). Flavonoids have been shown to be capable of scavenging free radicals including lipid peroxyl radicals (LOO·) and hydroxyl radicals (Lopaczynski and Zeisel, 2001).

The biological effect of anthocyans relies, in part, on their bioavailability. Anthocyanidins are anthocyanins without the sugar moiety and can be considered anthocyanin degradation products (Kern *et al*. 2007). Yet anthocyanidins have been shown to have greater potency in reducing cell survival than their glycosylated counterparts (Golijanin *et al*. 2006, Cooke *et al*. 2005). Wu *et al* (2007) demonstrated that a bilberry extract was able to decrease cell proliferation and induce apoptosis in the human colorectal cancer cell line HT29, at concentrations equivalent to 64µg/ml anthocyanins (10mg/ml bilberry extract) (Wu *et al*. 2007). A study by Talavéra *et al.* (2006) investigating the bioavailability of bilberry anthocyanins, concluded that the antioxidant capacity seen in plasma of rats fed on bilberry extract must be attributed to

anthocyanin metabolites or degradation products due to the absence of detectable anthocyanins in the plasma. Another interesting finding was that the anthocyanins were quickly absorbed and excreted into the urine (Talavéra *et al*. 2006). In a study by Cooke *et al*. (2006) APC^{Min} mice received cyanidin-3-glucoside (C3G), an anthocyanin purified from blackberries, or mirtoselect, a commercially available extract made up of 15 different anthocyanins, as part of their diet for 12 weeks to determine the effects these agents had on the development of intestinal adenomas. The investigators found that both C3G and mirtoselect were able to significantly reduce the number of adenomas but interestingly levels of anthocyanins were higher in the urine of the mice (7.2 and 12.3 µg/ml) compared to intestinal mucosa (0.043 and 8.1µg/g tissue). These anthocyans and in particular the commercially available bilberry extract mirtoselect, are now being investigated, in this study and another PhD project in this group, for their suitability as chemopreventive agents in bladder cancer in an attempt to reduce the high rate of recurrence and progression associated with the disease.

1.3.3.2.a Mirtoselect

Mirtoselect (Indena, Italy), a commercially available bilberry extract, contains a mixture of 15 different anthocyanins (25% anthocyanins), with delphinidin 3-D-arabinoside being the most abundant; mirtoselect contains mainly delphinidin and cyanidin with lower levels of peonidin. Some studies have shown that higher levels of delphinidin and cyanidin make an ideal mixture for the inhibition of cancer cell growth (Yi *et al*. 2005), whereas others believe it is the concentration of anthocyanins rather than composition which determines an extracts effectiveness (Wu *et al*. 2007). The benefits seen from a diet rich in fruits and vegetables might well be a result of the different vitamins and phytochemicals acting together, in potential synergy, rather one single

agent having an overall beneficial effect. As different anthocyanins have shown to have different effects depending on the cell line investigated (Reddivari *et al*. 2007, Lazze *et al*. 2004), it is felt a standardised mixture of anthocyanins such as that in mirtoselect, provides an opportunity to determine the potential combined effects of these different anthocyanins which could in combination have greater efficacy than as single agents. In the study by Cooke *et al*. (2006) mice fed a diet containing mirtoselect had levels of excreted anthocyanins in their urine (12.3µg/ml) (Cooke *et al*. 2006), which are capable of causing a 50% growth inhibition in colorectal cancer cells (Cooke unpublished data), and equates to a dose of 50µg/ml mirtoselect (based on the anthocyanin concentration of mirtoselect being 25%). Once potential mechanism by which mirtoselect is able to exert its growth inhibitory effects is through the suppression of protein tyrosine kinase activity of receptor tyrosine kinases (RTKs) (Teller *et al.* 2009). RTKs are receptors made up of extracellular ligand binding, *trans*-membrane and intracellular domains and are thought to have a crucial role in carcinogenesis and tumour progression. RTKs exist as monomers, forming dimmers upon ligand binding, which in turn initiates the protein tyrosine activity of these receptors bringing about autophosphorylation of specific tyrosine residues in the intracellular domain of the receptors. Once phosphorylated a cascade of signalling ensues which drives gene expression (Olayioye *et al.* 2001). One important example of a RTK in bladder cancer is the ErbB-family which includes the epidermal growth factor receptor (EGFR) and ErbB2-4. The presence of the EGFR in particular has been associated with high tumour stage and grade in bladder cancer and has been demonstrated to be a good predictor of tumour progression and poor long-term survival (Lipponen and Eskelinen, 1994, Mellon *et al.* 1995). Downstream signalling events of EGFR involves the mitogen-activated protein kinase (MAPK) pathway MAPK pathways have been associated with various different cellular responses

including cell growth, differentiation and apoptosis, resulting in the phophorylation of a number of transcription factors including, but not limited to, c-Jun, c-Fos, CREB, and Estrogen receptor (ER) (Baker and Reddy, 2004). Anthocyanins and anthocyanidins (including mirtoselect) have been shown to be able inhibit the EPGR in cell free systems and intact cells (Meires *et al.* 2001, Fridrich *et al.* 2008, Teller *et al.* 2009) as well as interfere with the activity of downstream signalling elements (Fridrich *et al.* 2008) and inhibit the phosphorylation of downstream transcription factors (Meiers *et al.* 2001) via MAPK pathway, therefore suggesting a role for anthocyans in interfering with the signalling events involved in cellular proliferation.

1.3.4 Chemoprevention in Combination with Cancer Treatments

Chemotherapeutic agents such alkylating agents and platinum compounds create DNA damage by initiating free radicals. Mechanistically one way chemopreventive agents, such as anthocyans, are thought to act is by reducing DNA damage, especially that generated by ROS, and therefore co-administration with genotoxic agents, such as MMC, could potentially result in reduced efficacy of the chemotherapeutic agent (Lamson and Brignall, 1999).

1.3.4.1 Studies Involving Combined Treatment Strategies

A few studies using flavonoids have been carried out investigating the interaction of dietary agents with chemopreventive properties upon subsequent treatment therapies. Sarkar and Li (2006), for example, summarized the findings of some recent investigations into the effect dietary chemopreventive agents had in combination with cancer treatment regimens. Overall from the *in vitro* and *in vivo* studies reviewed they concluded that dietary chemopreventive agents actually enhanced the effects of

treatment regimes such as chemotherapy and radiotherapy. The main agent investigated was genistein (soya extract), which was found to enhance the anti-tumour activities of chemotherapeutic agents such as cisplatin, Dox and tamoxifen as well as increase radiosensitivity in a non cell type specific manner (Sarkar and Li, 2006). However, not all studies have found such a clear enhancement of the chemotherapeutic activity; Sharma *et al.* (2004) investigated the effect of grape seed extract (GSE) on human breast cancer cells (MCF-7 and MDA-MB468) analysing cell proliferation, induction of apoptosis and cell cycle progression *in vitro* (Sharma *et al*. 2004). Cell growth experiments showed that both GSE and Dox alone caused cell growth inhibition and in combination the effect was approximately additive. Using a quantitative apoptotic cell assay the group also measured the induction of cell death by GSE and Dox alone and in combination. In MCF-7 cells the level of apoptosis was marginally increased upon treatment with either GSE or Dox alone and in combination, results were comparable to the agents alone. In MDA-MB468 cells both agents singularly increased the apoptotic cell population significantly compared to control, however, when cells were treated with the combined agents the level of apoptosis was significantly lower than in the Dox alone treated cells. Other studies have also seen an inhibition of apoptosis resulting from combined radiation based treatments and chemopreventive agents in human acute T lymphoblastic leukaemia cells (Lopaczynski and Zeisel, 2001).

Therefore prior to any clinical trial involving dietary agents with chemopreventive activity are undertaken it is essential to assess the potential benefit or hindrance these dietary agents may have as adjuncts to non-muscle invasive bladder cancer treatments. In order to assess the impact anthocyanins have on intravesical chemotherapeutic agents such as MMC for non-muscle invasive bladder cancer, DNA damage and cellular

response markers can be evaluated for indicators of tumour cell sensitivity towards the chemotherapeutic agents alone and in combination with chemopreventive agents.

1.4 Aims and Objectives

An *in vitro* study will be carried out to investigate the cytotoxic and genotoxic outcome of anthocyans administered singularly and in combination with a chemotherapeutic agent (MMC) in non-muscle invasive bladder cancer cell lines. The clonogenic assay will measure differences in the reproductive integrity of cells exposed to MMC that will either have been pre-treated with an anthocyanin mixture (mirtoselect) or not. Crosslink formation and repair in pre-treated and non-treated cells will be compared using the modified version of the alkaline comet assay in order to assess if anthocyans affect the DNA damaging potential of these genotoxic agents. Cell proliferation, cell cycle permutations and levels of apoptosis will be assessed in mirtoselect treated cells to determine if mirtoselect is capable of altering cellular response which may affect subsequent treatments.

Chapter Two

Materials and Methods

2.1 Chemicals, Reagents and Kits

All chemicals and reagents were purchased from Sigma Aldrich (Poole, UK) unless otherwise stated. Growth studies were carried out using the CyQUANT® NF cell proliferation assay kit, purchased from Invitrogen (Paisley, UK). The CFSE cell proliferation stain was also purchased from Invitrogen as was the Human Annexin V-FITC Kit and the TMRE assay for apoptosis studies; all reagents used were supplied by the manufacturer with the exception of the propidium iodide (PI) used in the Human Annexin V-FITC assay which was purchased from Sigma (Poole, UK). The ApoToxGlo Triplex assay was purchased from Promega (Southampton, UK).

Mirtoselect® was kindly supplied by Indena (Milan, Italy) and kept at -20ºC in the dark. Immediately prior to use the mirtoselect was dissolved in ddH_2O at an appropriate concentration of 1mg/ml. To ensure complete dissolution, the mirtoselect was sonicated for 1 minute at 10 amplitude microns on a soniprep 150 (MSE, Lower Sydenham, UK). The dissolved mirtoselect was then sterile filtered using a 0.2μM syringe filter. Mirtoselect solutions were either used immediately or stored at -20ºC for up to 1 month, undergoing only one freeze thaw cycle. Mirtoselect was adjusted to appropriate working solutions for experiments using the corresponding culture media for the cell line being investigated.

MMC was dissolved in filtered ddH_2O to a stock concentration of 1.5mM immediately prior to use and adjusted to the correct experimental concentration using the corresponding culture media for the cell line being investigated. Dissolved MMC was kept for up to 1 week at 4-7ºC.

2.2 Cell Culture

Three bladder cancer cell lines were used for this study all of which were of adherent cell type and epithelial morphology. RT112 cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK); RT4 cells were purchased from the American Type Culture Collection (ATCC, USA); HT1376 cells were purchased from Cancer Research UK (Cancer Research UK, London, UK).

RT112 cells were cultured and treated in Eagle's minimal essential medium (EMEM) supplemented with 10% foetal calf serum (FCS) and 1% non-essential amino acid (NEAA). RT4 cells were cultured and treated in McCoy's 5A medium supplemented with 1% glutamax and 10% FCS. Finally HT1376 cells were cultured and treated in Dulbecco's modified Eagle's medium (DMEM) with 1500mg glucose and supplemented with 10% FCS.

All cells were grown in an incubator at 37ºC with 5% CO_2 until 70-80% confluent after which they were sub-cultured by washing with phosphate buffered saline (PBS) followed by trypsinisation with trypsin/EDTA (TrpE) (0.05% trypsin, 0.7mM EDTA) at 37ºC for 5-10 minutes. Trypsinisation was stopped by adding 3 times the volume of culture media to TrpE. Cells were pelleted via centrifugation at 600g for 5 minutes, resuspended in culture media and tested for cell viability using the trypan blue exclusion assay (Section 2.3). Only cells having undergone fewer than 20 passages were used for experiments.

2.3 Trypan Blue Exclusion Assay

A small volume, typically 15µl, of trypsinised and resuspended cells were mixed with an equal volume of trypan blue before being placed on a haemocytometer using a cover

slip and capillary action. Both white (viable) and blue (non-viable) cells were counted within 2 minutes of mixing with trypan blue in order not to create too many false negatives. Cell number per ml of original cell suspension was calculated by the formula in Equation 2.3-1. Percentage cell viability was then calculated using the formula in Equation 2.3-2.

$$Number\ of\ cells\ per\ ml = \left(\frac{Number\ of\ cells\ counted}{Number\ of\ 4x4\ grids\ counted}\right) \times 2 \times 10^4$$

Equation 2.3-1 Cell Number
Where 104 is the conversion factor of cells according to the volume of the grid (0.1 mm3) and 2 is the dilution factor.

$$Percentage\ cell\ viability\ (\%) = \frac{Number\ of\ white\ cells}{Total\ number\ of\ cells} \times 100$$

Equation 2.3-2 Percentage Cell Viability

2.4 Cell Treatments

2.4.1 Repeat Mirtoselect Treatments

All experiments involving repeat mirtoselect dosing were conducted using the following schedule, unless otherwise stated. Cells were seeded at appropriate numbers and allowed 24 hours to attach in normal culture media. All media was then removed and an appropriate volume of freshly prepared mirtoselect containing media was added to the cells. Every 24 hours and for the duration of the experiment (72 hours, unless otherwise stated) all media was removed and freshly prepared mirtoselect in media was added to the cells.

Growth studies: mirtoselect (2, 20, 50, 75 and 100µg/ml) was added as 100µl.

Cell cycle and Comet assay (radiation): mirtoselect (20, 50 and 100µg/ml) was added as 4ml.

2.4.2 Single Mirtoselect Treatments

All experiments involving single mirtoselect dosing were conducted using the following schedule, unless otherwise stated. Cells were seeded at appropriate numbers and allowed 24 hours to attach in normal culture media. All media was then removed and an appropriate volume of freshly prepared mirtoselect containing media was added to the cells. This media was then left on the cells for the duration of the experiment (72 hours, unless otherwise stated).

Growth studies: mirtoselect (2, 20, 50, 75 and 100µg/ml) was added as 100µl.

Cell cycle, Annexin V FITC and TMRE assays: mirtoselect (20, 50 and 100µg/ml) was added as 4ml.

CFSE assay: mirtoselect (2, 20, 50, 75 and 100µg/ml) was added as a 6ml volume.

ApoToxGlo triplex assay: mirtoselect (50, 75 and 100µg/ml) was added as 50µl.

2.4.3 MMC Treatments

All experiments involving MMC treatments were conducted using the following schedule, unless otherwise stated. Cells were seeded at appropriate numbers and allowed 24 hours to attach in normal culture media. All media was then removed and an appropriate volume of freshly prepared media containing MMC was added to the cells for 1 hour, after which all media was removed. The cells were then washed with PBS and left for the duration of the experiments in fresh media free from any agent.

Growth studies: After the attachment period, media was removed and cells were given a further 24 hours in fresh media free from any agent. MMC was then added as a 20µl volume to achieve a final concentration of 10µM. Cells were left in the MMC containing media for the remainder of the assay.

Comet assay: MMC (50 and 100µM) was added as a 1ml volume.

2.4.4 Mirtoselect and MMC Combined Treatments

All experiments involving combined dosing were conducted using the following schedule, unless otherwise stated. Cells were seeded at appropriate numbers and allowed 24 hours to attach in normal culture media. All media was then removed and an appropriate volume of freshly prepared mirtoselect containing media was added to the cells for 24 hours. Media containing MMC was then added directly to the mirtoselect containing media for 1 hour to give the final appropriate concentration of MMC, after which all media was removed. The cells were then washed with PBS and left for the duration of the experiments in fresh media with or without mirtoselect.

Growth studies: mirtoselect (2, 20, 50, 75 and 100µg/ml) was added as 100µl, MMC was added as a 20µl volume to achieve a final concentration of 10µM, cells were left in this treatment media for the remainder of the assay.

Clonogenic assay: mirtoselect (20, 30 and 50µg/ml) was added as a 6ml volume, MMC was added as a 4ml volume at varying final concentrations for a period of 1 hour. The cells were then washed with PBS and left for the duration of the experiments in fresh media free of any agent.

Comet assay: mirtoselect (0, 20, 50, 100µg/ml) was added as a 4ml volume, fresh media containing mirtoselect was added every 24 hours for a total of 72 hours after which MMC was added directly to the mirtoselect media as a 1ml volume for a total of 1 hour. For DNA damage formation studies MMC was added at a final concentration of 5, 10, 20 and 50µM. For DNA repair studies MMC was added at a final concentration of 50µM. After the MMC media had been removed fresh mirtoselect containing media was added to the cells. Freshly prepared mirtoselect was added to the cells every 24 hours for the duration of the experiment.

Annexin V FITC and TMRE assays: mirtoselect (20, 50, 100µg/ml) was added as 4ml, MMC was added as a 1ml volume at a final concentration of 10µM. The cells were then washed with PBS and left for the duration of the experiments in fresh media free of any agent.

Cell cycle assay: mirtoselect (20, 50, 100µg/ml) was added as 4ml, MMC was added as a 1ml volume at a final concentration of 1µM. The cells were then washed with PBS and left for the duration of the experiments in fresh media free of any agent.

2.5 Cell Growth Assay

The effect of mirtoselect on growth was measured using the CyQUANT® NF cell proliferation assay kit (Invitrogen, Paisley, UK). The assay measures cellular DNA content, via binding of a fluorescent dye, which is closely proportional to cell number. The assay involves the lysing of cells with a buffer, supplied with the kit, to which the user adds the CyQUANT® GR dye. Once bound to cellular nucleic acids, the dye exhibits a strong fluorescence which can be read on a fluorescent plate reader. The assay does not require metabolic activation so is regarded as a more reliable technique

compared to other methods of cell growth measurement. The assay was carried out in 96 well plates, with at least 3 replicate wells for each treatment.

Optimisation experiments were carried out to determine appropriate cell seeding numbers to ensure the experiments was performed within the dynamic range of the assay. All three cell lines were plated in at least triplicate at numbers according to the optimisation experiment and left to attach for 24 hours in normal culture media. Fluorescence was measured at 0 hrs (immediately after the 24 hour attachment period), 24, 48, 72 and 96 hours (see Section 2.5.1).

2.5.1 Cell Staining and Measurement of Fluorescence

The dye binding solution was prepared immediately prior to use following the manufactures instructions. Briefly, a 1x HBSS buffer was prepared by diluting 1 part 5x HBSS buffer with 4 parts deionised water. The 1x dye binding solution was then prepared by adding 1 part CyQUANT® NF dye reagent to 500 parts 1x HBSS buffer. The binding solution was added directly to the cells and control wells, after removal of the culture media, using a multichannel pipette adding 50µl of 1x dye binding solution per well. The 96 well plate was then covered and incubated at 37ºC for 1 hour. After incubation, the plate was sealed using a SealPlate® adhesive sealing film suitable for use with fluorescence plate readers (Web SCIENTIFIC, Crewe, UK) and the fluorescence intensity was measured using a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, UK) with an excitation of 485nm and emission of 530nm. The gain value for each individual plate was set at 80% of the well with maximum fluorescence.

2.5.2 Data Analysis and Conversion

To allow for comparison between experiments obtained using different gain values, the data from the CyQUANT® NF proliferation experiments were standardised according to the negative control (media alone) included in each plate. After averaging the fluorescent values for each set of triplicate treatments, each averaged experimental fluorescent reading was divided by the average background fluorescent reading (obtained from the negative control) for that plate. Percentage cell number was then calculated by comparing the values obtained at varying time points to those obtained from a plate read at zero hours (i.e. after the 24 hour attachment period) (Equation 2.5-1)

$$Cell\ number\ (\%) = \frac{Background\ adusted\ fluorescent\ value\ at\ x\ hours}{Background\ adusted\ fluorescent\ value\ at\ 0\ hours} \times 100$$

Equation 2.5-1 Cell Growth

In order to determine the effect seen between the mirtoselect and MMC treated cells a correction factor was applied to the combined growth data. This correction factor was determined using an equation based on data obtained from growth studies treated with a single continuous dose of mirtoselect (Equation 2.5-2). The correction factor was then applied to the combined treatment data by multiplying each by the relevant correction factor for mirtoselect treatment and time point.

$$Correction\ factor = \frac{Cell\ number\ for\ 0\mu g/ml\ mirtoselect\ treatedcells\ at\ X\ hours}{Cell\ number\ in\ X\mu g/ml\ \text{mirtoselect treated cells}\ at\ \text{X}\ hours}$$

Equation 2.5-2 Correction Factor Equation for Combined Growth Studies

2.6 Clonogenic Cell Survival Assay

2.6.1 Cytotoxicity of MMC Alone

Cells were plated at appropriate numbers, dependant on subsequent treatments, in 9cm petri dishes in a final volume of 6ml culture medium. Each treatment combination was carried out in triplicate (for treatments used see Section 2.4.4). After treatment, cells were left in culture media free from any agent until colonies were visible, replacing the media every 7 days (RT112; 2-3 weeks, RT4; 3-4 weeks, HT1376; 4 weeks).

2.6.2 Fixing and Staining of Cells

At the end of the assay, culture media was removed and the colonies were fixed in 100% methanol, washed in dH_2O and left to dry. The colonies were then stained with a solution of crystal violet (Pro-lab, Cheshire, UK), washed again in dH_2O, dried and then counted. Results are expressed as the mean surviving fraction (SF) ±SD, where SF is the ratio between the number of colonies counted and the number of cells plated (Equation 2.6-1) taking into account the plating efficiencies (PE) of the different cell lines (Equation 2.6-2).

$$SF\ (\%) = \frac{Colonies\ counted}{Numbers\ cells\ seeded \times PE(\%) of\ control\ cells} \times 100$$

Equation 2.6-1 Percentage Cell Survival

$$PE(\%) = \frac{Colonies\ counted}{Number\ of\ cells\ seeded} \times 100$$

Equation 2.6-2 Plating Efficiency

2.7 Alkaline Comet Assay

MMC is a proven crosslinking agent. In order to measure the formation and repair of these crosslinks a modified version of the alkaline comet assay was used (Merk and Speit, 1999). Initial studies to determine optimal treatment conditions were performed after which the assay was used to measure crosslinks in cells pre-treated with mirtoselect.

2.7.1 Optimisation of Comet Assay Conditions

2.7.1.1 Determination of the Optimal Radiation Dose

Enough cells to make two gels were pelleted in 1.5ml eppendorfs and irradiated at 4, 6, 8 and 10Gy on ice, to inhibit repair, at a dose rate of 1Gy/minute with 250kV constant potential using a Pantak industrial X-ray machine. Control samples were kept on ice alongside irradiated cells. Cells were then processed for the comet assay following the normal procedure (Sections 2.7.3 to 2.7.5).

2.7.1.2 Determination of the Optimal MMC Dose

Cells were plated at a density of 1×10^5 (RT112) and 1.5×10^5 (RT4) in 12-well tissue culture plates allowing 24 hours for attachment. Cells were treated with a range of concentrations of MMC (0 to 200μM) for 1 hour at 37ºC after which they were washed with PBS and left in drug free media for 3 hours to allow for the formation of crosslinks. Cells were harvested, via trypsinisation with TrpE, pelleted at 2×10^4 cells per eppendorf, washed free of any media with PBS and re-pelleted. Cells were then resuspended in a minimal volume of PBS, irradiated on ice (10Gy at a rate of 1Gy/minute) and processed for the comet assay (Sections 2.7.3 to 2.7.5). Each

concentration of MMC was assessed in duplicate with un-treated non-irradiated controls being processed alongside the cell treated samples.

2.7.2 Cell Preparation

2.7.2.1 MMC Alone Experiments

Cells were plated at a density of 1×10^5 cells per well in 12-well plates and treated with MMC as detailed in Section 2.4.3. At each incubation time point, cells were collected via trypsinisation, cell number assessed using the trypan blue exclusion assay (Section 2.3) and frozen at –80ºC in culture media containing 10% FCS and 10% DMSO until all samples were collected.

Once all samples had been collected cells were thawed in a waterbath at 37ºC and immediately centrifuged at 350g at 4ºC for 5 minutes, washed free of freezing media with PBS and adjusted to $2x10^4$ cells, then placed on ice ready for irradiation. Some cells for each treatment dose were also kept for un-irradiated controls and were processed alongside other samples. Cells were then irradiated with 10Gy on ice at a dose rate of 1Gy/minute.

2.7.2.2 Combined Mirtoselect and MMC Treatments

Cells were plated at a density of 1×10^5 cells per well in 6-well plates (see Section 2.4.4 for cell treatments). At each incubation time point, cells were collected via trypsinisation, cell number assessed using the trypan blue exclusion assay (Section 2.3) and 3×10^4 cells pelleted for each concentration, also allowing for un-irradiated controls (the remainder of the treated cells were also retained as a back-up). All cells were frozen at –80 ºC in culture media containing 10% FCS and 10% DMSO until all samples were collected.

Once all samples had been collected cells were thawed in a waterbath at 37ºC and immediately centrifuged at 350g at 4ºC for 5 minutes, washed free of freezing media with PBS then placed on ice ready for irradiation. Cells were then irradiated with 10Gy on ice at a dose rate of 1Gy / minute.

2.7.2.3 Mirtoselect and Radiation Experiments

For mirtoselect pre-treated experiments RT112 cells were plated at a density of 1×10^5 cells per well in 6-well plates (see Section 2.4.1 for cell treatments). After 72 hours incubation cells were collected via trypsinisation, cell number assessed using the trypan blue exclusion assay (Section 2.3) and 3×10^4 cells pelleted in 1.5ml eppendorfs for each combination of mirtoselect and radiation dose. For non pre-treated experiments RT112 cells, growing in culture, were harvested following normal tissue sub-culturing techniques (Section 2.2) and 3×10^4 cells were pelleted in 1.5ml eppendorfs for each combination of mirtoselect and radiation dose. Cells were then irradiated on ice in 100µl of PBS alone or 100µl of PBS containing mirtoselect at a final concentration of 20, 50 or 100µg/ml mirtoselect. After irradiation cells were centrifuged at 350g at 4ºC for 5 minutes and the supernatant removed.

2.7.3 Slide Preparation

Twin frosted slides were pre-coated with 1% normal melting point agarose (1% agarose dissolved in ddH_20) and left to dry at 37ºC for 24 hours. The slides were then chilled on a metal tray resting on ice immediately prior to the addition of the cells. Cells were mixed with 170µl of warmed (37^{o}C) 0.6% low melting point agarose (0.6% low melting agarose dissolved in PBS) dispensing 80µl of the molten gel-cell suspension onto each of two pre-coated microscope slides and covered with a cover-slip. Slides were then left

on ice until the gels had solidified after which the coverslips were removed and the slides put into lysis.

2.7.4 Cell Lysis and Electrophoresis

Cells were lysed overnight in lysis buffer (100mM disodium EDTA, 2.5M NaCl, 10mM Tris-HCl, pH 10 with sodium hydroxide; adding 1% triton X-100 immediately prior to use) in coplin jars which were stored on ice and at 4ºC. Following lysis, the slides were washed twice with ice-cold ddH_2O for 10 minutes after which they were transferred to an electrophoresis tank and incubated in ice-cold alkaline electrophoresis buffer (300mM NaOH, 1mM disodium EDTA, pH 13) for 20 minutes. Duplicate slides were always placed in a random fashion in the electrophoresis tank to allow for variations in current across the tank. Electrophoresis was carried out for 20 minutes at a constant 30V and 300mA. After electrophoresis, the slides were neutralised with neutralisation buffer (0.4M Tris-HCl, pH 7.5) for 20 minutes followed by 10 minutes in ddH_2O. The slides were then left to dry at 37ºC. All procedures were carried out under subdued lighting in order to minimise the induction of further DNA damage.

2.7.5 Comet Visualisation and Analysis

To stain the slides, the gels were rehydrated in ddH_2O for 30 minutes after which they were stained with freshly made PI solution (2.5µg/ml) for 20 minutes in the dark at room temperature. The PI was removed by washing with ddH_2O for 20 minutes after which the slides were placed at 37ºC until dry and thereafter stored in the dark until analysed.

Comets were visualised using an Olympus fluorescence microscope, fitted with an excitation filter of 515-535nm and a barrier filter of 590nm, at ×200 magnification.

Images were captured using a charge-coupled device camera and analysed using the Komet Analysis software (version 5.5) from Kinetic Imaging (Nottingham, UK). Overall, 100 nucleoid bodies were analysed per time point, analysing 50 randomly chosen events per gel, with analysis restricted to the central region of the gel. Percentage tail DNA (%TD) was calculated by the Komet software for each comet. DNA crosslinking was expressed as the percentage decrease in mean %TD compared to the irradiated controls calculated by the formula in Equation 2.7-1.

$$\%decrease\ in\ \%TD = \left[1 - \left(\frac{TDdi - TDcu}{TDci - TDcu}\right)\right] \times 100$$

Equation 2.7-1 Percentage Decrease in %TD
TDdi = %TD of the drug treated irradiated sample, TDcu is the %TD of the non-drug treated unirradiated control and TDci is the %TD of the non-drug treated irradiated control (Hartley *et al*. 1999)

Repair capacity (at time (t)) was assessed as a percentage relative to maximum formation of crosslinks (Equation 2.7-2).

$$Repair = \frac{\%D\%TD\ at\ \max - \%D\%TD\ at\ Time}{\%D\%TD\ at\ max}$$

Equation 2.7-2 Repair Capacity
%D%TD = % decrease in %TD, calculated using Equation 2.7-1 (Spanswick *et al*. 2002).

2.8 Apoptosis Analysis

A Human Annexin V-FITC Apoptosis Kit was used to measure phosphatidylserine externalisation as a marker of apoptosis. Tetramethylrhodamine ethyl ester (TMRE) was also used to assess apoptosis, by measuring mitochondrial membrane potential.

The effect of mirtoselect alone and mirtoselect in combination with MMC were both analysed using the same protocol. Cells were plated at a density of 2×10^5 cells/well in

6-well plates. Cells were collected at various 24 hour time points (see Section 2.4 for cell treatments).

2.8.1 Collection and Staining of Cells for Apoptosis Assays

To harvest the cells, the medium containing any floating cells was retained and the adherent cells were detached via trypsinisation with TrpE (trypsinisation was as brief as possible, typically less than 5 minutes, to avoid false positives) and combined with the floating cells. The combined fractions were then centrifuged at 350g for 5 minutes, resuspended in 2ml of medium and incubated at 37ºC for 30 minutes. After incubation the cells were resuspended in the medium by gentle agitation and split equally between two FACS tubes. One tube was then used for the TMRE apoptosis assay and the other was used for the annexin V-FITC assay.

2.8.1.1 Annexin V FITC Assay

Cells were centrifuged in the FACS tubes at 600g for 5 minutes after which the medium was removed carefully, the pellets dislodged by gentle agitation and resuspended in 1ml of 1x annexin buffer. 4µl of annexin V-FITC conjugate was added to each sample, which were then vortexed before incubation at room temperature for 10 minutes. 1.5µg of PI was subsequently added and incubated for a further minute at room temperature. Samples were immediately analysed on the FACS, being kept on ice whilst being processed.

2.8.1.2 TMRE assay

After incubation at 37ºC (Section 2.8.1) cells were immediately placed in a waterbath at 37ºC and given 5 minutes to return to temperature. TMRE dye was then added to the culture medium at a final concentration of 50ng/ml immediately prior to analysis.

2.8.2 Data Analysis

All flow cytometry work was done using a Becton Dickinson FACScan flow cytometer with a 488-nm laser and CellQuest software (Becton Dickinson UK Ltd, Cowley, UK). 10,000 events per sample were plotted for each of the experiments.

2.8.2.1 Annexin V FITC assay

Apoptosis analysis using an annexin V FITC kit was performed using CellQuest software. Cells were primarily plotted according to their shape (side scatter, SSC-H) and size (forward scatter, FSC-H), a gate was then placed around the cells to disregard any debris. The gated cells were then used to plot the height of the fluorescent peak for FITC (green fluorescence) against the height of the peak for PI (red fluorescence).

2.8.2.2 TMRE assay

Membrane potential analysis using TMRE staining was performed using CellQuest software. Cells were primarily plotted according to their shape (SSC-H) and size (FSC-H), a gate was then placed around the cells to disregard any debris. The gated cells were used to plot a histogram of the number of cells versus the height of the fluorescent peak for TMRE (red fluorescence).

2.9 Cell Cycle Distribution Analysis

2.9.1 Cell Staining

Cells were plated at a density of 2×10^5 cells/well in 6-well plates (see Section 2.4 for cell treatments). At each time point the cells were collected via trypsinisation with TrpE. Cells were then centrifuged at 350g for 5 minutes, resuspended in 1ml of PBS, spun for a further 5 minutes and resuspended in 200µl PBS. Cells were then fixed in 2ml 70% ethanol, vortexing the samples as the ethanol was added, and kept at 4ºC until all samples were ready to be prepared for analysis and staining. All samples were left for a minimum of 24 hours in ethanol before analysis was performed.

Once all samples were collected the cells were centrifuged at 600g for 10 minutes at 4ºC and resuspended in 800µl PBS. 100µg RNase A was added to the cells and left at 37ºC for 10 minutes. Cells were then stained with 5µg PI and kept in the fridge overnight prior to flow cytometry analysis.

2.9.2 Data Analysis

All flow cytometry work was done using a Becton Dickinson FACScan flow cytometer with a 488-nm laser and CellQuest software (Becton Dickinson UK Ltd, Cowley, UK). 10,000 events per sample were plotted for each of the experiments.

For cell cycle experiments, cell shape (SSC-H) was plotted against size (FSC-H) and a gate was placed around the cells so as to disregard any debris. The gated cells were then used to plot the area of the fluorescent peak (FL2-A) against the width of the peak (FL2-W). A second gate was then placed around the cells with the appropriate FL2-A vs. FL2-W parameters. Finally, a DNA histogram using the second set of gated cells was plotted with the number of cells versus fluorescent intensity. Cell cycle distribution

data analysis was then performed using ModFit LT software (Becton Dickinson UK Ltd, Cowley, UK) which, using mathematical equations, determined the proportion of cells in the different stages of the cell cycle. Results are expressed as the mean ±SD of three independent experiments.

2.10 CellTrace CFSE Proliferation Assay

2.10.1 Cell Staining

Cells were stained with CFSE prior to being plated for the assay in small tissue culture flasks. Cells were trypsinised following normal sub-culturing techniques (Section 2.2) washed free of media in PBS and pelleted in falcon tubes at a density of $1x10^6$ cells/ml of warm (37ºC) PBS. The CFSE dye was resuspended in 18µl of DMSO (provided) and added to the cells at a final concentration of 10µM. Cells were mixed and left to stain for 10 minutes at 37ºC. After staining cells free CFSE dye was quenched by adding 5x volume of ice cold media and leaving on ice for 5 minutes. Cells were then pelleted and washed with culture media for a total of 3 washes. The cells were then plated ready for the experiment at appropriate cell densities. A period of 24 hour attachment was allowed before cells were exposed to mirtoselect (see Section 2.4 for cell treatments).

2.10.2 Data Analysis

At the appropriate time points cells were trypsinised and resuspended in PBS in FACs tubes. All flow cytometry work was done using a Becton Dickinson FACScan flow cytometer with a 488-nm laser and CellQuest software (Becton Dickinson UK Ltd, Cowley, UK). 10,000 events per sample were plotted for each of the experiments.

CFSE data analysis was performed using ModFit LT software. Cells were primarily plotted according to their shape (side scatter, SSC-H) and size (forward scatter, FSC-H), a gate was then placed around the cells to disregard any debris. The gated cells were then analysed using ModFits proliferation wizard. The position of parent peak was assessed using cells at 0 hours (i.e. after 24 hours attachment, before mirtoselect treatment), this was then used for all subsequent analysis. For each sample 8 generations were modelled with the background option enabled.

2.11 ApoTox-Glo Triplex Assay

2.11.1 Viability and Cytotoxicity Analysis

Cells were plated at a density of 1×10^4 cells/well in 96-well plates (see Section 2.4 for cell treatments). Viability/cytotoxicity reagent was made following the manufactures instructions (10µl of GF-AFC substrate and bis-AAF-R110 substrate were added to 2.5ml of assay buffer) this was kept at 4ºC for up to 7 days. At each time point 10µl of viability/cytotoxicity reagent was added to the wells and briefly mixed by orbital shaking (300rpm for 30 seconds). The cells were then incubated for 60 minutes at 37ºC. Fluorescence was measured using a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, UK) with an excitation of 400nm and emission of 505nm for viability and an excitation of 485nm and emission of 520nm for cytotoxicity.

2.11.2 Caspase Activity Analysis

Caspase-Glo 3/7 reagent was prepared by mixing the caspase-Glo 3/7 buffer with the caspase-Glo 3/7 substrate. The reconstituted caspase-Glo 3/75 reagent was then stored at 4°C for 3 days. After the fluorescent readings were taken for viability/cytotoxicity 50µl of caspase-Glo 3/7 reagent was added to the wells and briefly mixed by orbital

shaking. The cells were then incubated at room temperature, protected from light, for 60 minutes. Luminescence was measured using a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, UK).

2.12 Statistical Analysis

Statistical analysis was carried out using statistical software package SPSS version 12 (SPSS for Windows, Rel. 12.0.2. 2004. Chicago: SPSS Inc.). One-way analysis of variance (ANOVA) and post hoc Tukey test were performed to assess the significance of differences between groups. A p value of ≤ 0.05 was considered significant.

Chapter Three

Cell Growth and Survival in response to Treatment of Bladder Cancer Cell Lines with Mirtoselect Alone and in Combination with MMC

3.1 Introduction

As discussed in the introduction there is substantial preclinical evidence suggesting that anthocyans have cancer chemopreventive properties; the high concentration detected in mouse urine after administration suggests anthocyanins may potentially protect against bladder cancer development (Cooke *et al*. 2006). Although the exact mechanism of action of anthocyans is not yet apparent, other dietary chemopreventative agents have previously been shown to interfere with chemotherapeutic cytotoxic agents, some adventitiously (Hong *et al*. 1990) and some preventatively (Bairati *et al*. 2005a, Bairati *et al*. 2005b). In order to determine the possible effect of combining chemotherapy in the form of MMC with the putative chemopreventive agent mirtoselect, preclinical studies are needed before any clinical trials can be considered.

Three main questions were posed in order to try and determine the suitability of mirtoselect as a chemopreventative agent during the treatment of non muscle invasive bladder cancer; 1. What is the response of cells to mirtoselect alone? 2. What is the response of the cells to MMC alone? and 3. What is the effect of combining treatments in comparison to the individual treatments? As the particular anthocyan extract, mirtoselect, had not previously been investigated *in vitro* in bladder cancer cell lines it was important to firstly determine if mirtoselect was able to interfere with the promotion or progression of malignancy through the measurement of cellular growth and survival and if there was any impact, either positively or negatively, on the known anti-proliferative effect of MMC.

One of the six hallmarks of cancer is the ability of cancerous cells to grow in the absence of growth factors. One of the underlying principles of chemotherapy relies on the ability of the drugs to interfere with cellular proliferation and division; so enabling

the targeting of the rapidly growing cancerous cells, by inducing apoptosis or shrinking the tumour prior to surgery. It was crucial, therefore, to determine the effects of mirtoselect alone on the ability of cells to proliferate and divide and to then investigate if this had any influence on the known anti-proliferative effects of MMC. Assays used to measure cellular drug sensitivity belong to two different classes: those that measure cytotoxicity and those that measure cell survival. Measuring cytotoxicity has traditionally been done using shorter term assays such as the trypan blue exclusion assay and the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay whereas clonogenic assays are regarded as the gold standard for the measurement of cell survival (Blumenthal, 2005).

A wide number of cytotoxicity assays exist for the measurement of cellular drug sensitivity, many of which assess the structural integrity and metabolic function of the cells after drug exposure. If cells are allowed a period of growth post drug treatment then many of the cytotoxicity assays can also assess cellular proliferation. One of the easiest methods for the assessment of cellular sensitivity is by using the trypan blue exclusion assay. This method involves the harvesting and collection of cells post treatment, mixing with the trypan blue dye (a diazo dye), and counting the cells using a haemocytometer or cell counter. Viable cells are able to exclude the dye, due to the intact cellular membrane, however, dead cells take up the dye and stain blue, which can be seen under the microscope when placed on a haemocytometer. This method enables the user to distinguish between alive and dead cells so giving information on cell viability as well as the cytotoxicity of the drug. The major drawbacks to this method are associated with its difficulties in processing large numbers of samples at once, due to the fact the sample preparation is rather laborious on a large scale, this in turn can

lead to erroneous results and false positives if the dye is left on the cells for extended periods of time, with healthy cells taking up the dye.

Although seen as a rather crude way of assessing cytotoxicity the trypan blue exclusion assay is still one of the most commonly used assays and is usually used as a reference technique for other assays. In the drug development industry the need for fast reliable cytotoxicity assays are paramount, with automation and high-throughput techniques being most suited. The MTT assay was first reported in the 1980's (Mosmann, 1983) and can be adapted to 96 and 384 plate formats making it ideal for large scale cytotoxicity experiments (Vistica *et al*. 1991). The assay uses a tetrazolium dye (MTT) as an indicator of cell number using colorimetric based techniques. Cells, in the exponential growth phase, are exposed to the drug in question then allowed time to proliferate before the addition of the MTT dye. Assessment of the number of viable cells is possible due to the chemical reduction of the dye by live cells from a yellow, water soluble tetrazolium dye, to a purple formazan product that is water insoluble. The amount of formazan produced is directly proportional to the number of living cells and can be assessed, once solubilised in a suitable solvent, using a spectrophotometer assessing the absorbance between 540 and 570nm. Reduction of the MTT dye to formazan requires the action of mitochondrial dehydrogenases which cleave the tetrazolium ring, the amount of formazan is therefore dependant on the metabolic activity of the cell. Metabolic activity has been shown to differ vastly between cells and has been shown to be affected by some agents, the use of MTT is therefore considerably limited (Wang *et al*. 2010). Similar problems are associated with the measurement of ATP content (Niles *et al*. 2009).

Another method for the assessment of cell number is via the measurement of the incorporation of radio-labelled thymidine into DNA. Although highly sensitive, this method is associated with its own limitations, namely the safety and cost implications of using radioactive material (Blaheta *et al*. 1991). Cellular DNA content, however, is a very good indicator of cell number and so instead of a radioactive assay, measurement of cellular cytotoxicity for this project was carried out using a fluorescent based cellular DNA proliferation kit with cell survival being measured using a clonogenic assay.

3.1.1 Analysis of Cellular Growth using a Fluorescent DNA Dye

Cellular DNA is highly regulated within the cell and although DNA content of a cell does alter over the course of time, as long as the cell culture is not synchronous, the net nucleic acid content remains essentially constant. In non-synchronised cultures, cells are in all the various stages of the cell cycle at any given time, therefore the average cellular DNA content remains unchanged per cell as the cells proliferate (Jones *et al*. 2001). This, therefore, makes it a very good indicator of cell number with a direct relationship between total nucleic acid content and cell numbers. Development of DNA fluorescent molecules has meant that quantification of DNA has become much quicker and safer than previous applications using radioactive compounds. These dyes exhibit fluorescence enhancement upon binding to nucleic acids. One such class of these dyes are the cyanine dyes which have very low intrinsic fluorescence but a large fluorescent enhancement when bound to DNA, resulting in highly sensitive methods that can produce results from cell numbers as low as 100 (Blaheta *et al*. 1998). The CyQUANT NF cell proliferation assay uses the cyanine dye CyQUANT GR along with a plasma membrane permeabilisation reagent to produce a fluorescent based DNA binding assay with a dynamic range of 100-20,000 cells per well in a 96 well plate format (Jones *et al*.

2001). The assay does not rely on enzymatic or metabolic activity, which can affect results in a cell independent manner, yet gives information on the extent of growth when the fluorescence value of the treated samples is compared to the untreated control. Information can be gained on the cytotoxicity of a compound, if readings taken post treatment are related to values taken prior to treatment; cytotoxicity is distinguished by values being lower than the original readings. If, however, values do not decrease below the original readings, the assay is unable to distinguish between cytotoxic (cell killing) and cytostatic (reduced growth rate) events.

3.1.2 Clonogenic Assay

Clonogenic assays are seen as the 'gold standard' for measurement of cell sensitivity and survival. The technique was first described by Puck and Marcus (1955) but popularised some 22 years later by Hamburger and Salmon (Hamburger and Salmon, 1977). The assay assesses the clonogenic potential of cells; loss of clonogenic potential i.e. the inability to form a visible colony, is an indication of the loss of reproductive integrity. Clonogenic cell survival assays were originally used for the assessment of radiosensitivity, playing an essential role in radiobiology (Blumenthal, 2005). These days the technique is widely used in oncology research where it is used to assess the proliferative capacity of cancer cells after radiation and/or chemotherapy and also assess the effect of novel anticancer agents for drug discovery programmes (Fiebig *et al*. 2004).

In the standard clonogenic assay, cells are harvested and prepared as a single cell suspension, seeding at low densities onto tissue culture petri dishes or into soft agar. Treatment with the agent in question can then be applied for the duration of the experiment or for a specific time period, followed by incubation in drug free media.

Comparison of data from these different treatment regimes can help distinguish between the method of action of the agent, since cytostatic effects are likely to be lost once the drug is removed whereas cytotoxic effects will be maintained (Plumb, 2003). The formation of visible colonies usually takes between two to three weeks at which point the sensitivity/resistance of the cells to the compound can be assessed as a percentage of colonies in the treated samples compared to the untreated control. Each colony is visible proof that the original cell was able to grow into a large colony containing large numbers of progeny having retained its reproductive integrity. The major drawback to this technique is the length of time needed to produce colonies and therefore gain information on your compound.

3.1.3 Aims of this Chapter

In this results chapter cellular growth and cell survival assays were used to evaluate whether mirtoselect was able to induce any effects, consistent with chemoprevention, in the bladder cancer cell lines being investigated and then to evaluate whether it had any effect on the known anti-proliferative action of MMC.

3.2 Results

3.2.1 Analysis of Cellular Growth

Cellular growth was measured using the CyQUANT NF cell proliferation assay. The assay measures DNA content, via the binding of a fluorescent dye, and was proven to be directly proportional to cell number for each of the cell lines investigated, as determined through calibration curves and comparison of results to cell counts using the trypan blue exclusion assay (Appendix A). Optimal cell number for future experiments was determined based on these experiments.

In the growth graphs (Figure 3.2-1 to Figure 3.2-5) results are expressed as a percentage relative to the number of cells at the start of the experiment (0 hours). Cell cytotoxicity caused by mirtoselect and/or MMC is indicated by cell numbers falling below the initial number of cells seeded i.e. the fluorescence reading being lower than at the start of the experiment (however, cytotoxicity may still be taking place in experiments in which numbers do not fall below starting number, but to a lesser extent, as previously mentioned [Section 3.1.1]). Starting number is depicted by a solid line in the corresponding graphs.

3.2.1.1 Mirtoselect Treatments

Initial experiments were carried out in the presence of mirtoselect alone in order to determine the ability, if any, of mirtoselect to influence the proliferative capability of each of the 3 bladder cell lines. In the first instance treatment dosing regimens with mirtoselect were administered to mimic a scenario in which a patient would take a daily dose of mirtoselect.

3.2.1.1.a Repeat Dosing of Mirtoselect Every 24 Hours

The cytotoxic effect of mirtoselect treatment on RT112 cellular growth was seen as early as 24 hours for higher concentrations, although no statistically significant difference was observed at this time point (Figure 3.2-1A). Treatment with lower concentrations of mirtoselect, 2 and 20µg/ml, had little effect on the growth of cells compared to control, whereas the intermediary concentration of 50µg/ml mirtoselect did cause a decrease in cell number, but this was not statistically significant from control at any time point. Higher concentrations of 75 and 100µg/ml mirtoselect at 48 and 72 hours did, however, cause a significant decrease in the number of cells. At 48 hours cell

numbers were at 102% and 48% for 75µg/ml and 100µg/ml mirtoselect treatments, respectively, compared to control which was 336%; by 72 hours cell numbers had reduced further to 72% and 39% in 75µg/ml and 100µg/ml treated cells, respectively. Treatment of cells with 100µg/ml mirtoselect caused cell toxicity as early as 24 hours, cell numbers falling to 77%, 33% less than the original number seeded. In RT4 cells there was a noticeable dose dependent effect on cell numbers at 24 and 48 hours, however, by 72 hours the effect of the lower concentrations of 2 and 20µg/ml mirtoselect had been lost (Figure 3.2-1B). At no time point, or concentration used, did mirtoselect cause cell numbers to fall below starting numbers in RT4 cells, but at 48 and 72 hours, treatment with 100µg/ml mirtoselect did cause cell numbers to differ significantly from control. At 48 hours cell numbers were at 182% and 116% for control and 100µg/ml treated cells, respectively; by 72 hours control cells had increased to 264% whereas the 100µg/ml mirtoselect treated cells were at 132%, a 50% difference in cell number between treated and untreated cells and only a 32% increase in cell number compared to starting numbers. Compared to the obvious dose dependent decrease in cell growth seen in the other two cell lines, at 24 hours the effect in the HT1376 cell line was minimal (Figure 3.2-1C). A greater decrease in cell number in response to mirtoselect was seen at 72 hours, where there was a 34% difference in cell number between control cells and 100µg/ml treated cells, however none of these differences were statistically significant from control.

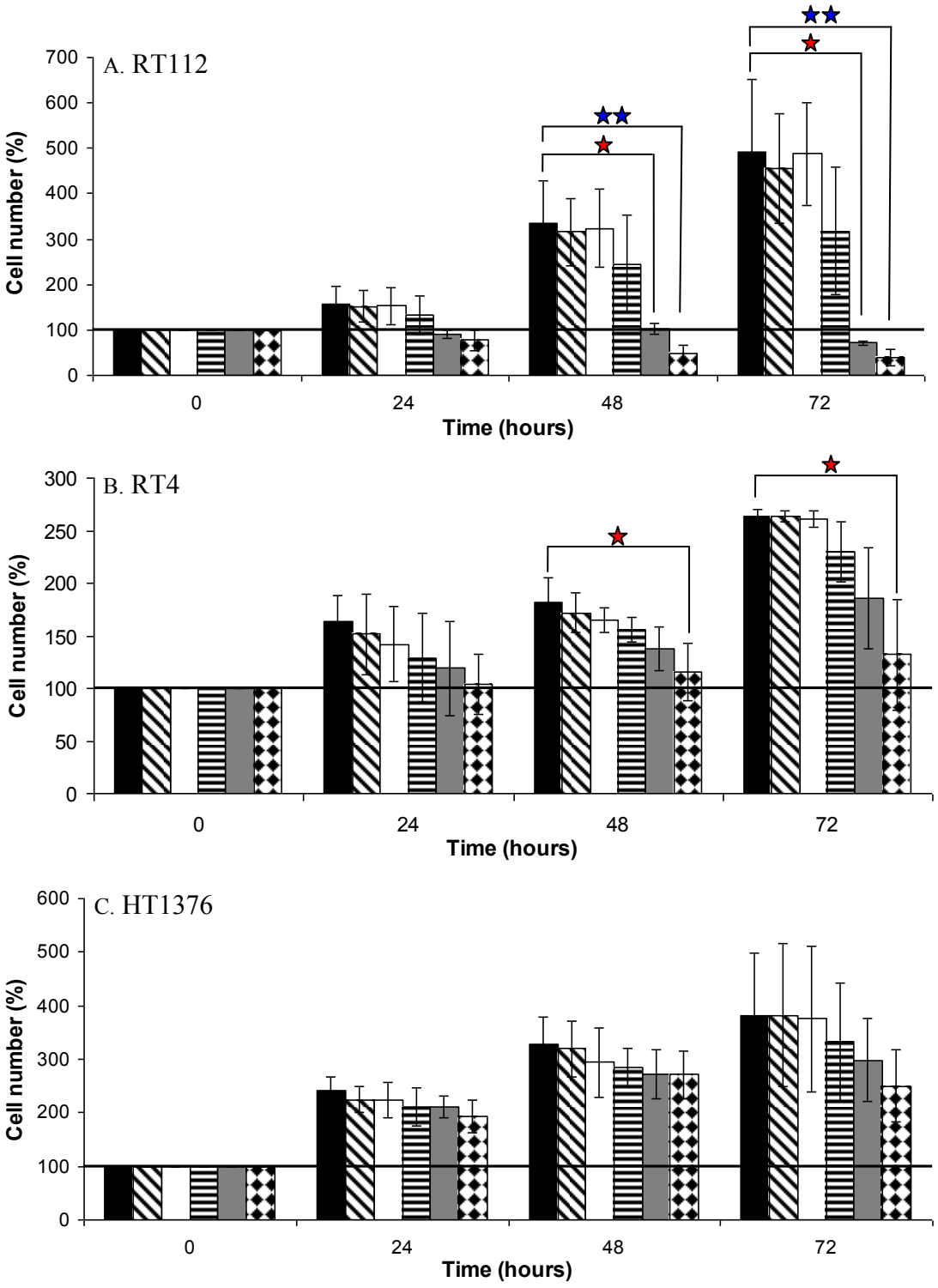


Figure 3.2-1 Cell Growth Graphs in Bladder Cells Treated with Repeat Doses of Mirtoselect.
Cell growth graphs for bladder cell lines treated over 72 hours with fresh mirtoselect being applied every 24 hours at 0 ■, 2 ◹ 20 □, 50 ▤, 75 ▩ and 100µg/ml ◪. Results are the mean of 3 independent experiments (n= 9) ±SD. Results are expressed as percentage cell number relative to the number of cells at 0 hours, $p \leq 0.05$ =★, $p \leq 0.01$= ★★.

In order to assess the interaction of mirtoselect and MMC, a period for pre-incubation with mirtoselect needed to be decided upon. In both RT4 and RT112 cells a dose response to mirtoselect was seen as early as 24 hours. This therefore indicated that at least part of the effects of mirtoselect were achieved in this initial 24 hour time frame and it is possible that subsequent treatments every 24 hours may not be causing any further significant anti-proliferative effect. A 24 hour pre-exposure to mirtoselect was chosen and assessments of cell growth were carried out with mirtoselect, treating the cells for 24 hours with mirtoselect then leaving the mirtoselect containing media on the cells for the duration of the experiment.

3.2.1.1.b Continuous Single Dose of Mirtoselect

Treatment of RT112 cells with a single continuous dose of varying concentrations of mirtoselect induced a dose response at the 24 hour time point, similar to that seen in the previous experiment (Figure 3.2-1) this was maintained throughout the experiment (Figure 3.2-2). Applying a single dose of mirtoselect to cells did not cause cell numbers to fall below the original number seeded, however, treatment with 75 and 100µg/ml mirtoselect did cause cells numbers to differ significantly from the control at 48 and 72 hours, respectively. Although treatment with 100µg/ml mirtoselect did not seem to cause cell toxicity, as indicated by cell numbers falling below starting numbers, cell numbers in this highest treatment group did not differ significantly from starting numbers over the time course of this experiment. A dose response was also seen in the RT4 cell line which was again maintained at the 72 hour time point even though no new mirtoselect had been applied. At all concentrations of mirtoselect, cells showed an ability to proliferate; only at the highest dose (100µg/ml) and latest time point (72 hours) did cell numbers differ significantly from the untreated control. Out of the three

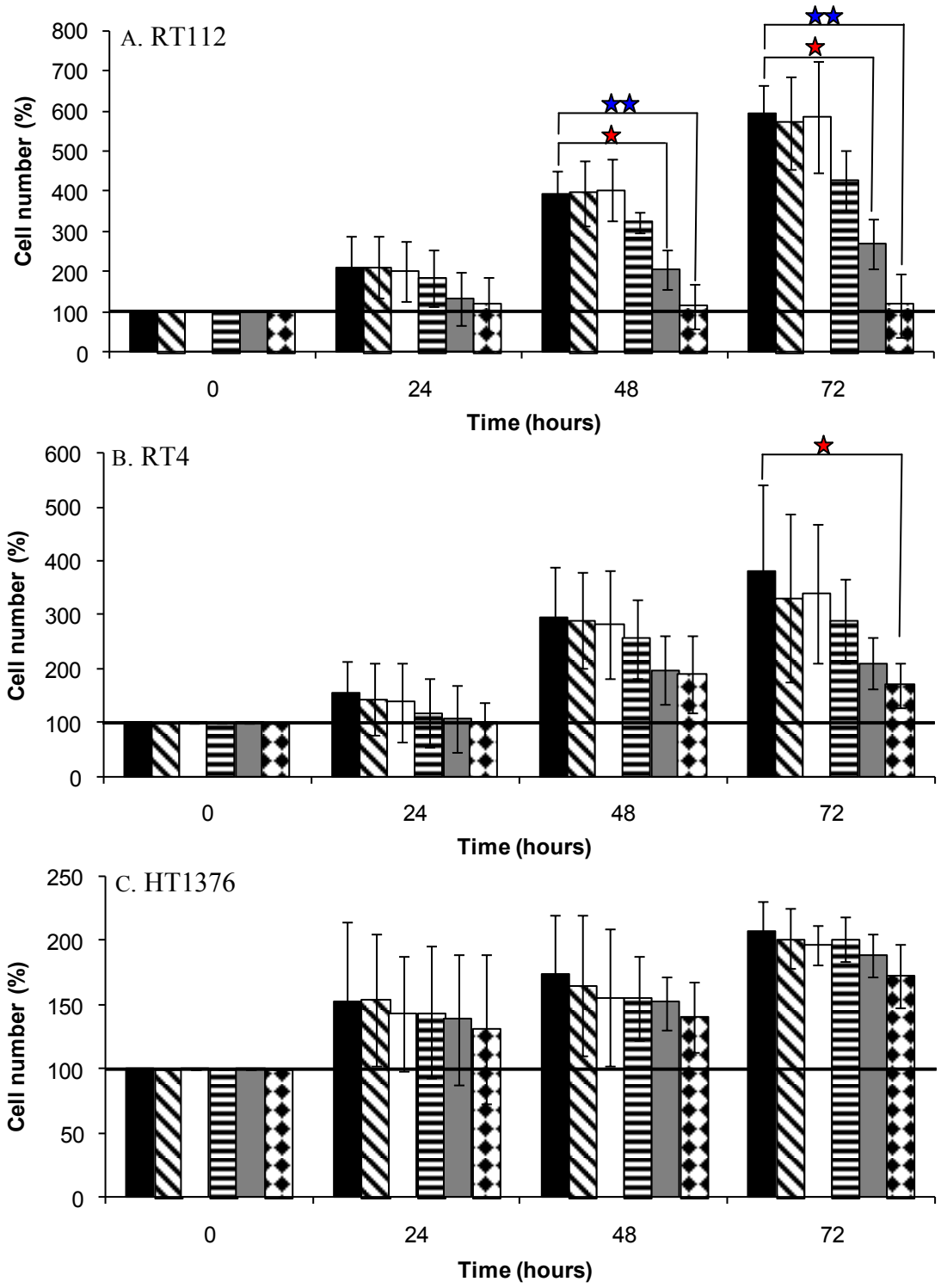


Figure 3.2-2 Cell Growth Graphs in Bladder Cells Treated with a Single Continuous Dose of Mirtoselect.

Cell growth graphs for bladder cell lines treated over 72 hours with a single dose of mirtoselect at 0 ■, 2 ◪, 20 □, 50 ▬, 75 ▩ and 100µg/ml ◘. Treatments were added to cells at 0 hours and then left on for the duration of the experiment with no change of media. Results are the mean of 3 independent experiments (n=9) ±SD. Results are expressed as percentage cell number relative to the number of cells at 0 hours, $p \leq 0.05 =$ ★, $p \leq 0.01 =$ ★★.

cell lines studied, mirtoselect had the least effect on growth in the HT1376 cells with none of the treatments differing significantly from control.

3.2.1.2 MMC Anti-Proliferative Effect

MMC is a proven anti-proliferative agent in bladder cancer cells (Volpato *et al*. 2005). Work from this current project has already established that mirtoselect was also able to cause a significant decrease in cell numbers. It was therefore important to investigate the potential impact mirtoselect may have on the standard response, in the bladder cancer cell lines used, of the chemotherapeutic agent MMC. The effect of MMC on these cell lines therefore had to be established. As for many different chemotherapeutic agents *in vitro* and *in vivo,* varying responses were seen in the three different bladder cell lines as a result of treatment with MMC alone (Figure 3.2-3). Sensitivity to MMC, measured via final cell number at 96 hours, indicated that RT4 cells were the most sensitive to MMC with cell numbers falling below the starting number. RT112 cells were also highly sensitive to MMC with cell numbers falling from 295% at 48 hours to 149% by 96 hours. HT1376 cell line had the greatest number of cells remaining at 96 hours compared to the other cell lines, cell numbers remained fairly constant over the 72 hours post MMC treatment, indicating it was the least sensitive to MMC-induced toxicity. At 48 hours, 24 hours after the addition of MMC, all three cell lines had reduced cell numbers in the MMC treated cells compared to their respective untreated controls, although no significant difference was observed. In RT112 and RT4 cell lines, treatment with MMC caused a significant difference in cell numbers at 72 hours and by 96 hours all cell lines differed significantly from their respective untreated controls.

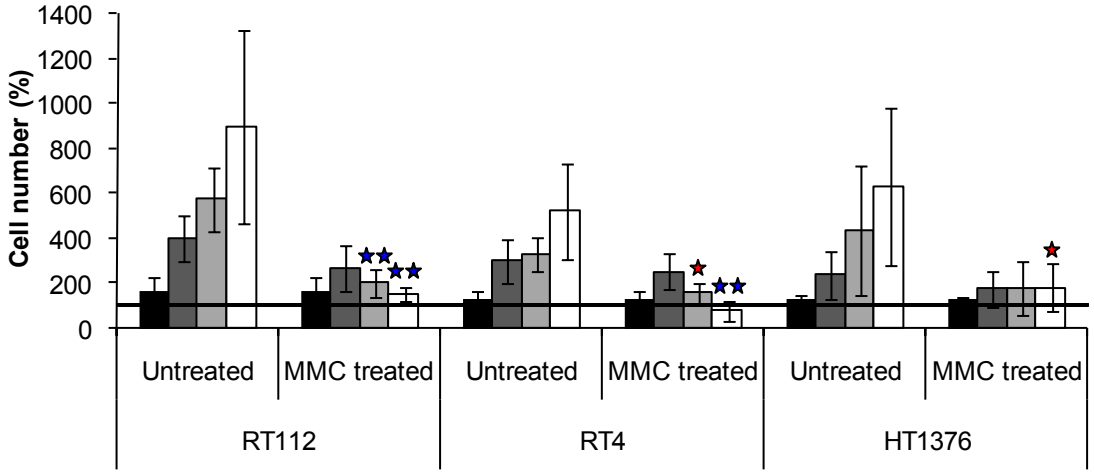


Figure 3.2-3 Cell Growth Data for Cells Treated with MMC Alone
Cell growth data for bladder cell lines treated with 10μM MMC. MMC was administered after the 24 hour time point and was left on the cells for the duration of the experiment, measurements were taken at 24 ■, 48 ■, 72 ■ and 96 hours □. Results are expressed as percentage cell number relative to the number of cells at 0 hours in untreated cells. Results are the mean of at least 3 independent experiments (n=9) ±SD, $p \leq 0.05$ =★, $p \leq 0.01$ =★★ compared to untreated control.

3.2.1.3 Combined Mirtoselect and MMC Treatments

Mirtoselect had shown an ability to cause significant dose dependent decreases in cell numbers in RT112 and RT4 cell lines (Figure 3.2-1 and Figure 3.2-2). The initial anti-proliferative effect seen in these cell lines after only 24 hours was maintained even without the addition of subsequent doses of mirtoselect. In designing combined treatment regimens with MMC and mirtoselect it was decided a pre-treatment period of 24 hours with mirtoselect was sufficient prior to the addition of MMC. Growth experiments were therefore carried out with a pre-incubation period of 24 hours with mirtoselect, followed by MMC, to determine whether the standard response to MMC seen in Figure 3.2-3 was altered by mirtoselect (Figure 3.2-4).

Prior to MMC treatment there was a clear dose dependent decrease in cell number in RT112 and RT4 cell lines, with a more subtle effect in HT1376 cells, in response to 24 hour mirtoselect treatment, as previously observed. A significant difference was

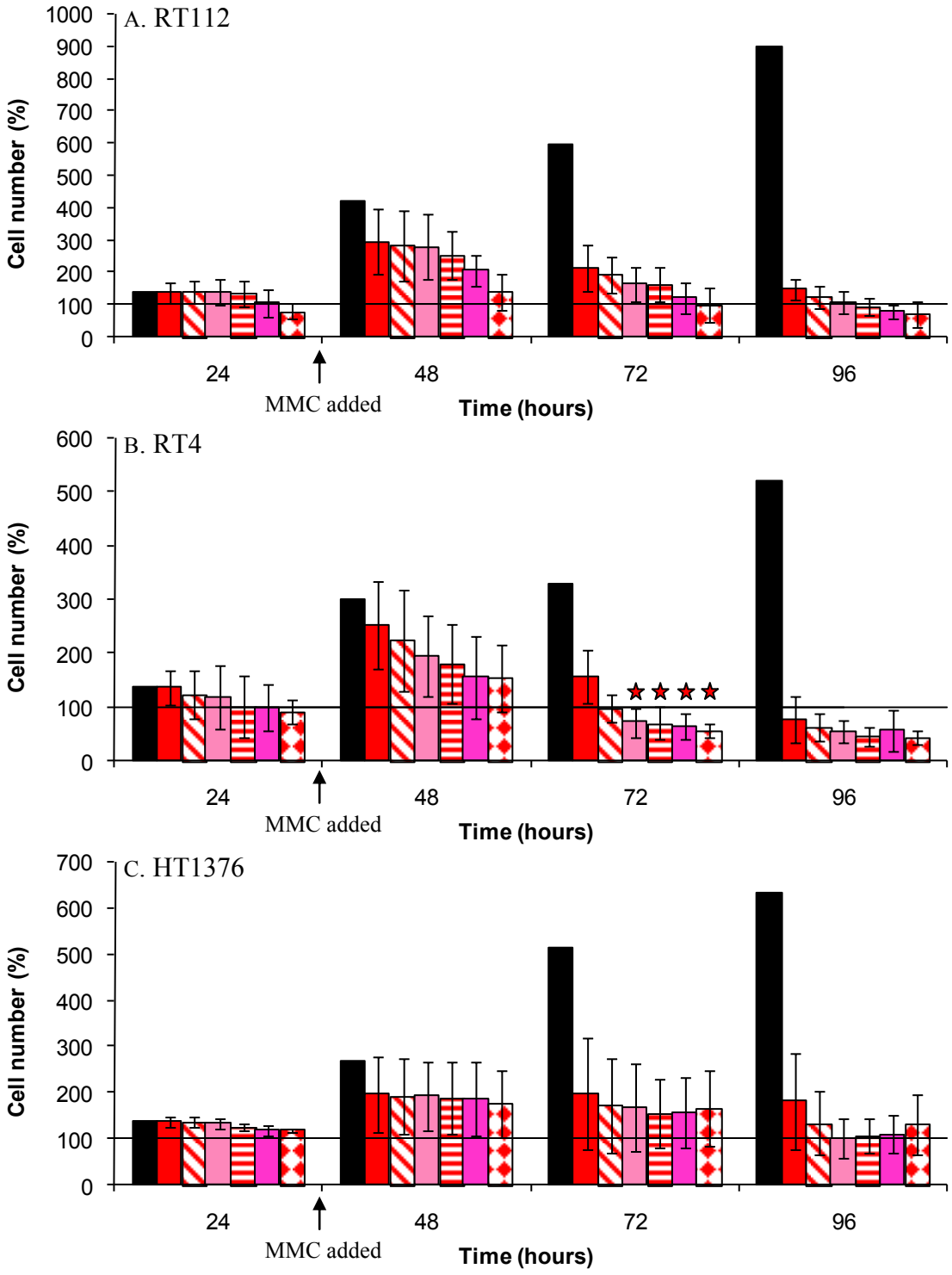


Figure 3.2-4 Combined Treatment Growth Experiments
Cell growth graphs for bladder cancer cell lines either untreated, no mirtoselect or MMC ■ , or treated for 24 hours with mirtoselect at 0 ■, 2 ■, 20 ■, 50 ■, 75 ■ and 100µg/ml ■ followed by 10µM MMC. Results are the mean of 3 independent experiments (n=9) ±SD. Results are expressed as percentage cell number relative to the number of cells at 0 hours in the control cells, $p \leq 0.05$ = ★ compared to the MMC treated cells alone.

observed at 72 hours between RT4 cells pre-treated with 20-100µg/ml mirtoselect compared to those treated with MMC alone. Although significance was not maintained at 96 hours, only 44% of cells remained in 100µg/ml mirtoselect pre-treated cells compared to 77% in MMC alone treated cells.

Whilst no significant differences were observed in RT112 or HT1376 cells, between combined treatment and MMC alone, there was, however, a non significant trend towards combined treatment enhancing the anti-proliferative effect of MMC. In previous experiments 2 and 20µg/ml mirtoselect had no effect on either cell line and 50µg/ml caused only a small decrease in cell numbers in RT112 and RT4 cell lines (Figure 3.2-2), yet in combined treatments (2, 20 and 50µg/ml mirtoselect at 72 and 96 hours) all three cell lines had clearly fewer cells compared to the MMC alone control. As mirtoselect had been shown to have a prolonged effect on cell growth even after a single administration (Figure 3.2-2), it was hypothesised that at these lower concentrations, which on their own were unable to cause significant differences in cell numbers, mirtoselect could be acting synergistically with the MMC to cause an enhanced anti-proliferative or cytotoxic effect. Although the appropriate experiments were not conducted in this study to actually prove synergy was occurring between mirtoselect and MMC (Pinmai *et al*. 2008), the data were adjusted to account for the observed mirtoselect anti-proliferative effect previously seen. This was performed to indicate whether the trend of decreased growth apparent with lower concentrations of mirtoselect might potentially be due to the mirtoselect acting in a synergistic manner with MMC (Figure 3.2-5). Data from each of the cell lines treated with a single continuous dose of mirtoselect (Figure 3.2-5A) was used to determine a correction factor for the effect the mirtoselect had at each concentration and time point (Equation 2.5-2).

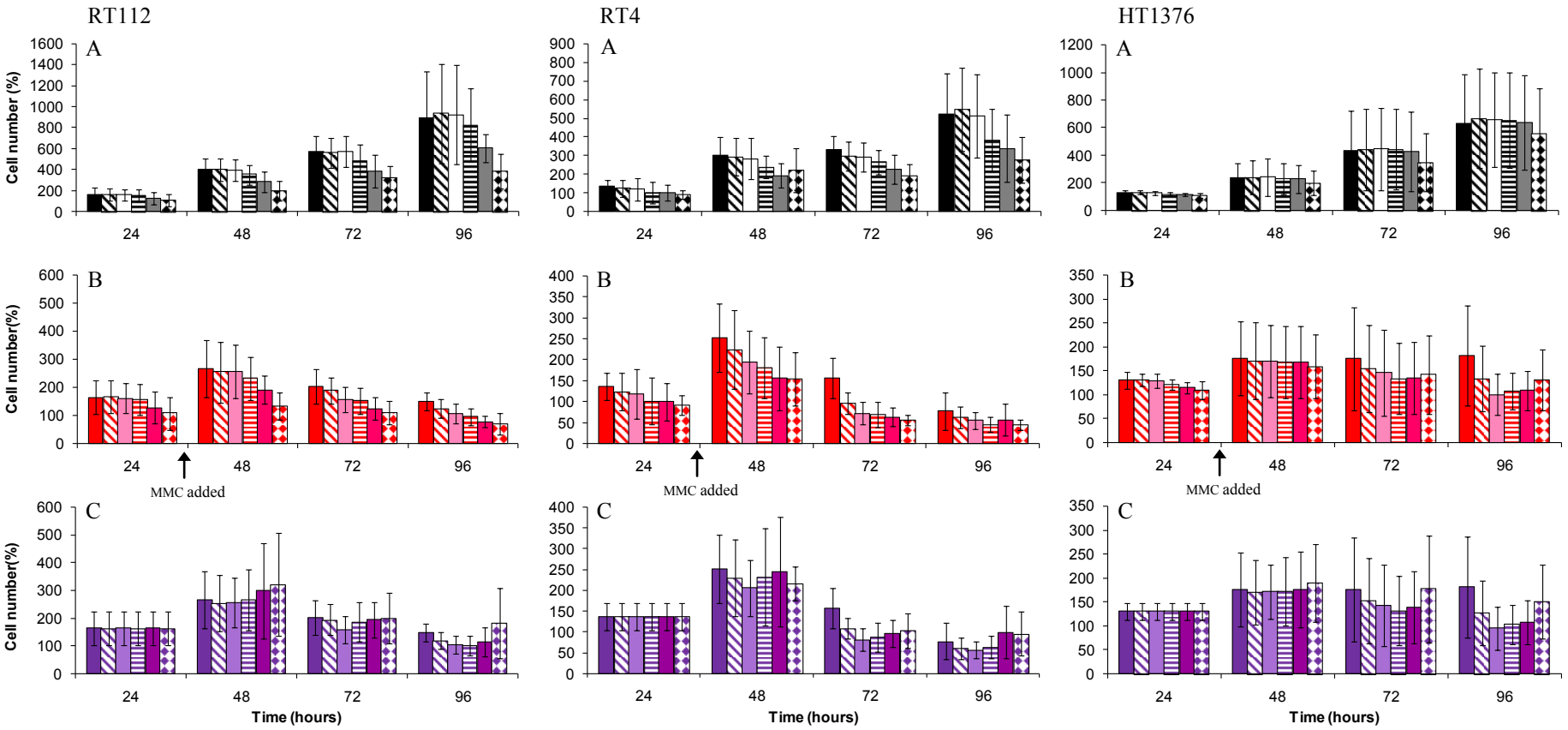


Figure 3.2-5 Adjustment of Data to Determine the Interaction between Mirtoselect and MMC

Cell growth graphs for RT112, RT4 and HT1376 cell lines. (A) Cells were treated with a single continuous dose of mirtoselect at 0 ■, 2 ◹, 20 □, 50 ▬, 75 ▩ and 100µg/ml ◪. (B) Cells were treated mirtoselect for 24 hours at 0 ■, 2 ◹, 20 ▩, 50 ▬, 75 ■ and 100µg/ml ◪ followed by 10µM MMC for the duration of the experiment. (C) Data from combined treatments (graph B) adjusted for the effect seen with mirtoselect alone (graph A); data refers to mirtoselect treatments of 0 ■, 2 ◹, 20 ▩, 50 ▬, 75 ■ and 100µg/ml ◪. Results are the mean of 3 independent experiments (n=9 in A and B, n=3 in C) ±SD. Results are expressed as percentage cell number relative to the number of cells at 0 hours in the control incubations.

This correction factor was then applied to the combined treatment data (Figure 3.2-5B) to determine whether the observed, matched the expected (Figure 3.2-5C). If the effect seen in the combined data was simply due to an additive effect, the correction for the mirtoselect would result in a flattening of the graphs, if there was a less than additive effect, the cell numbers would be higher than the MMC alone data and if there was any synergy between agents cell numbers would be lower than the MMC alone data. Analysis of the adjusted data set in each of the cell lines proved that mirtoselect did not adversely interfere with the anti-proliferative effect of MMC with the possible exception for RT112 and RT4 where a less than additive response was observed at some time points and concentrations of mirtoselect. At 96 hours a trend towards a U-shaped dose response curve was seen. Lower concentrations of mirtoselect (2 and 20μg/ml) displayed a trend towards a synergistic effect in combined treatments, notably in the HT1376 cell line. The highest dose of mirtoselect, 100μg/ml, although the most significant in causing decreases in cellular growth when applied singularly, may in the instance of RT112 and RT4 only be additive in combination. None of the data points, however, were significantly different from control cells, treated with MMC alone, in the adjusted data.

3.2.2 Clonogenic Cell Survival Assay

Cell survival using the clonogenic assay was used to measure the long term replicative capacity of the cells after treatment. MMC is a known cytotoxic agent able to severely affect a cell's long term proliferative ability; the clonogenic assay was used as a way of assessing the impact that pre-treatment with mirtoselect may have on cells over a longer time frame. A large amount of preliminary work determining adequate cell numbers and incubation techniques was needed before a working protocol could be established.

All three cell lines were assessed for their ability to form colonies, differing incubation periods were required with each cell line with the fastest growing cell line, RT112, able to form visible colonies within 3 weeks whereas the slower growing RT4 and HT1376 cell lines took between 4-5 weeks. Plating efficiency of each of the three cell lines was also assessed with the aim of achieving between 50-200 visible colonies per dish at the end of the experiment. Based on preliminary experiments carried out to determine a dose response to MMC, a concentration range of 0-3μM was chosen.

Growth studies had shown a 24 hour treatment with mirtoselect was sufficient to cause non significant differences in a cells ability to proliferate, clonogenic experiments were therefore carried out treating cells with mirtoselect alone for 24 hours and leaving them to form visible colonies. The three bladder cell lines being investigated behaved very differently to the varying concentrations of mirtoselect (Figure 3.2-7A). HT1376 cells were the least affected by mirtoselect, with no clear dose dependent decrease in cell survival. RT112 and RT4 cell lines displayed similar responses to mirtoselect treatment, with even the lowest of concentrations causing ~20% decrease in cell viability. Both RT112 and RT4 proved to be highly sensitive to mirtoselect treatment with only ~7% and 1% of cells surviving, respectively, after a 24 hour treatment with 50μg/ml mirtoselect.

Combined treatments of mirtoselect and MMC were performed for each of the cell lines over a concentration range of 0-50μg/ml mirtoselect and 0-2μM MMC. For RT112 and RT4 cell lines the highest dose of mirtoselect (50μg/ml) could not be achieved due to the low numbers of surviving cells in the mirtoselect alone treatments, which could not be rectified by increasing cell numbers.

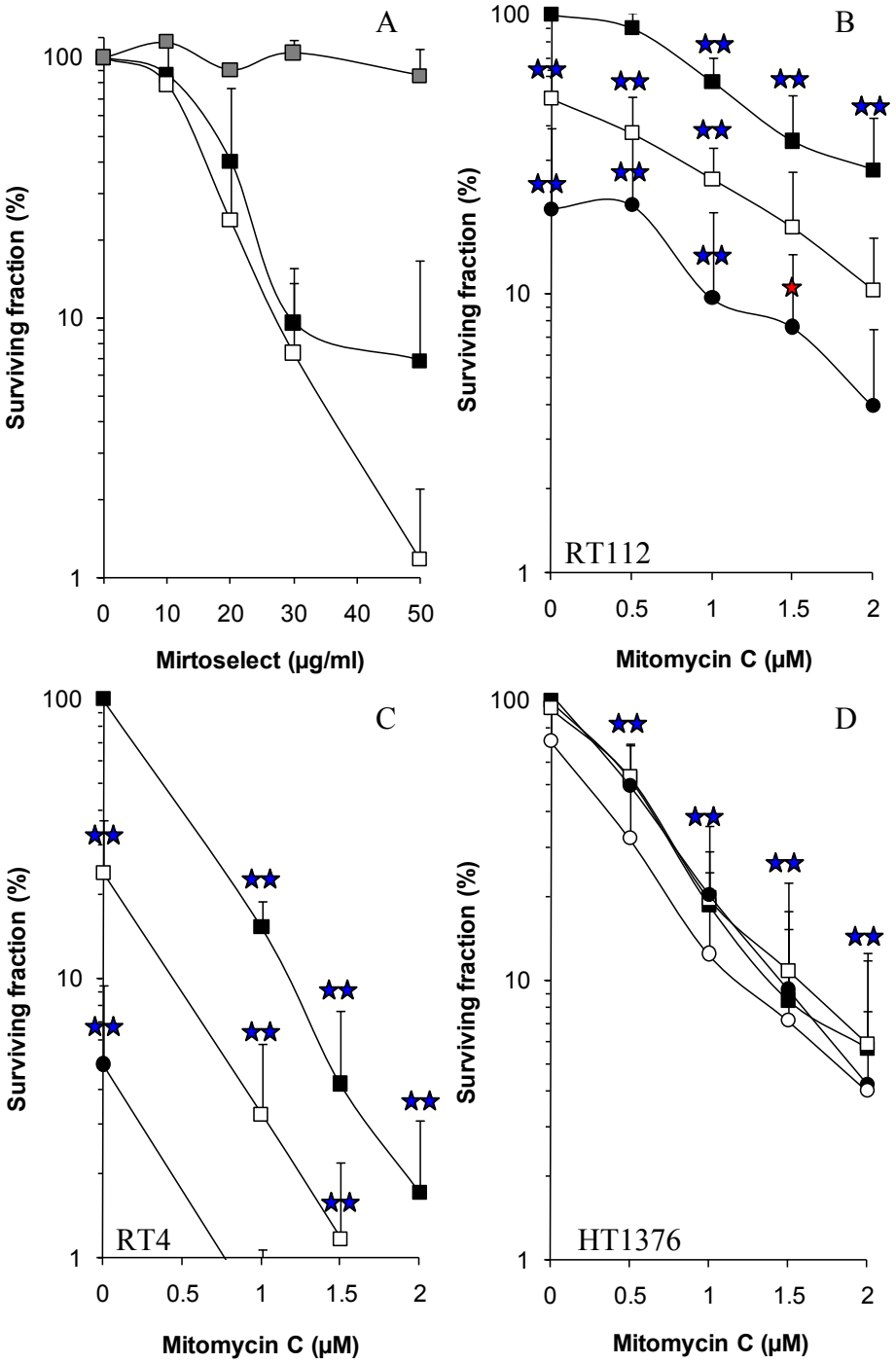


Figure 3.2-6 Clonogenic Survival of all Bladder Cancer Cells

Clonogenic cell survival graphs. A; Treatment of cells: RT112 ■, RT4 □ and HT1376 ▩ with varying concentrations of mirtoselect for 24 hours. B-D; Treatment of RT112, RT4 and HT1376 cells, respectively, with 0 ■, 20 □, 30 ● and 50µg/ml ○ mirtoselect for 24 hours followed by 1 hour treatment with varying concentrations of MMC. Results are the mean of 3 independent experiments (n=9) ±SD, $p \leq 0.05$ = ★, $p \leq 0.01$ = ★★.

In RT112 cells, MMC treatment caused a significant decrease in the surviving fraction (SF) of cells at concentrations >1μM (1, 1.5 and 2μM p=0.000) compared to control (Figure 3.2-6B). Treatment with mirtoselect alone also caused a significant decrease in SF (20μg/ml and 30μg/ml p=0.000). In combination with MMC 20μg/ml pre-treatment with mirtoselect resulted in significantly fewer colonies at 0.5 and 1μM MMC compared to MMC alone whilst 30μg/ml pre-treatment also caused significantly fewer colonies at 1.5μM. RT4 cells had significantly fewer cells in MMC alone treated cells compared to control and were more shown to be more sensitive to MMC than RT112 cells with ~2% cell survival after treatment with 2μM MMC (Figure 3.2-6C). Mirtoselect treatment also resulted in significantly fewer colonies in 20 and 30μg/ml treated RT4 cells compared to control. As RT4 cells were far more sensitive to MMC than RT112 cells and also showed a significant inhibition on survival by mirtoselect only a few combinations of MMC and mirtoselect could be achieved. In 20μg/ml pre-treated cells a significant difference in surviving colonies was observed between pre-treated cells and MMC alone treated cells at 1 and 1.5μM (p=0.000). In HT1376 cells MMC caused a significant decrease in SF at concentrations >0.5μM (0.5μM p=0.003, 1, 1.5 and 2μM p=0.000) (Figure 3.2-6D). Mirtoselect treatment, however, did not cause a significant difference in SF even at 50μg/ml. There was also no significant difference in SF in combined MMC and mirtoselect treated cells compared to MMC alone.

In order to determine if any enhancement had occurred in combined treatment because of the mirtoselect pre-treatment, in RT112 and RT4 cells the known effect of the mirtoselect was adjusted for by comparing each of the MMC treatments to the mirtoselect control rather than the untreated control (Figure 3.2-7). This was not done for HT1376 cells as no significant effect by mirtoselect had been seen previously (Figure 3.2-6D).

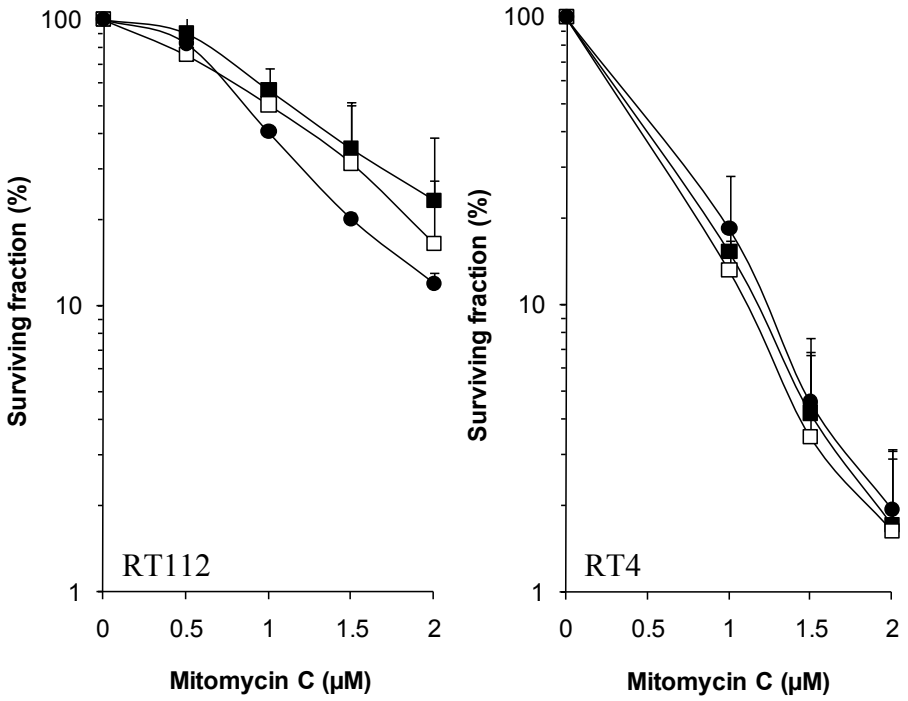


Figure 3.2-7 Assessment of the Effect of Mirtoselect Pre-Treatment on MMC Cytotoxicity
Clonogenic cell survival graphs were adjusted to account for the effects of mirtoselect alone to determine if in combination the cytotoxicity of MMC was in anyway affected. A-C; Treatment of RT112, RT4 and HT1376 cells, respectively, with 0 ■, 20 □, 30 ● and 50µg/ml ○ mirtoselect for 24 hours followed by 1 hour treatment with varying concentrations of MMC. Results are the mean of 3 independent experiments (n=9) ±SD.

In RT112 cells combination of 30µg/ml mirtoselect and 1µM MMC resulted in ~40% of cells surviving compared to 57% of the MMC alone treated cells and at higher MMC concentrations 30µg/ml mirtoselect resulted in ~12% survival compared to 23% in MMC alone treated cells (Figure 3.2-7). This indicates that in 30µg/ml pre-treated cells the combination of MMC and mirtoselect causes a decrease in SF greater than either agent alone, however, no data point was significantly different from the MMC alone control.

Although RT4 cells were found to be more sensitive to mirtoselect (Figure 3.2-6C) and MMC in measures of cell survival (Figure 3.2-7C), they showed a more subtle enhancement effect in combined treatments compared to RT112 cells. No significant

difference was observed between MMC alone cells and pre-treated cells adjusted for the mirtoselect effect, indicating little interaction between the two agents.

3.3 Discussion

3.3.1 Cellular Growth and Cell Survival in Response to Mirtoselect

Short term measures of cellular growth were performed to assess whether mirtoselect possessed an anti-proliferative capacity in bladder cancer cells, as reported for other anthocyan containing compounds in various cell lines. To our knowledge this is the first reported investigation into the effect of anthocyans in bladder cancer cell lines. Growth was measured using a fluorescent nucleic acid binding dye, over a concentration range of 2-100µg/ml. Initial studies using a repeat dosing strategy proved that mirtoselect did in fact possess anti-proliferative activity in all three cell lines investigated and had a significant effect on two of the three cell lines at concentrations of 75-100µg/ml. The vast majority of studies in the literature investigating the effects of anthocyans on cell growth *in vitro* have been conducted using single agents, a small number of investigators have used mixtures of anthocyans but to our knowledge only two of these (Wu *et al*. 2007, Zhao *et al*. 2004) reported using a commercially available anthocyanin-rich extract (ARE) that could be comparable to mirtoselect. In the study by Zhao *et al* (2004), however, the commercial AREs were semi-purified by the investigators, using solid phase extraction, isolating the anthocyanins and other phenolics. The anthocyanin content of the AREs were then assessed based on the content of the anthocyanin cyanidin-3-glucoside. The bilberry ARE was able to significantly inhibit growth of HT29 colon cancer cells at concentrations of 25-75µg/ml. Mirtoselect is a mixture of 15 different anthocyanins (Cooke *et al*. 2006) containing 25% anthocyanins (w/w) (Indena data sheet) therefore the concentrations used here that

gave a significant anti-proliferative effect (75-100µg/ml) equate to a anthocyanin content, of ~19-25µg/ml. At the corresponding 25µg anthocyanin/ml concentration, the bladder cancer cell line RT112 used in this study exhibited greater levels of growth inhibition to that seen by Zhao *et al*. for HT29 cells, however, inhibition in RT4 cells (36% at 48 hours and 50% at 72 hours) was comparable to that seen in the HT29 cells (25% at 48 hours and 53% at 72 hours). Wu *et al*. (2007) used an alternative anthocyanin mixture containing the same 15 anthocyanidin glycosides found in mirtoselect, however, the exact composition was not stated. This mixture of anthocyanins was found to be significantly anti-proliferative in HT29 cells at concentrations ≥75µg/ml, which is in agreement with the results seen in RT112 and RT4 cell lines, however, Wu *et al.* observed this at the earlier incubation time point of 24 hours. Interestingly Wu *et al*. also found that the mixture of anthocyanins was far more effective at inducing growth inhibition than the same concentration of the single, cyanidin-3-glucoside, anthocyanin, achieving 20% greater inhibition of cell growth at concentrations of 100µg/ml. The effects of a number of different anthocyanidins, anthocyanins and AREs on cancer cell growth *in vitro* have been reviewed elsewhere (Cooke *et al*. 2005). In the majority of the reports using anthocyanins, concentrations in the region of >400µM were required to obtain growth inhibition of ~80% (Katsube *et al*. 2003, Olsson *et al*. 2004, Meiers *et al*. 2001, Shih *et al*. 2005). As mirtoselect is a mixture of 15 different anthocyanins comparison between these studies and ours is somewhat difficult, however, using the molecular mass of the most abundant anthocyanin, delphinidin-3-galactoside (16% of mirtoselect anthocyanins), as a means of determining a relative anthocyanin concentration, the highest concentration of 100µg/ml which caused 80% growth inhibition in RT112 with single dosing regimen after 72 hours exposure (Figure 3.2-2) equates to ~72µM anthocyanins. Katsube *et al.*

(2003) found that a concentration of 431μM delphinidin-3-galactoside was needed in order to induce growth inhibition of ~80% in HL60 promyelocytic leukaemia cells. Even though 80% growth inhibition was not achieved in RT4 cells, 100μg/ml mirtoselect did achieve ~40% growth inhibition, indicating that the bladder cancer cell lines investigated are far more sensitive to the anti-proliferative effects of anthocyanins than previously reported cell lines.

Both dosing strategies gave similar profiles in relation to the anti-proliferative effect of mirtoselect in the cell lines investigated, with a sensitivity ranking of RT112 > RT4 > HT1376. HT1376 cell line originated from a primary bladder cancer, histological grade 3 and clinical stage T2 (Masters *et al*. 1986). Of the three bladder cancer cell lines investigated it was the least representative model of superficial disease, however, RT4 cell line represents a more accurate model of non-muscle invasive bladder cancer (G1 pTa) (Crallan *et al*. 2006). It is therefore possible that the differential anti-proliferative effects seen within these cells could be due their stage and grade, with mirtoselect only being able to have a significant impact on low grade well differentiated tumours.

Clonogenic cells survival indicated that in two of the three cell lines, RT112 and RT4, mirtoselect caused a significant reduction in the number of surviving cells after a 24 hour treatment with concentrations of mirtoselect ≥20μg/ml compared to control. These results are more than striking when compared to the growth assay results in which 20μg/ml had no effect. Clonogenic survival and growth assays also differed in the rank order of mirtoselect sensitivity for these cell lines, with growth studies ranking RT112 the most sensitive whereas the clonogenic assay ranked RT4 highest. Previous reports have also revealed that correlation between short term measures of proliferation, using the MTT assay, and clonogenic assays is not always established (Lazze *et al*. 2004).

Investigators reported that in MTT assays cyanidin had no effect on cell viability at concentrations up to 200μM in three different cell lines (normal human fibroblasts [NHF], uterine carcinoma cells [HeLa] and colon adenocarcinoma cells [CaCo-2]), whereas in clonogenic assays the same cell lines all exhibited a dose dependent decrease in cell survival over the same concentration range. In the same study delphinidin was shown to cause cytotoxicity at the highest concentrations (150 and 200μM) in HeLa cells, which correlated with clonogenic cell survival. At the non cytotoxic concentration, as determined by viability being greater than 80% in the MTT assay, of 100μM, however, the rank order of sensitivity to delphinidin in MTT measures (Hela > CaCo-2 > NFH) did not correlate with clonogenic assays (NFH > HeLa > CaCo-2). The clonogenic assay is considered to be the more accurate determinant of cytotoxicity since it takes into account any initial anti-proliferative effects, that can be measured using proliferation assays such as the MTT and CyQUANT NF assays, as well as assess a cells long term survival and capacity to overcome that proliferative inhibition (Skehan, 1999). On the basis of the clonogenic data, therefore, mirtoselect is extremely cytotoxic in RT112 and RT4 cell lines and warrants further investigation into its mechanism of action especially in light of previous reports highlighting the antioxidant properties of anthocyanin containing agents.

As expected, corresponding responses to mirtoselect were observed in growth studies between the two dosing regimens, in all three of the cell lines, over the initial 24 hours, supporting the robustness of the assay. Comparison of the growth data obtained from the two different dosing strategies suggested that repeat dosing of mirtoselect caused a greater inhibition of growth at the later time points, however, even a single dose of mirtoselect applied to cells was able to cause a prolonged inhibition of cellular growth up to 72 hours. At the highest concentration of mirtoselect, repeat administration

induced cytotoxicity in RT112 cells, as indicated by the relative cell number falling below the starting value, and although this is not seen in the cells treated with a continuous exposure to a single dose of mirtoselect, there is no significant difference ($p \leq 0.05$) in cell number at any time point at any concentration of mirtoselect between the two different treatment techniques indicating a prolonged anti-proliferative effect by mirtoselect up to 72 hours even after a single dose of mirtoselect. The majority of reports in the literature looking at the potential growth inhibitory properties of chemopreventive agents *in vitro* use a single dose regimen, requiring relative high concentrations of agents in order to achieve growth inhibition at 24 hours (Katsube *et al*. 2003, Olsson *et al*. 2004). In studies in which investigators have used longer exposure periods or longer incubation periods much lower concentrations of agents are required to induce similar levels of growth inhibition (Chen *et al*. 2005, Moiseeva *et al*. 2007). The bioavailability and stability of anthocyans has been reported to be low (Talavéra *et al*. 2006, Seeram *et al*. 2001, Fleschhut *et al*. 2006, McDougall *et al*. 2007). In culture, degradation of anthocyanidins occurs very quickly, after 3 hours of incubation Kern *et al.* (2007) were only able to detect <10% of the applied concentration of anthocyanidins, and so many investigators have assumed the effects of anthocyans to also be short lived *in vitro*. Along with the disappearance of the parent anthocyanidins Kern *et al*. also found there to be an increase in anthocyanidin breakdown products (phenolic acids), which themselves have been shown to posses anti-proliferative effects (Wu *et al*. 2007), however, these were judged to be of an insufficient level to account for the complete loss of the applied anthocyanidins (Kern *et al*. 2007). Kern *et al*. therefore hypothesised that confounding factors such as protein binding could account for the disappearance of the anthocyanidins from culture media but also for their prolonged anti-proliferative as a result of later release from those

proteins. In experiments to determine whether the effects of delphinidin were reversible Afaq *et al*. (2008) demonstrated that removal of delphinidin from the culture media resulted in a gradual recovery from the growth inhibitory effects in AU565 breast cancer cells, whereas in experiments where the media was replaced with fresh delphinidin, there was a time dependent decrease in growth (Afaq *et al*. 2008). These findings alongside our own, in which a repeat exposure to 50μg/ml resulted in cell numbers that were statistically insignificant ($p \leq 0.05$) from cells exposed to a single prolonged concentration of 75μg/ml mirtoselect, suggest that repeat dosing regimens of lower concentrations of agents can result in similar levels of growth inhibition to single exposures of higher concentrations.

3.3.2 Cellular Growth and Cell Survival in cells Treated with Mirtoselect and MMC

Measurement of cellular growth after treatment with MMC revealed a difference in the response of the three cell lines to MMC. HT1376 was the least sensitive to MMC and RT4 the most sensitive, as determined via final remaining numbers at 96 hours. Overall response is also an important factor in determining the effectiveness of a cytotoxic compound. RT112 and RT4 both reveal an initial increase in cell number 24 hours post MMC treatment, with RT112 numbers doubling and RT4 almost doing so, followed by a steady decrease, with cell numbers returning to similar values prior to treatment for RT112 and falling below starting values for RT4. In the HT1376 cell line it appears MMC acts slightly differently, potentially causing cellular growth to cease as cell numbers remain unchanged over the period analysed. Clonogenic cell survival in combined treatments was investigated in each of the cell lines. In combined treatments, where the effect of the mirtoselect is adjusted for (by comparing each combined

treatment to a mirtoselect alone treated control) some differences were visible in the clonogenic data. In relation to the MMC alone treated control, RT112 cells exhibited the most substantial effect as a result of the combination, followed by RT4 and with HT1376 having little or no effect in combination. In growth studies it is the lower concentrations of mirtoselect that appear to have the most impact in combination with MMC, possibly acting synergistically to cause a decrease in cell numbers. As previously mentioned the appropriate experiments were not performed to confirm that synergy is actually taking place in these samples, however, in HT1376 cells, in which the mirtoselect had no substantial effect and MMC caused cell numbers to remain unchanged, combination of the two agents causes a decrease at 96 hours with a 40-45% inhibition of growth in 20-75μg/ml treated combinations compared to cells incubated with MMC alone, implying a positive interaction between the two agents. A number of different chemopreventive agents and chemotherapy drugs have shown to have growth inhibitory effects in combination greater than either agent alone (Sharma *et al*. 2004, Tharakan *et al*. 2010, Li *et al*. 2005, Sarkar and Li, 2004, Cover *et al*. 1999) and some have shown the interaction to be synergistic (Sharma *et al*. 2004, Tanos *et al*. 2002, Lev-Ari *et al*. 2005). In a study using chemopreventive agent proanthocyanidin (PA) at concentrations unable to cause any growth inhibition, the investigators demonstrated that in combination with a chemotherapeutic agent, doxorubicin (DOX), the inhibitory effect of DOX was enhanced (Zhang *et al*. 2005b, Zhang *et al*. 2005a). In a bladder cancer xenograft tumour bearing model Xin *et al.* (2005) were able to show that at concentrations unable to cause growth inhibition, suramin, an anticancer agent, caused a significant enhancement on the activity of MMC. Previous studies using suramin clinically, have found concentrations of 100-200μM which gave only modest activity were severely toxic (Rapoport *et al*. 1993), however, investigators using low dose

suramin saw chemosensitization in human tumours without enhancing toxicity (Xin *et al*. 2005). Our data also suggests that using lower concentrations of mirtoselect in combination with MMC may be potentially more beneficial clinically than concentrations that can themselves cause cytotoxicity.

3.3.3 Conclusion

Studies of mirtoselect treatment have shown that in clonogenic studies mirtoselect is cytotoxic in RT112 and RT4 cells causing significant decreases in surviving fraction of cells at concentrations ≤20µg/ml. In both measures of cellular response to combined treatment, the three cell lines displayed varying degrees of sensitivity to the agents. However, at no concentration or time point investigated did combination of the two agents have an overall adverse effect i.e. in growth studies cell numbers were consistently lower in combined treated samples than MMC alone and similarly the percentage of surviving cells in combined treatments were lower than the MMC control in clonogenic studies. In most instances pre-treatment with mirtoselect slightly enhanced the cytotoxicity of MMC. Adjusted growth data did show a trend towards the highest dose of 100µg/ml mirtoselect possibly inhibiting the action of MMC; this dose could not be achieved in clonogenic experiments due to the mirtoselect itself decreasing cell survival too drastically to achieve any visible colonies, however an enhancement was seen in clonogenic experiments in RT112 cells at 30µg/ml although this was not significant. We therefore believe lower concentrations of mirtoselect (20-50µg/ml) may be more promising for investigation clinically.

Chapter Four

The Effect of Mirtoselect Pre-Treatment on MMC-Induced DNA Crosslink Formation and Repair as Determined by the Alkaline Comet Assay

4.1 Introduction

The majority of the cytotoxicity associated with MMC is attributed to the formation of crosslinks in DNA, which bring about cell cycle arrest and, if are not repaired, cell death. It is therefore possible, based on the results from growth and clonogenic assays (Chapter Three) that mirtoselect is enhancing MMC crosslink formation or modulating repair.

MMC is an alkylating agent and much of its toxicity and cytotoxicity is attributed to the formation of inter-strand DNA crosslinks. Methods for the detection of DNA crosslinks include alkaline elution (Kohn, 1991) and the Comet assay. DNA filter elution (alkaline elution) has been used successfully in the measurement of crosslinks, however, it has low sensitivity, the results are easily influenced by several unrelated variables and it is time consuming (Pfuhler and Wolf, 1996, McKelvey-Martin *et al*. 1993). The Comet assay on the other hand is a sensitive, inexpensive and rapid method for measuring, not only crosslinks, but many other types of DNA lesions when minor adjustments are applied to the standard protocol. (Collins, 2004). Studies have shown that measurement of DNA crosslinks, formed as a result of chemotherapeutic drugs, using the alkaline comet assay is a good method for detecting such lesions at clinical relevant doses (Merk and Speit, 1999, Hartley *et al*. 1999, Spanswick *et al*. 2002). Since formation and subsequent repair of crosslinks *in vitro* have been shown to have a positive correlation with patient response *in vivo* (Reed *et al*. 1986, Gupta-Burt *et al*. 1993, Parker *et al*. 1991) it was important to measure these parameters in relation to MMC in combination with mirtoselect, to assess if mirtoselect had any effect on the toxic lesions formed by MMC.

4.1.1 The Comet Assay

The single-cell gel electrophoresis assay (SCGE), or as its more widely known as the 'Comet assay', can be used to measure a number of different DNA lesions through variations in the assay's protocol. The Comet assay protocol was first reported by Östling and Johanson (Ostling and Johanson, 1984). In this original version, a neutral electrophoresis buffer was used, which allowed for the detection of double strand breaks (DSB), induced via ionising radiation, in single cells imbedded in agarose on glass slides. Modifications to the original protocol, including the introduction of electrophoresis under alkaline conditions by Singh *et al.* (1988), meant that a greater number of lesions could be detected including the more abundant single strand breaks (SSB), alkali labile sites (ALS) and incomplete excision repair sites, as well as the original DSB (Singh *et al*. 1988).

4.1.1.1 Alkaline Comet Assay

The most frequently used protocol for the detection of DNA lesions is the alkaline version of the assay; the key stages of this protocol are shown in Figure 4.1-1. This protocol involves the preparation of a single cell suspension which is then mixed with low melting point (LMP) agarose before being placed onto microscope slides, pre-coated with 1% normal melting point agarose. The gels are then allowed to set under coverslips; the coverslips being removed once the gels are set. The cells are lysed in the gels to remove cellular contents i.e. membranes and proteins (including histones) leaving behind a structure referred to as the nucleoid body. The nucleoid body is composed of supercoiled DNA held together with a number of scaffolding proteins. During the unwinding step of the protocol the nucleoid bodies are left in a buffer with a pH of greater than 13, resulting in a relaxation and denaturation of any of the strand

break damaged DNA. This is then followed by electrophoresis in the same buffer. Electrophoresis causes any strand broken DNA to migrate towards the anode of the electrophoresis tank, away from the undamaged DNA, which is retained in the nucleoid body, forming something which visually resembles a comet. The 'tail' of the comet is made up of damaged DNA and the 'head' undamaged DNA (Figure 4.1-1), therefore, the more DNA damage a cell contains the greater the amount of DNA there will be in the tail of the comet following electrophoresis. The gels are then neutralised before visualisation of the comets in order that the electrophoresis buffer does not interfere with the staining of the DNA later on. The gels are then washed free of all buffers in pure water and left to dry, ensuring all the nucleoid bodies are in the same plane of vision in the gel. A DNA-binding dye is then used (*e.g.* propidium iodide [PI] or ethidium bromide [EtBr]) to stain the gels prior to analysis using fluorescent microscopy. The comets can then either be scored visually using a visual classification system (Collins, 2004) or by using a computer, linked to a camera, which uses specifically designed image analysis software to determine particular parameters associated with the comets. The most commonly and widely accepted parameters used are Tail length, %Tail DNA (%TD) Tail Moment (TM) and Olive Tail Moment (OTM). Of the three parameters %TD has now become the measure of choice in published works as a result of its linearity with damage levels, its wide range over which damage can be measured, the fact that it bears a visual relevance to the comets and so allows for easy comparison of data between laboratories (Kumaravel and Jha, 2006). Whichever scoring method is used, a defined number of nucleoid bodies are scored per gel (usually 50 per gel, with 2 gels per sample (Collins *et al*. 1997) and the results are traditionally expressed as the mean value of the collective nucleoid body scores ± the standard error of the mean (SEM).

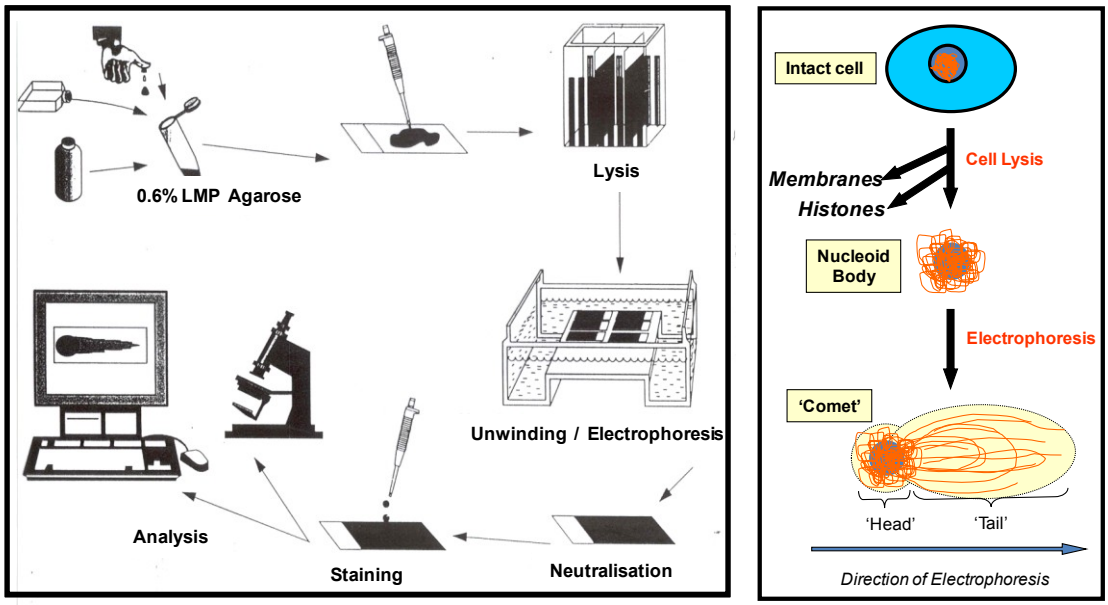


Figure 4.1-1 Comet Assay Outline
Outline showing the work flow involved in the standard alkaline Comet assay. A single cell suspension (possibly isolated from either blood, cells in culture or tissues) is embedded in low melting point agarose and placed onto a pre-coated microscope slide. The slides are then lysed, a process by which membranes and histones are removed, resulting in the formation of nucleoid bodies. The nucleoid bodies are then subjected to unwinding and electrophoresis (in an alkaline buffer). Neutralisation of the electrophoresis buffer is performed before staining with a DNA intercalating dye. Slides are analysed using fluorescent microscopy either visually or with the use of image analysis software. (Adapted image by McKelvey-Martin V *et al.*)

The main advantages to the Comet assay are its low requirements for test material, its speed, low experimental costs and high sensitivity. A number of studies and workshops have investigated parameters which affect variability within the assay and have prepared detailed protocols in order to help minimise inter-experimental variability in order to aid in inter-laboratory collaborations (Tice *et al*. 2000). In order to minimise variability it is recommended that conditions for the Comet assay are kept constant between repeats, these include temperature (room and tank), % agarose for the gels and electrophoresis conditions, namely voltage, amperage and duration.

4.1.2 Measuring Crosslinks using the Alkaline Comet Assay

As mentioned in Chapter One, MMC introduces crosslinks into the DNA of treated cells. Crosslinks cannot be measured directly via the standard comet assay protocol so further modifications have to be introduced into the standard alkaline protocol (Hartley *et al*. 1999, Olive *et al*. 1992, Olive and Banath, 1995). The measurement of MMC interstrand crosslinks has been previously shown to be easily measurable using a modified version of the comet assay (Merk and Speit, 1999, Volpato *et al*. 2005, Suggitt *et al*. 2003). Formation of crosslinks causes the DNA to have reduced electrophoretic mobility upon alkaline gel electrophoresis. To measure crosslink-induced decreased DNA mobility, a fixed level of damage is introduced into the cells immediately prior to analysis. SSB introduced into the DNA, usually via X-ray irradiation, results in a relaxing of the DNA and upon electrophoresis the damaged DNA is freed from the nucleoid body. As crosslinks cause the DNA to have reduced electrophoretic mobility, their presence in the DNA leads to a decrease in the measured extent of comet formation, in this case %TD, of a drug treated irradiated cell compared to the non-drug treated irradiated control. To facilitate the representation and interpretation of the data, decreases in the %TD are expressed as a percentage of the non-drug treated irradiated control (Hartley *et al*. 1999) whereby increasing values of '% decrease in %TD' are indicative of the formation of crosslinks (Figure 4.1-2). Furthermore, if cells are given a period of time post-treatment, in drug free media, repair of the crosslinks can be measured as a decrease in '% decrease in %TD' or increase in %TD (Figure 4.1-3). Interpretation of the data is once again achieved by comparing treated cells to non-drug treated irradiated controls, whereby levels of damage returning to that of the irradiated control are indicative of the removal of the crosslinks. Using this modification, the comet assay can be used to measure both the formation and repair of crosslinks and

assess a cell's intrinsic ability to repair DNA damage-induced via crosslinking agents. This method has also proved sensitive enough to assess crosslinks *in vivo* (Hartley *et al*. 1999, Almeida *et al*. 2006)

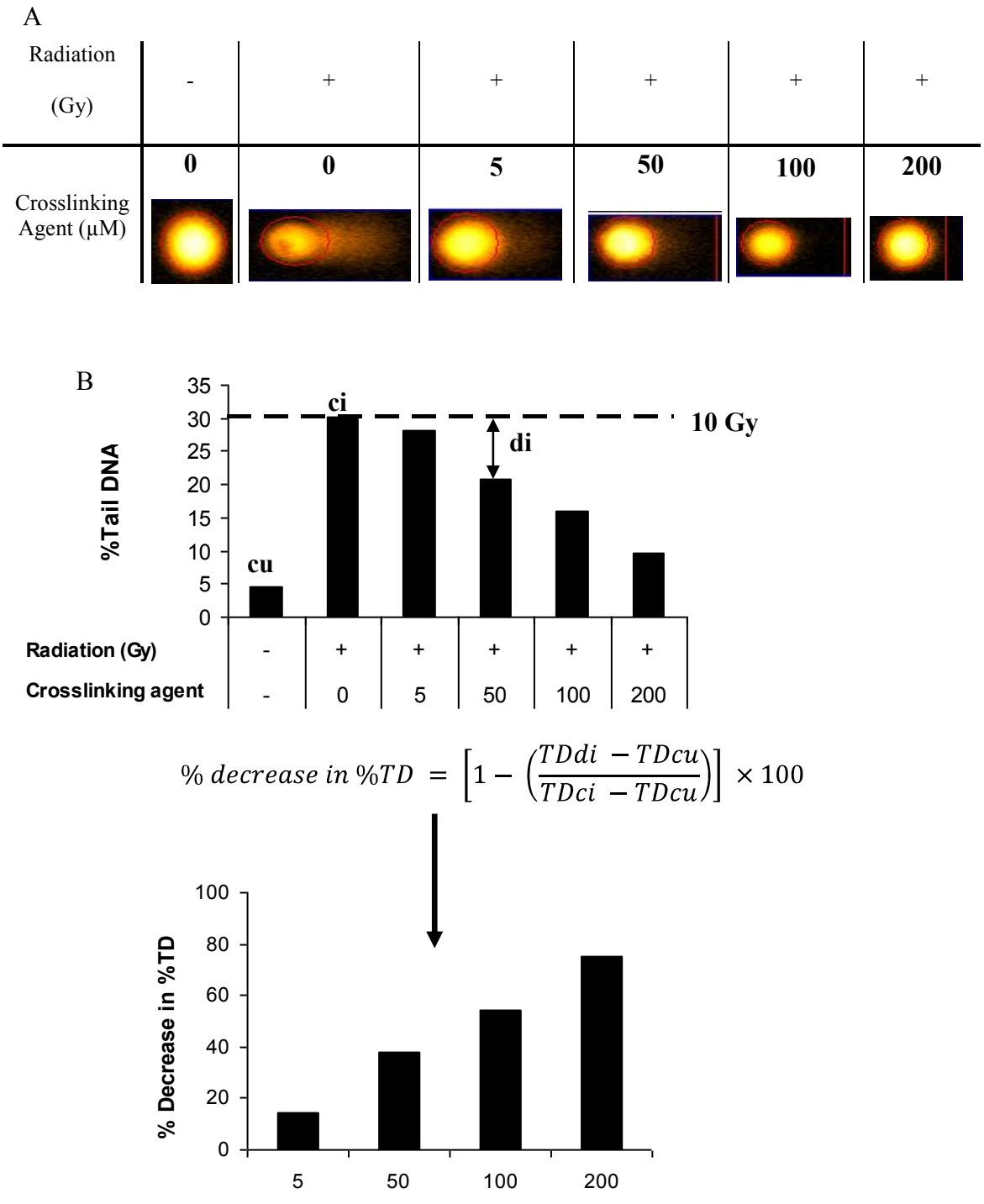


Figure 4.1-2 Crosslink Formation
Measurement of crosslink formation using a modified version of the alkaline comet assay. (A) Cells treated with a crosslinking agent are irradiated prior to analysis. Formation of crosslinks is measured as an inhibition in DNA migration (i.e decrease in amount of DNA in the tail of the comet) in treated samples compared to the irradiated control. (B) For ease of interpretation results are expressed as the percentage decrease in %TD as calculated by the above formula where cu = untreated uniradiated, ci = untreated irradiated and di = treated irradiated.

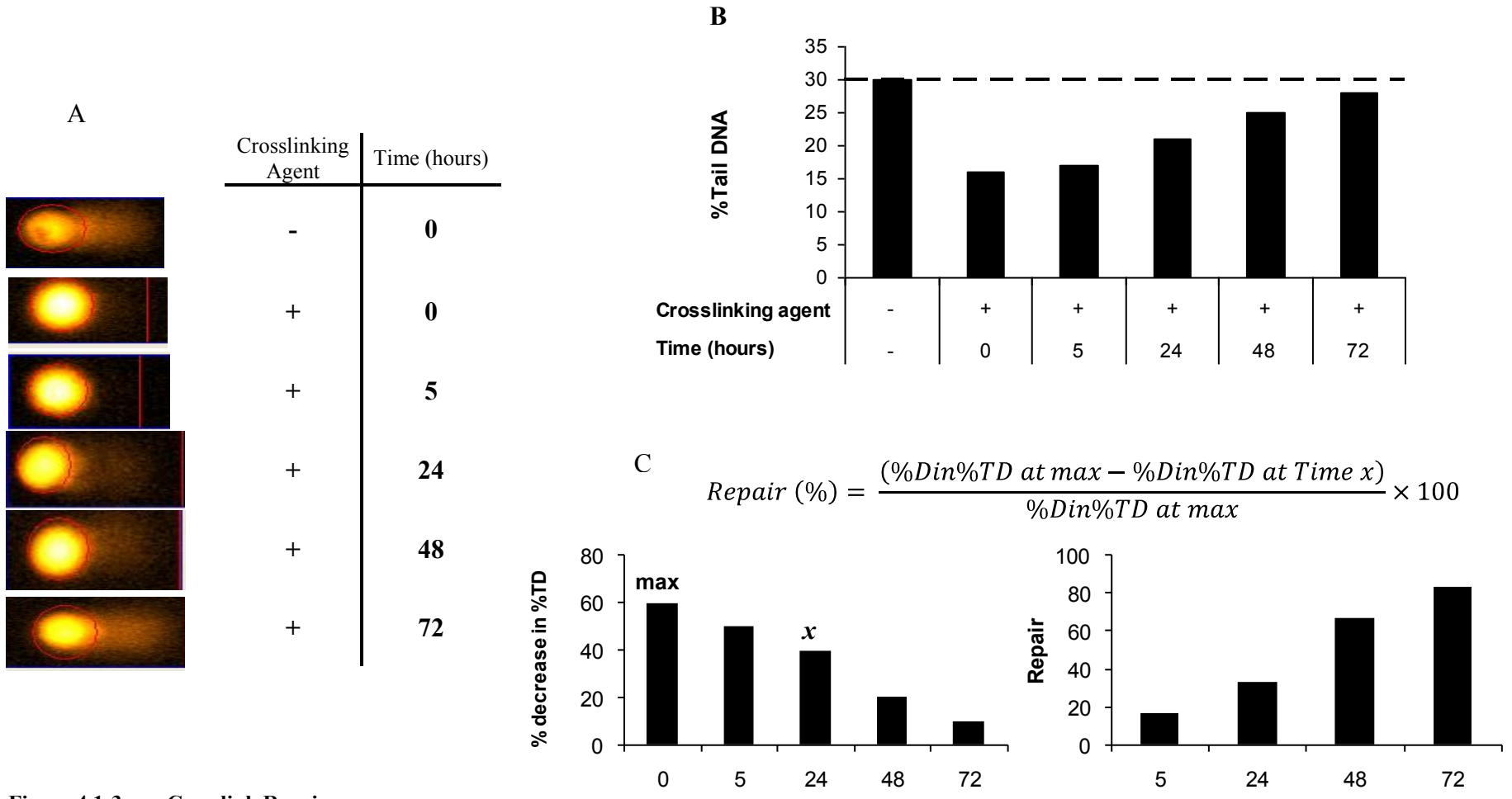


Figure 4.1-3 Crosslink Repair

Measurement of crosslink repair using a modified version of the alkaline comet assay. (A) Cells treated with a crosslinking agent are incubated in drug free media before being irradiated prior to analysis. (B) An increase in %TD over time, compared to the irradiated control is an indication of crosslink repair. (C) For ease of interpretation, results are expressed as percentage repair as calculated by the above formula where %Din%TD = % decrease in % Tail DNA.

4.1.3 Aim of this Chapter

In this results chapter a modified version of the Comet assay was used to evaluate whether mirtoselect had any effect on the formation and repair of the lesion thought to be mainly responsible for the cytotoxicity induced by MMC, interstrand crosslinks. Investigations into the properties of anthocyans have shown that they can protect cells against global (comet assay) and specific (M_1dG) DNA damage (Cooke *et al*. 2006, Duthie *et al*. 2005). Genotoxic chemotherapeutic drugs, such as MMC, are DNA damaging in their nature, although their modes of action differ, these agents rely on the induction of sufficient levels of damage in cancer cells to be insurmountable by repair systems, thereby causing toxicity and cell death. Therefore, a chemopreventive agent that was able to generally protect against all types of DNA damage would be potentially counterproductive in the clinical setting, limiting the efficacy of the treatment being given to treat the patient. Chemotherapeutic agents also exert their therapeutic advantage by targeting the faster growing cancer cells, therefore an agent that can decrease growth could potentially interfere with the formation or repair of any DNA damage. In the previous chapter, results showed that mirtoselect was able to cause significant decreases in the proliferative capability of the cell lines being investigated. There is therefore a potential risk that mirtoselect could directly inhibit MMC-induced DNA, via inhibition crosslink formation and/or enhancement of repair, whilst also indirectly effecting the level and consequence of crosslink formation through alterations in cellular growth.

4.2 Results

4.2.1 Optimisation of the Modified Comet Assay for Crosslink Measurement

Prior to analysis of the combined treatments (mirtoselect and MMC) on the formation and repair of crosslinks, a number of preliminary experiments were necessary to establish the optimal conditions for measurement of crosslinks in the cell lines being investigated. Initial studies included establishment of the optimal radiation dose and MMC treatment conditions. This work was only carried out using the RT112 and RT4 cell lines.

4.2.1.1 Radiation Dose Response Experiments

To measure crosslinks using the comet assay a standardised level of DNA damage has to be introduced into the cells in order to be able to detect the decreases in DNA mobility as a result of crosslink formation. Radiation is an ideal source for inducing DNA damage, compared to chemical sources, as it is a direct and immediate DNA damaging agent, making it ideal for use in the modified comet assay. Both RT112 and RT4 cell lines were exposed to varying doses of radiation and analysed by the standard comet assay to identify an appropriate level of radiation-induced DNA damage (i.e. a dose that gave a damage response that was within the limits of detection of the comet assay) (Figure 4.2-1).

Over a dose of 0-8Gy both cell lines exhibited similar responses with around 19% tail DNA at 8Gy. At the highest dose of 10Gy an increase in % tail DNA could be seen in the RT112 cell line, however, there was a flattening of the graph in RT4 cells indicating that further increases in radiation were not able to induce further DNA damage. For future comet experiments using the crosslinking agent MMC a dose of 10Gy was

chosen as this gave appropriate levels of DNA damage for the measurement of the crosslinks with this being within the linear portion of the comet assays limit of detection. Saturation of the RT4 cell line was not deemed to be a confounding factor as the damage induced was consistent between experiments for each cell line and as crosslink formation is assessed as a relative value to the radiation-induced DNA damage it was important that levels were consistent.

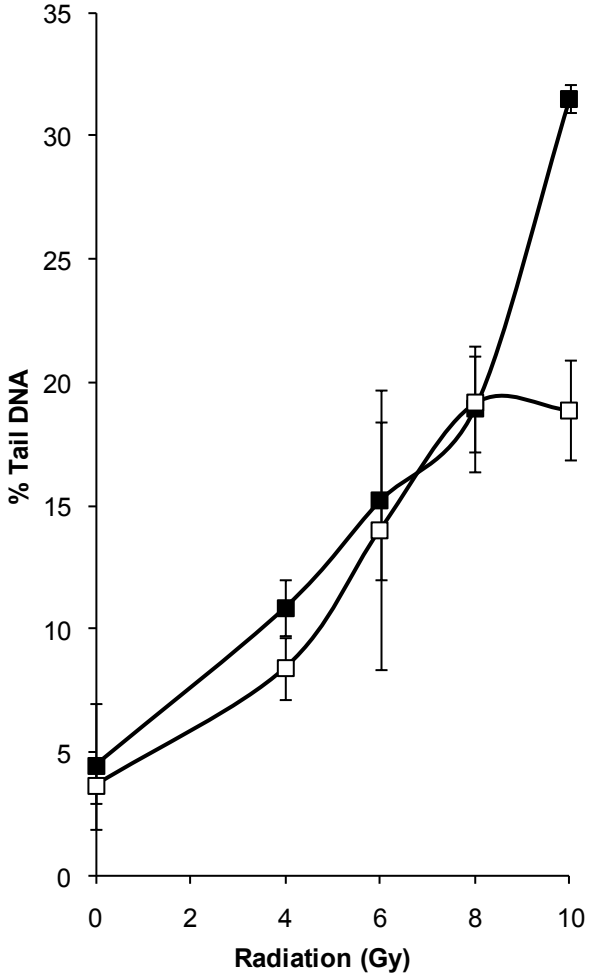


Figure 4.2-1 Measures of Radiation-Induced DNA Damage
Radiation dose response graph for RT112 ■ and RT4 □ cell lines as measured using the alkaline comet assay. Results are the mean of three independent experiments (n=6) ±SD.

4.2.1.2 MMC Dose Response

Prior to any combined studies it was important to determine if the cell lines being used were damage sensitive to MMC and that crosslinks were measurable using the modified version of the comet assay. A clear dose response was seen in both cell lines (Figure 4.2-2), with RT4 cells exhibiting a slightly more damage sensitive profile than RT112, indicating the assay was able to detect the crosslinks in a dose dependant manner. For many future experiments the use of a range of doses of MMC would be unfeasible, it was important therefore to choose a dose of MMC that gave a substantial level of crosslinks without exhausting the assay. Both 50 and 100μM MMC gave appropriate levels of crosslinks, decreasing the %TD between 44-67%, and were therefore chosen as suitable concentrations for future experiments.

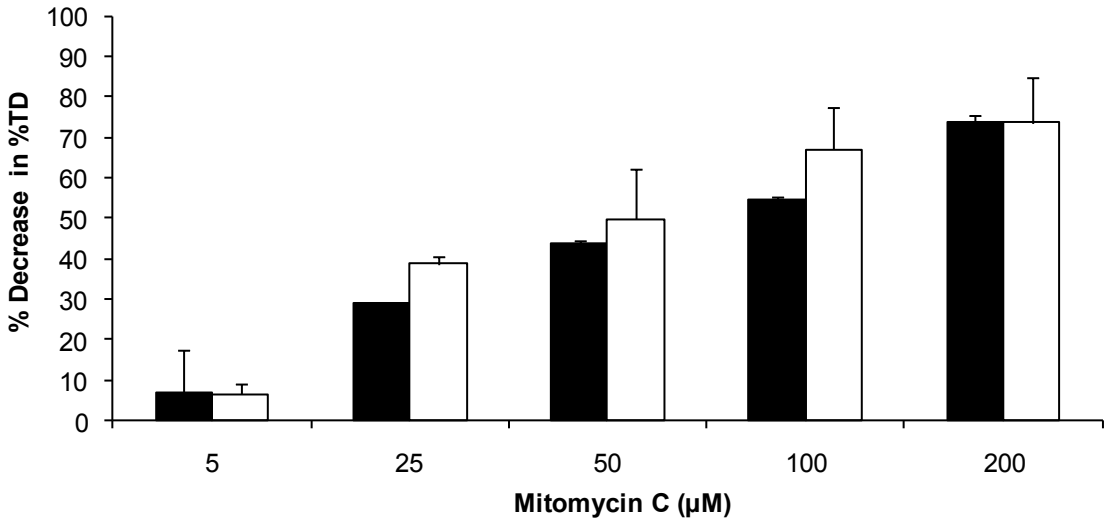


Figure 4.2-2 Measures of Crosslink-Induced DNA Damage
Bladder cell lines RT112 ■ and RT4 □ were treated for 1 hour with MMC then left in drug free media for 3 hours, to allow for crosslink formation, after which the numbers of crosslinks were measured as a percentage decrease compared to the radiation control. Results are the average of three independent experiments (n=3) ±SD.

4.2.2 Measures of MMC Crosslink Formation

Previous studies have shown that maximum crosslink formation by chemotherapeutic agents, such as cisplatin and oxaliplatin, in some instances occurs some hours post treatment, after a period in drug free media. In order to assess the formation of crosslinks induced by MMC in the bladder cancer cell lines, cells were treated for 1 hour with MMC followed by a period of up to 5 hours in drug free media.

Using RT112 and RT4 cell lines, two concentrations (50 and 100μM) of MMC were applied for 1 hour, and the formation of crosslinks was measured immediately after the removal of the MMC (0 hours) and up to 5 hours post removal. Initial levels of crosslinks (0 hours) were similar between the two cell lines and there was a visible dose response with the 100μM dose introducing ~14% more crosslinks than the 50μM dose (Figure 4.2-3). Over the 5 hours studied, crosslink numbers differed only marginally in RT112 cells, falling by 4% and 6% in the 50 and 100μM treated cells, respectively. The RT4 cells showed a slight indication of crosslink repair over the first 3 hours but at 5 hours there was no significant difference in numbers of crosslinks in the 50 or 100μM treated cells compared to immediate crosslink induction at 0 hours. Maximum crosslink formation was therefore established to occur during the one hour treatment period with no further crosslink formation post treatment and only a little repair of the crosslinks over 5 hours in drug free media.

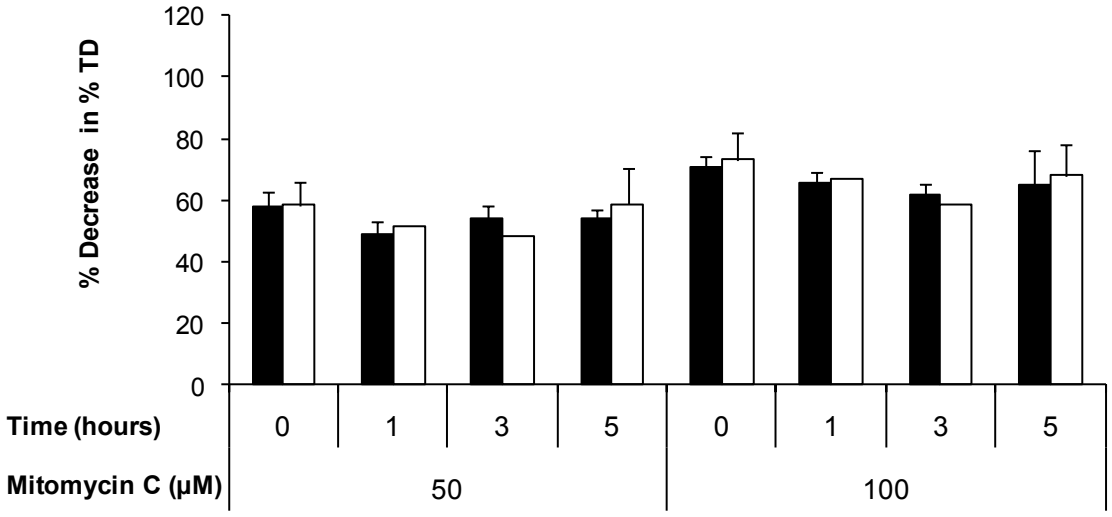


Figure 4.2-3 Crosslink Formation in Bladder Cancer Cell Lines
Bladder cell lines RT112 ■ and RT4 □ were treated for 1 hour with MMC then left in drug free media for up to 5 hours to establish the rate at which crosslinks were formed. Results are the average of three experiments (n=3) ±SD.

4.2.3 MMC Crosslink Repair

No substantial evidence of repair had been shown to occur over the 5 hours studied during the crosslink formation experiments (Section 4.2.2). Therefore, the extent to which the crosslinks were repaired and the time frame over which this occurred was investigated over longer periods in drug free media. Bladder cells were treated for one hour with MMC, immediate crosslink formation was measured after MMC treatment (0 hours) and repair was measured over the following 72 hours. Measurement of the crosslinks once again showed little difference in damage levels over the initial 5 hours post treatment in either cell line (Figure 4.2-4). Overall there was evidence of more repair in the RT112 cell line compared to RT4, for example, by 72 hours there was only a 15% decrease in % tail DNA in the 50µM RT112 treated cells compared to 36% in RT4.

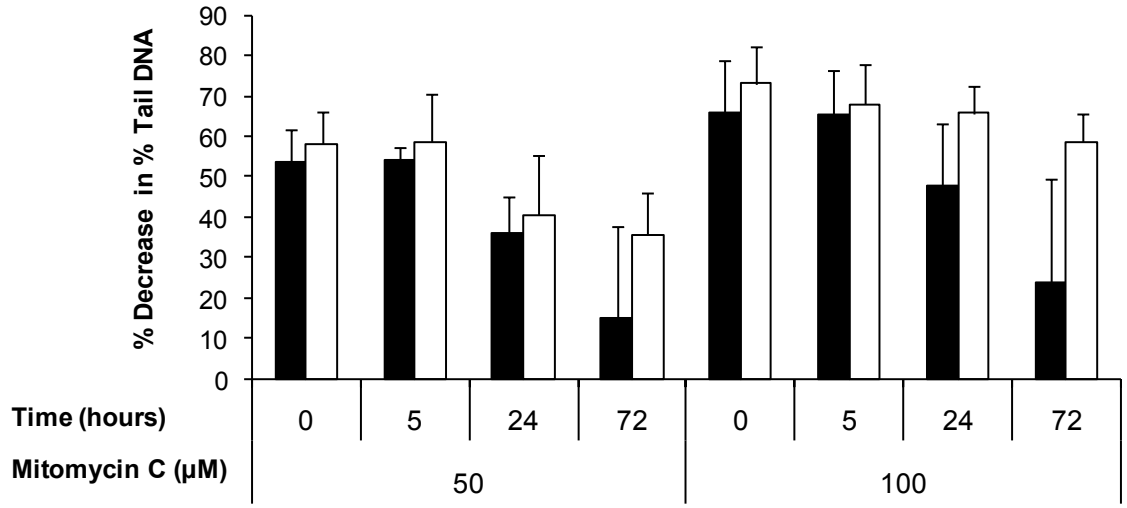


Figure 4.2-4 Measurement of Crosslinks in Bladder Cells over Time in Drug Free Media
Bladder cell lines RT112 ■ and RT4 □ were treated for 1 hour with MMC then left in drug free media for up to 72 hours to establish the extent to which crosslinks were repaired. Results are the average of three experiments (n=3) ±SD.

In measures of the extent of repair, similar amounts of crosslinks had been repaired in the first 24 hours in both cell lines, however, over the subsequent 48 hours the number of RT4 crosslinks repaired was minimal with ~7% repair occurring over this time frame (Figure 4.2-5). The RT112 cell line exhibited a more damage resistant phenotype with slightly fewer crosslinks being induced initially and also having a greater capacity to repair, the crosslinks with between 65-70% repair occurring by 72 hours. In comparison, RT4 cells exhibited a more damage sensitive phenotype with slightly more crosslinks induced and a poorer capacity to repair the crosslinks, with only 20-40% repair by 72 hours. The extent of repair was also affected by the initial treatment concentration of MMC in both cell lines, with those cells treated with 50µM showing a greater extent of repair compared to those treated with the higher 100µM dose; this may be due to the higher concentration saturating the cells repair capacity. For combined mirtoselect/MMC experiments, 50µM MMC was therefore chosen as a suitable

concentration of MMC, in order that any potential effect by mirtoselect could be measured without the assay becoming saturated.

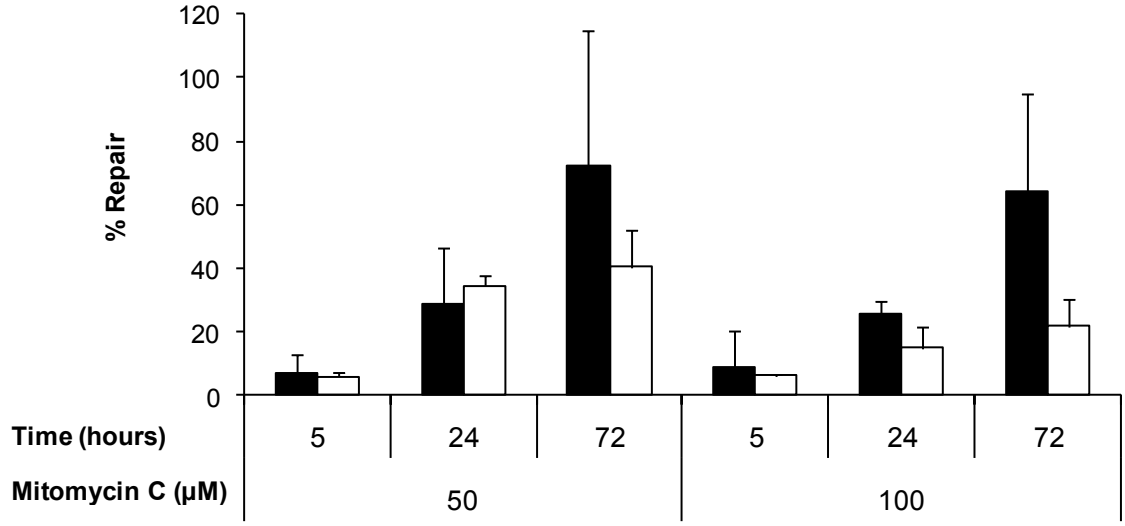


Figure 4.2-5 Extent of Crosslink Repair in Bladder Cancer Cell Lines
Bladder cell lines RT112 ■ and RT4 □ were treated for 1 hour with MMC then left in drug free media for up to 72 hours to establish the extent to which crosslinks were repaired. Results are the average of three experiments (n=3) ±SD.

4.2.4 Effect of Mirtoselect on MMC Crosslink Formation

Prior to the analysis of the effect of mirtoselect on the formation and repair of MMC-induced crosslinks it was essential to ensure that mirtoselect did not itself cause decreased DNA mobility or interfere with the formation of radiation-induced DNA damage needed to successfully measure the decreased DNA mobility caused by MMC. Measures of endogenous levels of DNA damage in all three bladder cancer cell lines were relatively low, with ~5% TD. Treatment with mirtoselect did not cause endogenous levels to differ significantly in any of the cell lines (Figure 4.2-6). Measures of radiation-induced DNA damage also revealed no significant differences between mirtoselect pre-treated cells and control cells. So, mirtoselect neither hindered the measurement of radiation-induced DNA damage or caused measureable differences

in endogenous levels of DNA damage using the standard alkaline comet assay and could therefore be used in combination with MMC in the modified comet assay for measuring crosslinks.

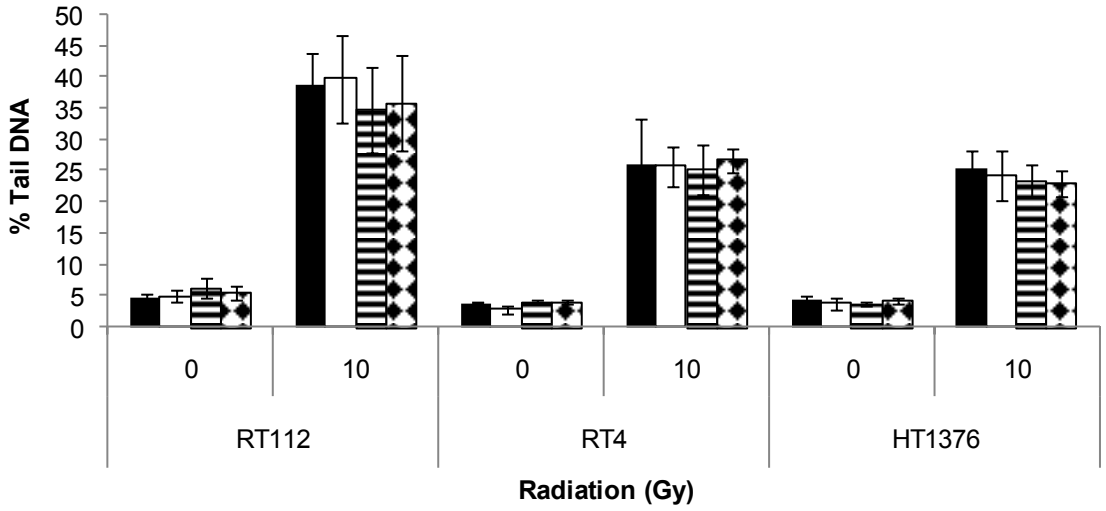


Figure 4.2-6 Measurement of DNA Damage caused by Mirtoselect.
Bladder cancer cell lines RT112, RT4 and HT1376 were treated for 72 hours with repeat doses of mirtoselect 0 ■, 20 □, 50 ▬, and 100µg/ml ▫ and then irradiated with 0 or 10Gy irradiation to assess whether any effect could be seen on the levels of endogenous or induced damage levels by mirtoselect. Results are the average of three experiments (n=3) ±SD.

4.2.4.1 DNA Damage Levels

Initial experiments were carried out to assess the effect mirtoselect had on the formation of crosslinks in the three bladder cancer cell lines. Combined mirtoselect and MMC comet data was initially assessed based on the raw %TD data for each cell line before conversion was performed for representation as % decrease in %TD in order to assess the numbers of crosslinks.

In all three cell lines a dose dependant decrease in %TD, as a result of MMC treatment, was measurable in all treatment combinations (Figure 4.2-7). In the RT112 and RT4 cell lines mirtoselect pre-treatment of 50µM MMC treated cells caused an enhancement of

the decrease in %TD in a dose dependent manner. Indeed, in the RT112 cells there was a significantly lower measure of %TD in 100µg/ml pre-treated cells compared to MMC alone treated cells (Figure 4.2-7A), i.e. there was greater decrease in DNA mobility in the 100µg/ml mirtoselect pre-treated cells. Assessment of HT1376 cells revealed only a very slight difference in measures of %TD in combined treated cells compared to MMC alone cells and was the cell line least affected by the combined treatment (Figure 4.2-7 C).

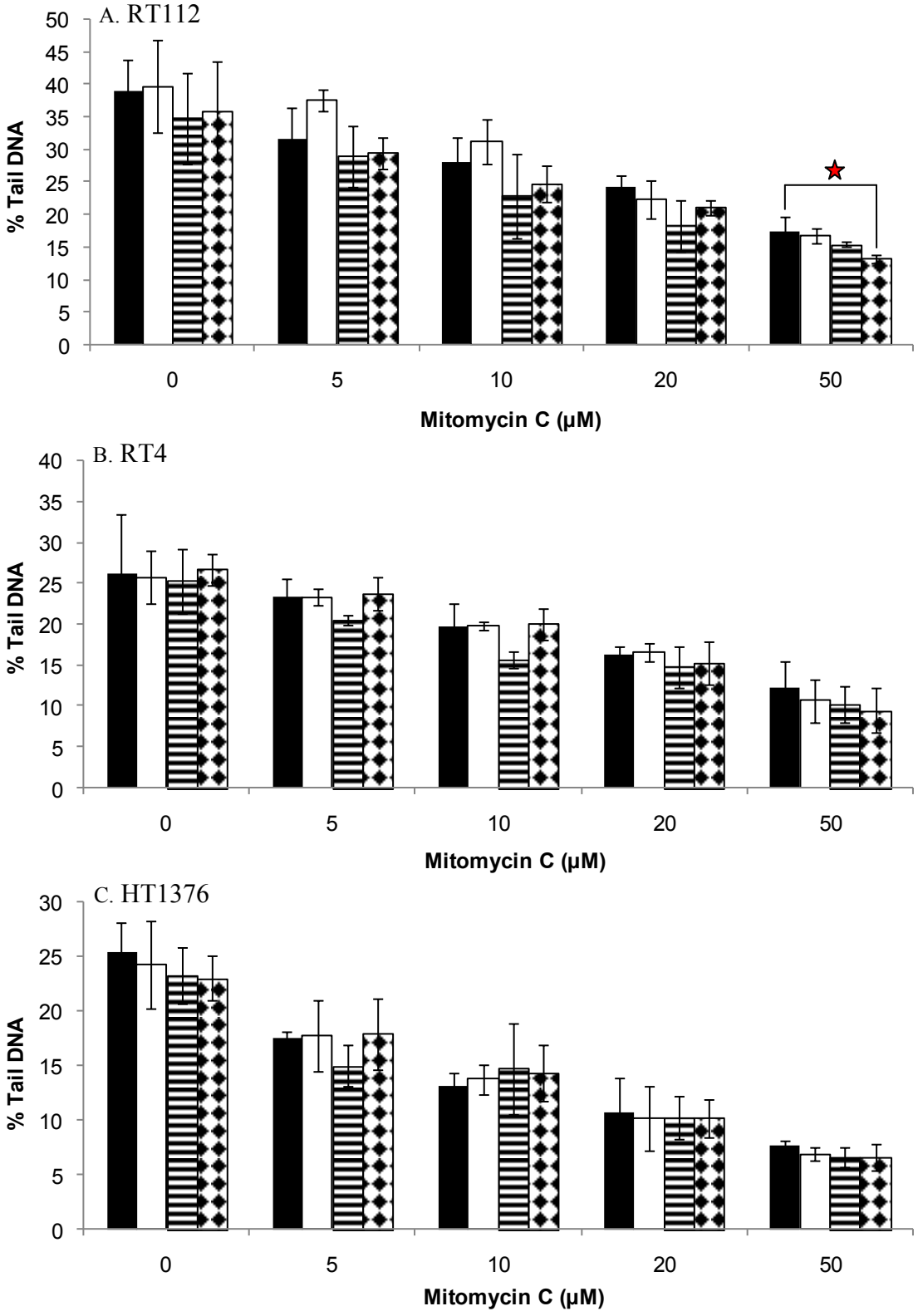


Figure 4.2-7 Assessment of DNA Damage with Combined Mirtoselect and MMC Treatments. Bladder cancer cell lines RT112 (A), RT4 (B) and HT1376 (C) were pre-treated with repeat doses of mirtoselect at 0 ■, 20 □, 50 ▬, and 100µg/ml ⊠ followed by varying concentrations of MMC in order to assess DNA damage using a modified version of the comet assay. Results are the mean of three independent experiments (n=3) ±SD, $p \leq 0.05 =$ ★.

4.2.4.2 Measurement of Crosslinks

For the assessment of the extent of crosslinks, data from Figure 4.2-7 was converted using Equation 2.7-1 (Chapter Two). In the 50μM MMC treated cells, RT112 and RT4 cell lines had shown a dose dependent effect due to mirtoselect pre-treatment. In measures of the numbers of crosslinks both cell lines exhibited a dose dependent increase in crosslink numbers due to mirtoselect pre-treatment (Figure 4.2-8). In RT112 cells, 100μg/ml pre-treatment caused a 74.9% decrease in %TD compared to 50μM MMC alone which caused 62.8% (Figure 4.2-8A). In RT4 cells the enhancement was greater with 50μM MMC resulting in 59.5% decrease in %TD and 100μg/ml pre-treated cells causing a 75.9% decrease (Figure 4.2-8B). HT1376, on the other hand, showed no enhancement by the mirtoselect on MMC-induced crosslink numbers (Figure 4.2-8 C).

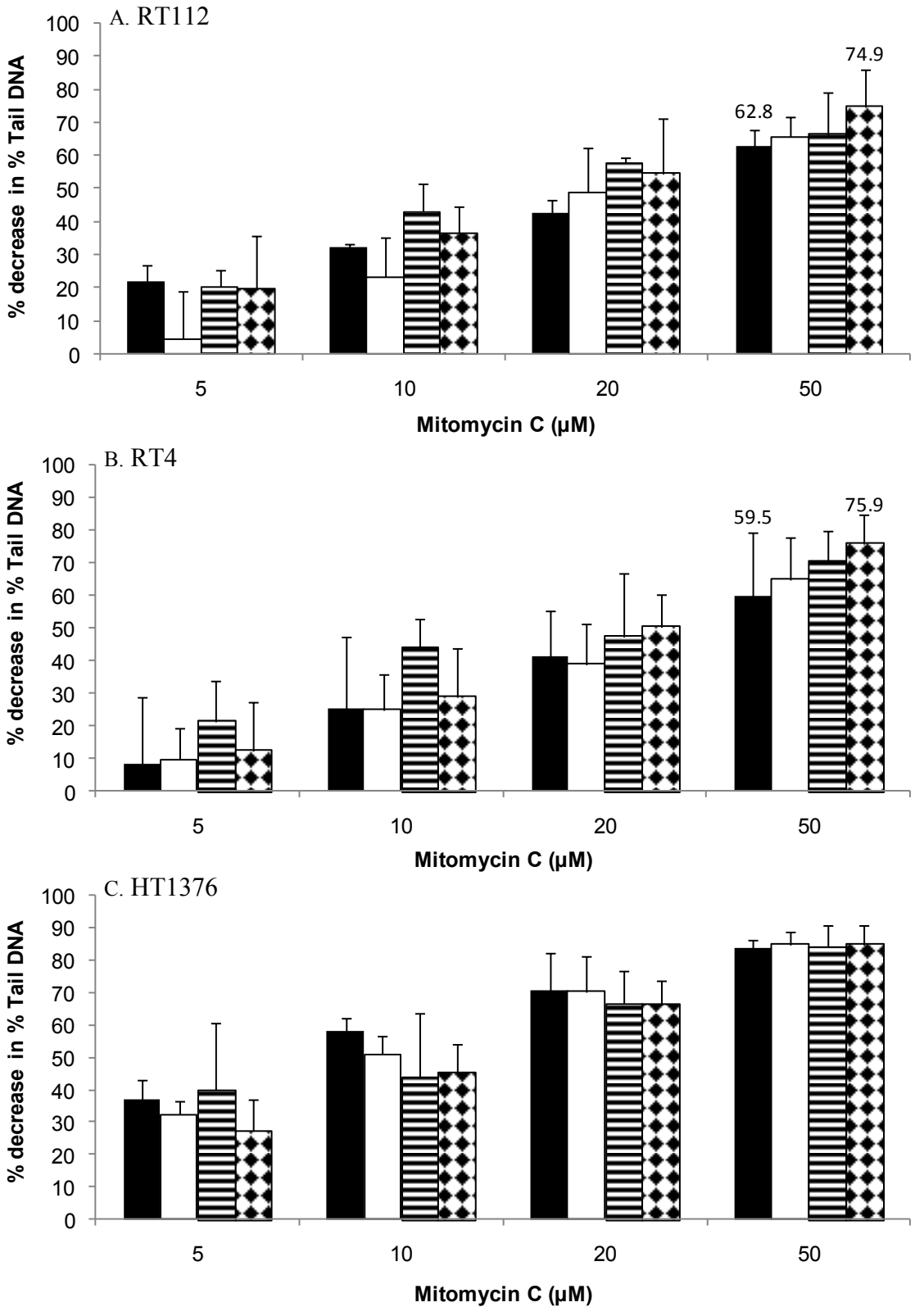


Figure 4.2-8 Measure of Crosslinks in Combined Mirtoselect and MMC Treated Cells.
Bladder cancer cell lines RT112 (A), RT4 (B) and HT1376 (C) were pre-treated with repeat doses of mirtoselect at 0 ■, 20 □, 50 ▬ and 100µg/ml ◪ followed by varying concentrations of MMC. Crosslink formation was assessed using the Equation 2.7-1 and displayed as a percentage decrease in % Tail DNA compared to an irradiated control. Results are the mean of three independent experiments (n=3) ±SD.

4.2.5 Effect of Mirtoselect on the Repair of MMC Crosslinks

In two of the three cell lines investigated mirtoselect increased crosslink levels. To assess crosslink repair, immediate crosslink induction using 50μM MMC was monitored over 72 hours in drug free media. Repair of the crosslinks is indicated by an increase in % tail DNA as the DNA damage induced by the radiation is no longer withheld in the head of the comet. When data is represented as % decrease in %TD, repair is measured/visualised as the removal of the crosslinks and transpires as decreasing values of %D%TD. This can subsequently be converted to % repair to better understand the extent of repair.

4.2.5.1 Measurement of Crosslinks over Time in Drug Free Media

In RT112 cells, immediate crosslink numbers were higher in mirtoselect pre-treated cells compared to MMC alone treated cells, as previously observed (Figure 4.2-8). At each subsequent time point measured, numbers of crosslinks were consistently higher in pre-treated cells compared to the MMC alone control (Figure 4.2-9 A). Numbers of crosslinks did not alter significantly over the initial 24 hours investigated; by 72 hours, however, the % decrease in %TD in the non mirtoselect pre-treated cells had fallen to ~10% from ~59% at 0 hours. In the highest (100μg/ml) mirtoselect treated cells, removal of the crosslinks followed a similar pattern with the majority of the crosslinks being removed between 24 and 72 hours, with only ~21% decrease in %TD remaining at 72 hours. In RT4 cells, crosslink numbers were also highest immediately after the removal of the MMC, i.e. at the 0 hour time point. A significantly higher number of

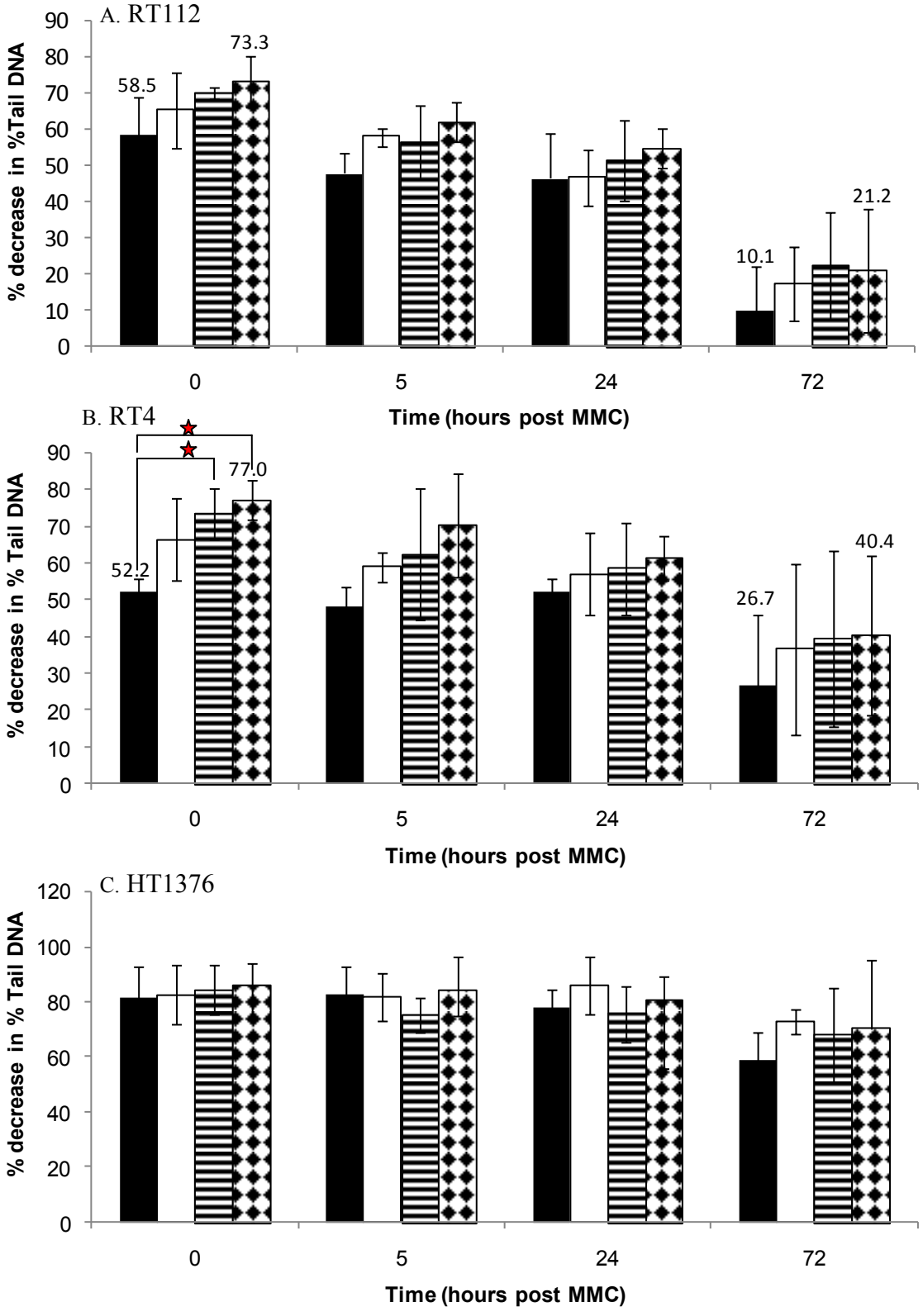


Figure 4.2-9 Measurement of the Disappearance of Crosslinks

RT112 (A), RT4 (B) and HT1376 (C) cells were treated for 72 hours with repeat doses of 0 ■, 20 □, 50 ◘ and 100µg/ml ◘ mirtoselect followed by 1 hour with 50µM MMC and a further 72 hours post treatment in drug free media. 0 hours is representative of immediately after the removal of the MMC. Results are the mean of three independent experiments (n=3) ±SD, $p \leq 0.05$ =★(based on values of % decrease in %TD).

crosslinks were measured in the 50 and 100µg/ml mirtoselect pre-treated cells compared to the MMC alone cells (Figure 4.2-9 B). In the MMC alone treated cells a similar pattern to the RT112 cell line was observed where the majority of the crosslinks were removed post 24 hours, with a ~27% decrease in % TD resulting at 72 hours compared to ~52% at 0 hours. Crosslink numbers remained higher in mirtoselect pre-treated cells compared to MMC alone treated cells at each time point investigated, e.g. in 100µg/ml mirtoselect pre-treated cells a 40% decrease in %TD remained at 72 hours. HT1376 cells were the least sensitive to mirtoselect pre-treatment in combination with MMC (Figure 4.2-8C) with no observable enhancement on crosslink numbers; this was maintained over the duration of the experiment with crosslink numbers remaining similar amongst treatment groups (Figure 4.2-9C). However, similar to RT112 and RT4 cells, removal of the crosslinks did not occur until after the 24 hour time point, with a small decrease in crosslink numbers observable at 72 hours in all treatment groups.

4.2.5.2 Extent of Crosslink Repair

Repair of the crosslinks, calculated as a percentage of the maximum number of crosslinks formed, was assessed using Equation 2.7-2 (Chapter Two). In all three cell lines mirtoselect treatment did not affected the rate at which crosslinks had formed i.e. maximum formation occurred immediately after the 1 hour MMC incubation in MMC alone treated cells and mirtoselect pre-treated cells (Figure 4.2-9). In RT112 MMC alone treated cells ~80% repair occurred over the 72 hours investigated (Table 4.2-1A). Although more crosslinks were measurable in pre-treated cells compared to MMC alone treated cells, over the course of the 72 hour experiment, mirtoselect pre-treatment did not significantly affect the extent of repair with ~68-72% repair occurring in the pre-treated groups. RT4 cells were not as efficient at repairing the crosslinks compared to

RT112 cells, with only ~47% of the crosslinks repaired by 72 hours (Table4.2-1B). Although mirtoselect pre-treatment did significantly increase the number of crosslinks at 0 hours, pre-treatment did not affect the extent to which the crosslinks were repaired with all treatment groups exhibiting similar levels of repair at 72 hours. HT1376 cells were the least efficient at repairing MMC-induced crosslinks, with only 26% repair having occurred by 72 hours (Table 4.2-1C). Mirtoselect pre-treatment did not, however, affect the repair of crosslinks and all treatment groups exhibited similar levels of repair.

A	**Repair of Crosslinks (%)**		
Time (hours post MMC)	**5**	**24**	**72**
0µg/ml	**17.00** ± 12.81	**16.34** ± 34.26	**80.06** ± 23.88
20µg/ml	**9.92** ± 12.51	**27.02** ± 17.80	**72.01** ± 18.64
50µg/ml	**19.59** ± 14.39	**26.64** ± 15.83	**67.76** ± 21.10
100µg/ml	**14.65** ± 13.07	**25.09** ± 3.83	**71.15** ± 24.05

B	**Repair of Crosslinks (%)**		
Time (hours post MMC)	**5**	**24**	**72**
0µg/ml	**7.58** ± 15.57	**-0.44** ± 11.88	**47.81** ± 38.28
20µg/ml	**9.98** ± 12.54	**13.77** ± 11.07	**45.89** ± 28.64
50µg/ml	**15.67** ± 18.33	**20.43** ± 13.82	**47.58** ± 28.02
100µg/ml	**8.61** ± 19.02	**20.44** ± 6.20	**47.16** ± 27.57

C	**Repair of Crosslinks (%)**		
Time (hours post MMC)	**5**	**24**	**72**
0µg/ml	**-2.02** ± 10.69	**3.70** ± 11.56	**26.44** ± 17.47
20µg/ml	**0.18** ± 11.58	**-6.66** ± 6.45	**11.03** ± 6.52
50µg/ml	**10.61** ± 3.40	**10.28** ± 8.75	**18.58** ± 21.43
100µg/ml	**2.42** ± 9.52	**6.31** ± 4.72	**18.97** ± 22.14

Table 4.2-1 Repair of Crosslinks
Percentage repair of crosslinks in 50µM MMC alone treated RT112 (A), RT4 (B) and HT1376 (C) cells and those pre-treated with varying concentrations of mirtoselect. Results are the mean of three independent experiments (n=3) ±SD.

4.3 Discussion

Measurement of DNA damage levels was performed using the alkaline comet assay to assess whether pre-treatment with mirtoselect would alter the formation or repair of the lesion thought to be responsible for MMC cytotoxicity. Initial experiments to optimise assay conditions for the measurement of MMC-induced crosslinks were performed and proved that crosslinks could be measured in a dose dependant manner in the bladder cancer cell lines being investigated. It was also shown that mirtoselect would not interfere with the measurement of crosslinks as it failed to alter background levels of DNA damage or damage induced by X-rays.

4.3.1 Effects of Mirtoselect on MMC-Induced Crosslink Formation

In combined mirtoselect/MMC experiments, varying responses were observed between the three different cell lines being investigated. Following exposure to MMC alone, crosslink numbers were highest in the HT1376 cell line and lowest in RT4 cell line, indicating a damage sensitivity ranking of HT1376 > RT112 > RT4. This order is the opposite of that based on the cell growth data, where MMC alone had very little impact on the growth of HT1376 cells. Pre-treatment with mirtoselect caused a dose dependent increase in crosslink numbers in two of the three cell lines. The greatest enhancement was observed in RT4 cells with 16% more crosslinks occurring in 100µg/ml pre-treated cells compared to 50µM MMC alone cells. In the MMC sensitive cell line HT1376, there was no measureable enhancement by mirtoselect on crosslink numbers. This data is in agreement with the growth data where a significant difference was measurable in RT4 cells in combined treatments and, of the three cell lines, HT1376 cells showed the least response to combined treatment in relation to cell numbers. It can therefore be

concluded that pre-treatment did not negatively interfere with the formation of crosslinks and in two of the three cell lines being investigated pre-treatment actually enhanced the numbers of crosslinks formed in a dose dependant manner.

4.3.2 Effects of Mirtoselect on MMC-Induced Crosslink Repair

Although initial formation of crosslinks is an important measure of cellular response and sensitivity to MMC (Moneef, 2002), how the cells manage the crosslinks and higher levels of residual damage could also be of great importance with measurement of DNA damage using the comet assay being a potential predictive test of patient chemosensitivity, however, little work has been carried out using tumour cells isolated from biopsy material for the prediction of tumour chemosensitivity, with the majority of studies using surrogate cells, such as peripheral blood lymphocytes (PBL) to assess indirect levels of DNA damage in patients undergoing chemotherapy (Almeida *et al*. 2006, McKenna *et al*. 2008). In the few studies using tumour cells, however, tumour cells isolated from ovarian cancer patients were treated ex vivo with cisplatin and assessed for crosslink formation and repair using the modified version of the comet assay (Wynne *et al*. 2007). The investigators were interested in determining the cause of resistance to cisplatin, which results during the course of treatment. Although no significant differences in terms of crosslink formation were observed between newly diagnosed patients and those who had previously been treated with platinum-based therapy, repair was greater in the group of patients who had previously been treated. The investigators concluded that clinical acquired resistance to cisplatin could therefore be a result of increased repair of cisplatin-induced DNA crosslinks in ovarian tumour cells. However, it should be noted that correlation to patient response *in vivo* was not carried out in this study.

The three bladder cell lines used in this project exhibited varying abilities to repair MMC-induced crosslinks, RT112 cells were the most efficient at repairing the crosslinks whereas the HT1376 cell line was the least efficient with only 26% repair occurring after 72 hours in drug free media. Mirtoselect pre-treatment was shown to enhance the level of crosslinks in RT4 cells the greatest and this enhancement persisted over the 72 hours post MMC treatment. In measures of the repair capacity of the three cell lines, although each differed in their ability to repair the crosslinks, with varying levels of residual crosslink damage remaining at 72 hours, mirtoselect pre-treatment did not alter the percentage of crosslinks repaired. It can therefore be concluded that mirtoselect pre-treatment does not adversely interfere with the repair of crosslinks, in that it does not enhance their removal.

Clinical response to MMC post TURBT varies considerably. Studies have shown that response can be significantly correlated to repair, with patients with lower levels of repair enzyme mRNA (ERCC1 and XPAC) having improved survival due to enhanced tumour cell sensitivity (Bellmunt *et al*. 2007, Dabholkar *et al*. 1994, Shirota *et al*. 2001). As mirtoselect was unable to influence the repair of MMC-induced crosslinks, other relationships, between cellular response and molecular mechanisms of dealing with the DNA damage, need to be investigated.

Chapter Five

The Effect of Pre-Treatment with Mirtoselect on MMC-Induced Apoptosis and Cell Cycle Distribution Changes

5.1 Introduction

Previously in this study mirtoselect alone was shown to cause significant decreases in cellular growth and inhibit clonogenic cell survival (Chapter Three). In combination with MMC, growth results pointed towards an enhanced effect and in the comet assay, mirtoselect enhanced the formation of MMC-induced crosslinks in two of the three cell lines. MMC has been shown to induce an apoptotic response and bring about cell cycle arrest in cell lines and cells derived from tumours (Kawasaki *et al*. 1996, Kelly *et al*. 2000). Measurement of apoptosis and cell cycle changes in combined treatments of MMC and mirtoselect were therefore important in determining whether mirtoselect disrupted the standard treatment regimens for non-muscle invasive bladder cancer.

In the previous results chapter it was shown that in two of the three cell lines being investigated there was an increase in the number of crosslinks in cells treated with both mirtoselect and MMC. A vast amount of evidence supports the theory that the critical cytotoxic lesion of MMC is DNA crosslinks (Dronkert and Kanaar, 2001). It is therefore possible that combined treatment of mirtoselect and MMC could increase the potency of chemotherapeutic treatment with MMC and induce higher levels of cell killing. So, for the next part of this study, analysis of apoptosis was carried out to determine whether increases in crosslink numbers previously measured could be correlated with increases in the amount of apoptosis measured. Cell cycle analysis was also performed to reveal whether MMC-induced cell cycle blocks and/or the bladder cancer cell lines ability to recover from those blocks, was altered in anyway which could account for the greater anti-proliferative effect seen in the combined treated cells compared to those incubated with MMC alone.

5.1.1 Flow Cytometry

Flow cytometry is a fast, high throughput technique for the measurement and analysis of fluorescent signals generated from particles, such as cells, chromosomes or beads, which flow in a liquid stream through a beam of light. Complete reviews on all aspects of flow cytometry techniques, from the fundamentals of how the instrumentation operates to the variety of assays that can be implemented, can be found elsewhere (Givan, 2001); however, a brief synopsis of the technique will be given here.

As particles, suspended in the fluid stream, are accelerated through the laser beam, light signals are given off which are detected using a system of photodetectors, which convert the light signals into electrical impulses, and filters, which make the photodetectors colour specific. The detected light signals give information on the physical characteristics of the particle. For cells, information can be gained on the size and volume, or diameter (as a result of the forward scatter, FSC) and also surface texture and internal structure (based on side scatter, SSC). If the cells are also fluorescently labelled or contain endogenous fluorescent compounds then the colour and intensity of the fluorescence emitted can be detected, assuming the correct configuration of filters and photodetectors are in place. The data collected from light hitting the photodetectors can then be used to analyse individual cells within the sample, in relation to each other, using appropriate computer software.

5.1.2 Apoptosis Analysis by Flow Cytometry

In normal tissues apoptotic cell death is an important process, having two major functions; tissue remodelling and removal of damaged cells. The most striking example of the role of cell death in tissue remodelling is during embryogenesis; here for example the elimination of interdigital cells results in the formation of separated digits in vertebrates

(Zuzarte-Luis and Hurle, 2005). Apoptosis, also referred to as programmed cell death or cell suicide, plays an important role throughout the lifetime of an organism. In order to maintain the normal homeostasis of an organism there must be a balance between proliferation and death, if this equilibrium is altered then abnormal growth occurs (King, 2000). Cells must be able to coordinate proliferation and apoptosis in response to varying stimuli in order to maintain homeostasis within the tissue. In carcinogenesis, unregulated proliferation or decreased cell death contribute to the formation of a tumour; the objective of treatment, therefore, is to achieve the converse. In many cancers defects in the apoptotic pathway have been found, which contribute to the increases in cell numbers observed during tumour development. These defects can also determine the response of a cancer to treatment, conferring resistance to treatments such as chemotherapy and radiation (Mattson, 2000, Yu and Zhang, 2004).

As previously mentioned, apoptosis is a tightly controlled event and as such it consists of a number of biological events which occur in an orderly sequence. A number of these biological events can be exploited to aid in the measurement of cell death and help discriminate the mode of action between apoptosis and necrosis. The events that can be exploited for flow cytometry analysis of cell death are extensively reviewed elsewhere (van Engeland *et al*. 1998, Vermes *et al*. 2000, Darzynkiewicz *et al*. 1992), however, one of the major differences between apoptosis and necrosis is that initially the integrity of the cell membrane is retained in apoptotic cells. In the process of apoptosis, cells sequentially undergo activation of caspases, loss of mitochondrial membrane potential and finally redistribution of phosphatidylserine and the formation of apoptotic bodies. Necrosis on the other hand is a passive event resulting from cellular injury, resulting in cell and mitochondrial swelling and ending in plasma membrane rupture and release of

the cytoplasmic contents. This in turn evokes an inflammatory response which is absent during an apoptotic event. Of the many different markers of the apoptotic process, two protocols were used in this chapter to assess three different cell death markers, which can be measured using flow cytometry. In the first protocol, initially annexin V-FITC was used to as a marker of one of the final stages in early apoptosis, phosphatidylserine exposure, followed by propidium iodide (PI) which was used as a marker of membrane permeability, which occurs during late apoptosis or necrosis. The second protocol used loss of mitochondrial membrane potential; an early marker in the apoptotic pathway occurring prior to phosphatidylserine exposure (Zamzami *et al*. 1995). The two different protocols were chosen in order to detect different stages of the apoptotic pathway and to potentially provide mechanistic information on the means by which mirtoselect and MMC affect the apoptotic pathway.

5.1.2.1 Annexin V-FITC/PI

Under normal viable conditions the plasma membrane of cells retains an asymmetric distribution between the inner and outer leaflets of the membrane. During apoptosis the architectural arrangement of phospholipids in the plasma membrane undergoes redistribution. The most prominent of these is phosphatidylserine which, under viable conditions, is almost completely confined to the inner leaflet of the plasma membrane due to the constant translocation of phosphatidylserine from the outer leaflet (Bretscher, 1972, Op den Kamp, 1979). During the early stages of apoptosis phosphatidylserine is relocated to the outer leaflet providing a sustained presence for proteins to bind. Annexin V was discovered to only bind to the phospholipid membrane when phosphatidylserine was exposed on the outer leaflet in the presence of calcium ions, thus giving it the power for the discrimination of phosphatidylserine exposing and non-

exposing cells (Vermes *et al*. 1995). Annexin V is unable to penetrate the phospholipid bilayer of live cells, so when conjugated to a fluorescent marker such as fluorescein-5-isothiocyanate (FITC), fluorescent activated cell sorting (FACS) can be used to distinguish between live and early apoptotic cells (Figure 5.1-1).

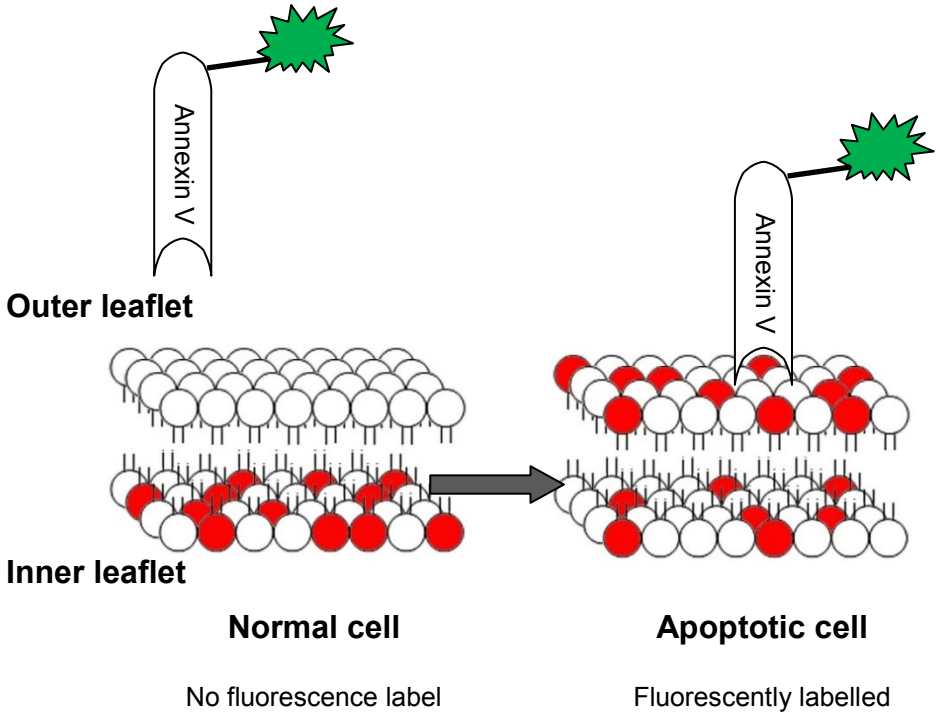


Figure 5.1-1 Schematic of Annexin V-FITC binding
Schematic representation of the loss of membrane lipid asymmetry during early apoptosis. Membrane asymmetry, present in normal cells, is lost during early apoptosis leading to the externalisation of phosphatidylserine (red circles) to which the annexin V – FITC conjugate will bind. (Picture reproduced with permission of John Wiley and Sons and published in van Engeland *et al*. 1998).

In order to further distinguish early apoptotic from late apoptotic or necrotic cells it is necessary to counterstain the cells with an alternative DNA dye which is membrane impermeable. PI is one such dye, meaning that viable and early apoptotic cells are not stained as they are able to exclude the PI, yet late apoptotic or necrotic cells, which have lost this ability, are doubly stained. Discrimination of cells can then be made based on various states of staining between viable (annexin V and PI negative), early apoptotic (annexin V positive, PI negative) and late apoptotic or necrotic cells (annexin V and PI

positive). Therefore, using this method it is possible to identify the percentage of cells within a population undergoing apoptosis at a specific point in time.

5.1.2.2 Mitochondrial Membrane Potential

Loss of mitochondrial membrane potential is also an event involved in apoptosis which can be measured using flow cytometry. Mitochondria are made up of an inner and outer leaflet with megachannels (also known as permeability transition pores) that allow membrane proteins to flux into and out of the cytosol. During the early stages of apoptosis there is a reduction in mitochondrial transmembrane potential ($\Delta\Psi_m$). Loss of $\Delta\Psi_m$ is associated with opening of the megachannels to allow greater outer mitochondrial membrane permeability and is induced by physiological effectors such as ROS. Anthocyanins are hypothesised to reduce cellular ROS (Section 3.4.2) and therefore could potentially moderate the induction of apoptosis. One mechanism of action of chemotherapeutic agents, including MMC, is to cause cells to over generate ROS inducing apoptosis (Lopaczynski and Zeisel, 2001). Any agent that has the potential to reduce ROS could therefore interfere with the activity of MMC, making measurement of loss of $\Delta\Psi_m$ an appropriate marker of apoptosis in combination treated cells, as well as those incubated with mirtoselect alone, in order to determine that mirtoselect does not interfere with MMC activity.

In measures of mitochondrial transmembrane potential using flow cytometry, cationic lipophilic fluorochromes are used. The inner side of the inner mitochondrial membrane is negatively charged, therefore, the fluorochromes distribute to the mitochondria and accumulate in the matrix. Along with loss of $\Delta\Psi$m there is an associated reduction of

fluorescence in the mitochondria as the cell losses its ability to accumulate the fluorochromes. In this study the highly fluorescent dye tetramethylrhodamineethyl (TMRE) was used to stain cells. The TMRE accumulates in the mitochondria of vital cells and the efflux of the dye, measured as a reduction in fluorescence intensity of the cells, is a marker of apoptosis (Figure 5.1-2).

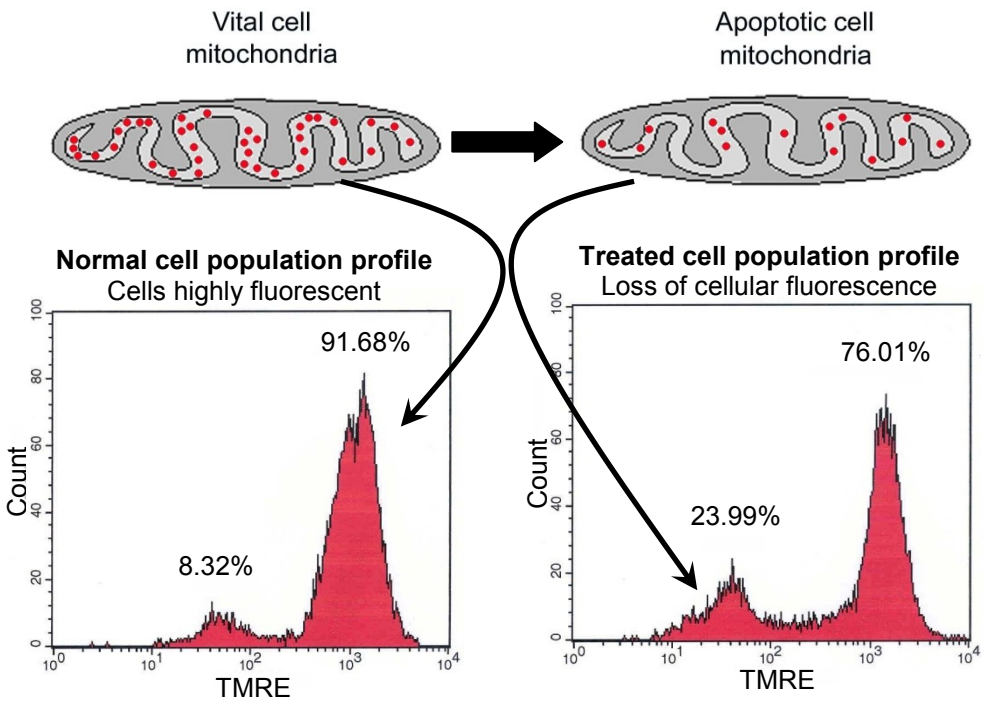


Figure 5.1-2 Schematic of the loss of $\Delta\Psi_m$ as a marker of apoptosis
Schematic representation of the loss of mitochondrial transmembrane membrane potential ($\Delta\Psi_m$) as a marker of apoptosis. Membrane potential, present in normal cells, is lost during apoptosis leading to the efflux of TMRE which under normal conditions accumulates in the mitochondrial matrix. Apoptosis is indicated by a loss of fluorescence.

5.1.3 Cell Cycle Analysis by Flow Cytometry

As covered in detail in Chapter One, during cellular division there are distinct phases where the DNA content of the cell changes. Collectively the phases are referred to as the cell cycle and can be measured using flow cytometry by exploiting these changes in DNA content. In an asynchronous population during the first gap phase (G_1), including cells in

G_0, the DNA content of the cell is normal, i.e. for human somatic cells there are 23 pairs of chromosomes (2n). Cells that have gone through replication and are in the second gap phase (G_2) or are undergoing mitosis, possess double the amount of DNA (4n) and those in S phase have varying amounts of DNA ranging between 2n and 4n. It is therefore possible to measure cell cycle distribution within a cell population by staining the cellular DNA and grouping the cells, based on their DNA content, into three distinct groups G_0/G_1, S and G_2/M.

In order to be able to stain nuclear DNA the cell first has to be permeabilised to allow the fluorophore in use, access across the cell membrane. PI is routinely used in flow cytometry analysis as it only fluoresces when bound to double stranded nucleic acids but in a viable cell it is excluded from the cells due to the cell membrane. The cell membrane can be permeabilised through the use of alcohol, which creates tiny holes in the cell membrane allowing PI access to the cell nuclei but leaving the cell intact so that it can still be analysed using flow cytometry (Givan, 2001). In order that only the DNA is stained with the PI, after permeabilisation but before staining, RNAse is used to digest the RNA so that any resulting fluorescence can be solely attributed to the DNA content of the cell. Once stained and analysed with a flow cytometer the nuclei of the cells fluoresce red with an intensity that is proportional to their DNA content (Givan, 2001). Using the analysis software associated with the flow cytometer, gates can be placed around the cells to aid in the analysis of the proportion of cells in the varying phases of the cell cycle (Figure 5.1-3). Initial gating is done according to the shape (SSC) and size (FSC) of the cells, allowing for the exclusion of cellular debri with the majority of the cells being included in the gating at this stage (Figure 5.1-3A). Any cells included in the initial gating are then analysed according to their fluorescence and a plot based on the area (FL2-A) and width (FL2-W) of the fluorescent peak is produced (Figure 5.1-3B). In order to eliminate any cells that have

not fully disaggregated and have passed through the flow cytometer clumped together, a gate is placed around the cells with only a defined area and width. This therefore excludes any aggregated cells, as although 2 cells clumped together, which each posses a DNA content of 2n, would fluoresce at the same fluorescent intensity as a 4n cell, they would have double the cell area and width (Figure 5.1-3B). A DNA histogram is then produced, based on the cells included in this second gating, plotting the number of cells versus PI fluorescence (Figure 5.1-3C). The data obtained in this final DNA histogram can then be analysed by appropriate software packages (*e.g.* ModFit) to estimate the proportion of cells in each of the various groupings of the cell cycle (G_0/G_1, S and G_2/M.) (Figure 5.1-3D).

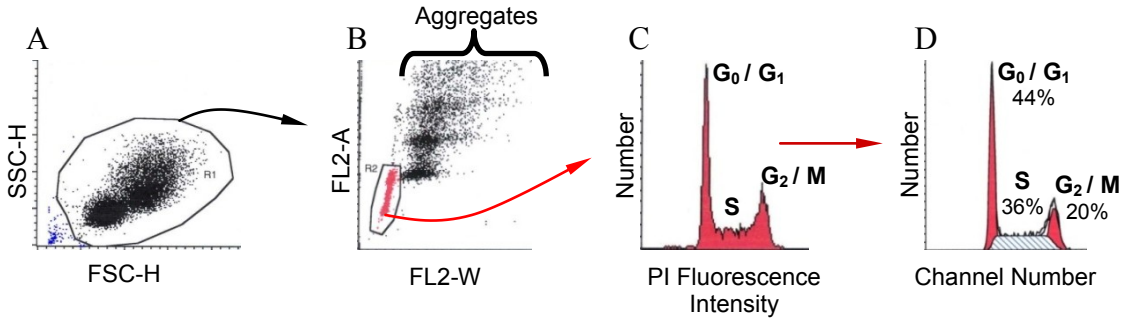


Figure 5.1-3 Cell Cycle Gating using a Flow Cytometer for the Analysis of Cellular Distribution
Flow diagram showing the process involved in gating cells for cell cycle analysis using flow cytometry; (A) cells are plotted according to their size and shape based on FSC and SSC parameters and gated to exclude cellular debris (blue cells), (B) gated cells are then plotted in a second chart and gated according to their fluorescence peak area (FL2-A) and width (FL2-W), thus allowing for the removal of clumped cells (black cells), (C) a DNA histogram of these gated cells is then plotted as the number of cells per fluorescent intensity, (D) and finally, appropriate computer software is used to estimate the proportion of cells in the various phases of the cell cycle.

5.1.4 Aims of this Chapter

Flow cytometry techniques were used in this chapter to study cell death and cell cycle distribution changes in the three bladder cancer cell lines in order to determine whether previous anti-proliferative results (Chapter Three) and enhanced crosslink formation (Chapter Four) had any biological significance in terms of changes in the cellular

responses known to be induced by MMC alone. All techniques were initially undertaken using mirtoselect alone treated cells, to determine if mirtoselect itself had any impact on cellular behaviour and subsequently in mirtoselect and MMC combined treated cells, to establish if combined treatment had any measurable beneficial or detrimental effects on bladder cancer treatment regimens.

5.2 Results

5.2.1 Cell Death

In analysis of cell death, cells were initially treated with mirtoselect alone to assess whether the decreases in cell number seen in the growth studies (Section 3.2) could be accounted for by induced cell death. Cells were also treated with mirtoselect and MMC in combination to assess whether the increase in crosslinks measured using the comet assay (Section 4.2) lead to a greater amount of cell death.

5.2.1.1 Annexin V – FITC Apoptosis Assay

Apoptosis was measured in the three bladder cancer cell lines by analysing the binding of annexin V to the surface of cells using flow cytometry. Gating of the cells for analysis of apoptotic response was performed, based on their fluorescence, using CellQuest software with early apoptotic cells (annexin V positive, PI negative) appearing in the top left quadrant and late apoptotic or necrotic cells (annexin V positive, PI positive) in the top right quadrant. Figure 5.2-1 gives an example of the differing profiles observed in RT112 cells treated with mirtoselect (B), MMC (C) and mirtoselect and MMC combined (D).

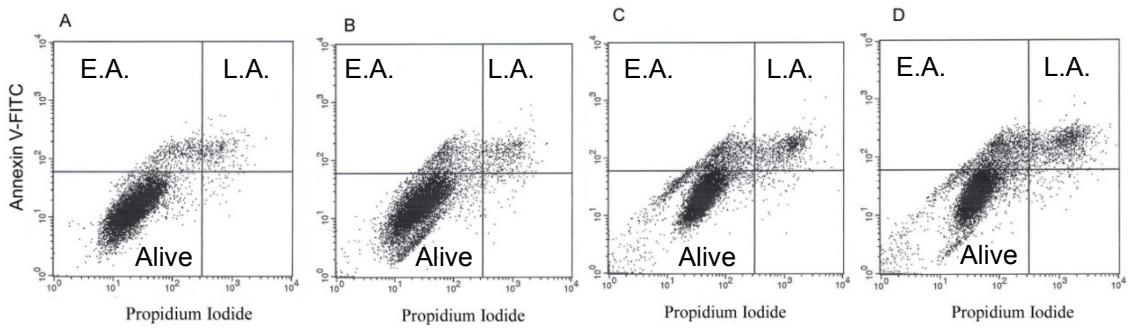


Figure 5.2-1 Annexin Profiles
Detection of the percentage of cells in early (E.A) (annexin V positive and PI negative; top left quadrant) and late apoptosis (L.A.) or necrosis (annexin V and PI positive, top right quadrant) by flow cytometry and CellQuest software, analysing 10,000 events per sample, in RT112 cells in response to mirtoselect and/or MMC. All samples were analysed at 72 hours post mirtoselect treatment, **A** control sample; **B** cells treated with 100µg/ml mirtoselect; **C** cells treated with 0µg/ml mirtoselect for 24 hours followed by 10µM MMC for 1 hour and incubated in drug free media; **D** cells treated with 100µg/ml mirtoselect for 24 hours followed by 10µM MMC for 1 hour and incubated in drug free media.

5.2.1.1.a Mirtoselect Alone Treatments

In mirtoselect alone studies the three bladder cancer cell lines were treated with mirtoselect at varying doses which was left on the cells for the duration of the experiment. This was to ensure that any apoptotic cells, induced due to mirtoselect treatment, were not lost through changing of the mirtoselect media as would of happened had a repeat dosing protocol had been followed.

The RT112 cell line exhibited a small dose dependent increase in the percentage of dead cells at each time point, with a significant level of apoptosis occurring at 48 hours in 50 and 100µg/ml mirtoselect treated cells (Figure 5.2-2A). There was not, however, a time dependent increase in apoptosis and at 72 hours there was no significant difference in the percentage of early or late apoptotic cells in mirtoselect treated cells compared to control. The RT4 cells also revealed a dose dependent increase in apoptosis in response

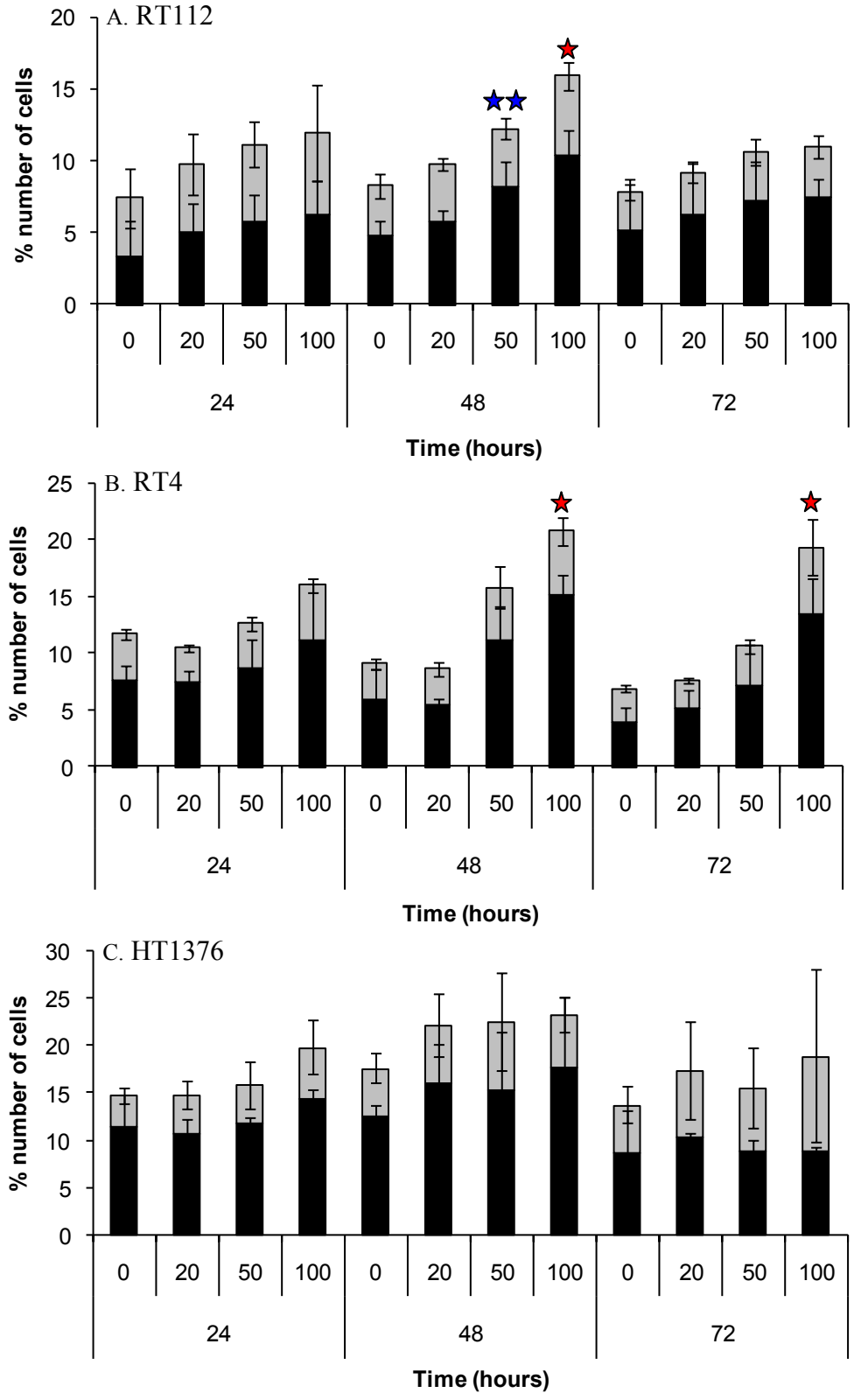


Figure 5.2-2 Annexin V-FITC Results for Mirtoselect Alone Treated Cells
Bladder cancer cell lines RT112 (A) RT4 (B) and HT1376 (C) were treated with varying doses of mirtoselect and analysed every 24 hours for a period of up to 72 hours. Percentage of early apoptotic (■) and late apoptotic/necrotic cells (□) were assessed based on annexin V-FITC and PI staining. Results are the mean of three independent experiments (n=3) ±SD, $p \leq 0.05$ =★, $p \leq 0.01$= ★★ as compared to the total number of apoptotic cells in the control.

to mirtoselect, with significant levels of apoptosis occurring in 100µg/ml treated cells at 48 and 72 hours, with the latter time point having ~3 times the amount of apoptosis compared to control (Figure 5.2-2B). The HT1376 cells did not have any consistent or significant increase in levels of apoptosis in response to mirtoselect (Figure 5.2-2C). This cell line did, however, contain higher levels of apoptotic cells in the control samples compared to the other two cell lines which potentially could have masked any small increases in apoptosis, as seen in the other cell lines. There was, however, a trend at 24 and 48 hours towards mirtoselect causing a dose dependent increase in early apoptotic cell numbers.

5.2.1.1.b Combined Studies

In combined studies, cells were treated for 24 hours in the presence of mirtoselect followed by a one hour treatment with MMC, all media was then removed and the cells were left in drug free media, measurement of apoptosis was done at 48 and 72 hours post mirtoselect treatment.

Measurement of apoptosis in combined treatments is shown in Figure 5.2-3. MMC-induced apoptosis in all three cell lines which was measurable using the annexin V-FITC apoptosis assay (Figure 5.2-3). All three cell lines exhibited a time dependent increase in apoptosis in MMC alone treated cells; RT4 cells exhibited the largest number of apoptotic cells at 72 hours whilst RT112 and HT1376 both had smaller numbers, indicating RT4 cells were more sensitive to MMC. In combined treatments, at 72 hours in RT112 cells (Figure 5.2-3A) and at 48 hours in HT1376 cells (Figure 5.2-3C) there was a clear dose dependent increase in apoptosis, though neither of these were statistically significant compared to the MMC treated control.

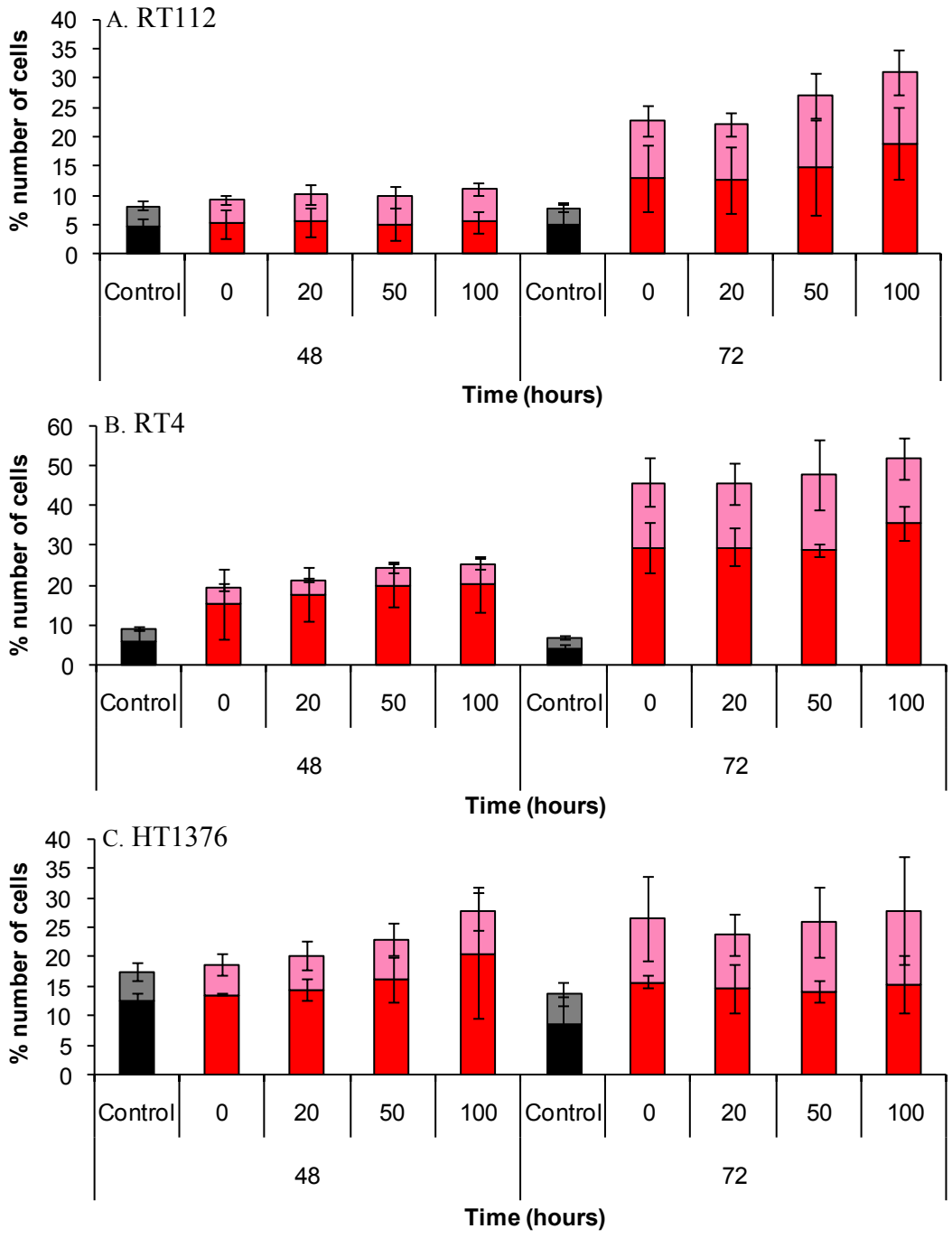


Figure 5.2-3 Annexin V-FITC Results for Combined Treatments
Bladder cancer cell lines RT112 (A) RT4 (B) and HT1376 (C) were treated with varying doses of mirtoselect for 24 hours followed by a one hour incubated with 10μM MMC. Cells were analysed 48 and 72 hours post mirtoselect treatment. Percentage of early apoptotic (control ■, MMC treated ■) and late apoptotic/necrotic cells (control ■, MMC treated ■) were assessed based on annexin V-FITC and PI staining. Results are the mean of three independent experiments (n=3) ±SD.

Pre-treatment of cells with mirtoselect did not adversely interfere with the apoptotic response of cells to MMC, and indeed appeared to mildly amplify the apoptotic

response of cells, though mostly in a sub-additive manner, with the exception of the 100µg/ml pre-treated cells which produced levels of apoptosis above that expected for an additive response in HT1376 cells at 48 hours (Table 5.2-1) and RT112 cells at 72 hours (Table 5.2-2).

Treatment	RT112		RT4		HT1376	
	Observed (%)	*Expected (%)*	*Observed (%)*	*Expected (%)*	*Observed (%)*	*Expected (%)*
Control	8	-	9	-	18	-
Mirtoselect 20µg/ml	10	-	9	-	22	-
Mirtoselect 50µg/ml	12	-	16	-	23	-
Mirtoselect 100µg/ml	16	-	21	-	23	-
MMC	**9**	**-**	**19**	**-**	**19**	**-**
MMC + Mirtoselect 20µg/ml	10	11	21	19	20	23
MMC + Mirtoselect 50µg/ml	10	13	24	26	23	24
MMC + Mirtoselect 100µg/ml	11	17	25	31	**28**	24

Table 5.2-1 Apoptosis Measurements using the Annexin V-FITC Assay at 48 hours
Observed measures of apoptosis from Annexin V-FITC experiments and expected results based on an additive relationship between mirtoselect and MMC. Results in red indicate an observed result exceeding expected.

Treatment	RT112		RT4		HT1376	
	Observed (%)	*Expected (%)*	*Observed (%)*	*Expected (%)*	*Observed (%)*	*Expected (%)*
Control	8	-	7	-	14	-
Mirtoselect 20µg/ml	9	-	8	-	17	-
Mirtoselect 50µg/ml	11	-	11	-	16	-
Mirtoselect 100µg/ml	11	-	19	-	19	-
MMC	**23**	**-**	**46**	**-**	**27**	**-**
MMC + Mirtoselect 20µg/ml	22	24	46	47	24	30
MMC + Mirtoselect 50µg/ml	**27**	26	48	50	26	28
MMC + Mirtoselect 100µg/ml	**31**	26	52	58	28	32

Table 5.2-2 Apoptosis Measurements using the Annexin V-FITC Assay at 72 hours
Observed measures of apoptosis from Annexin V-FITC experiments and expected results based on an additive relationship between mirtoselect and MMC. Results in red indicate an observed result exceeding expected.

5.2.1.2 TMRE

Externalisation of phosphatidylserine occurs in cells once they have reached the point of no return and are committed to undergoing apoptosis (Vermes *et al*. 2000). Prior to this there are a number of other events which can be used as markers of cell death, one of which is the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). Figure 5.2-4 gives an example of the differing profiles observed in RT112 cells stained with TMRE (A) after treatment with mirtoselect (B), or MMC (C) or mirtoselect and MMC combined (D).

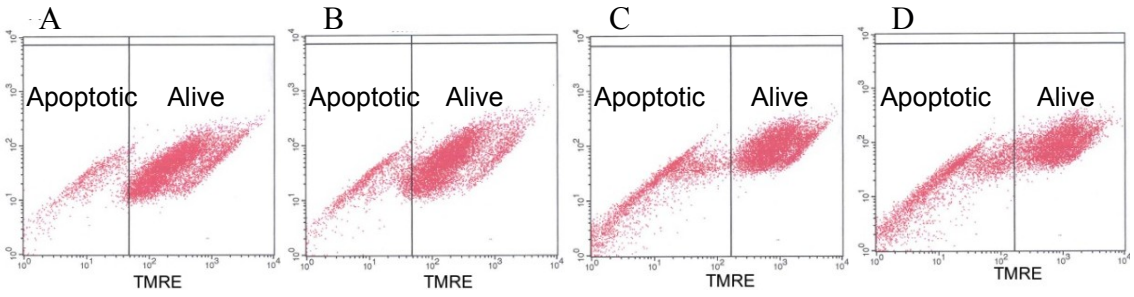


Figure 5.2-4 TMRE Profiles
Detection of the percentage of dead cells by flow cytometry using CellQuest software in RT112 cells in response to mirtoselect and/or MMC. Cell death was indicated by a reduction in fluorescence as cells were no longer able to accumulate TMRE in their mitochondria. All samples were analysed at 72 hours post mirtoselect treatment, **A** control sample; **B** cells treated with 100µg/ml mirtoselect; **C** cells treated with 0µg/ml mirtoselect for 24 hours followed by 10µM MMC for 1 hour and incubated in drug free media; **D** cells treated with 100µg/ml mirtoselect for 24 hours followed by 10µM MMC for 1 hour and incubated in drug free media.

5.2.1.2.a Mirtoselect Alone

Mirtoselect studies were carried out using a single dose of mirtoselect which was left on the cells for the duration of the experiment. Apoptosis was assessed in all three cell lines, analysing the cells every 24 hours over a 72 hour time period. RT112 cells exhibited a dose and time dependent increase in the percentage of apoptotic cells and at 72 hours there were significantly more apoptotic cells in the 100µg/ml mirtoselect treated sample compared to control (Figure 5.2-5A). RT4 cells did not show a clear

relationship between mirtoselect treatment and induction of apoptosis, however, a trend towards mirtoselect generally increasing the percentage of apoptotic cells was visible, with consistently higher measures in 100µg/ml treated cells (Figure 5.2-5B). In HT1376 the opposite was evident with a trend towards mirtoselect treatment causing a decrease in the percentage of apoptotic cells compared to control, though this was not significant (Figure 5.2-5C).

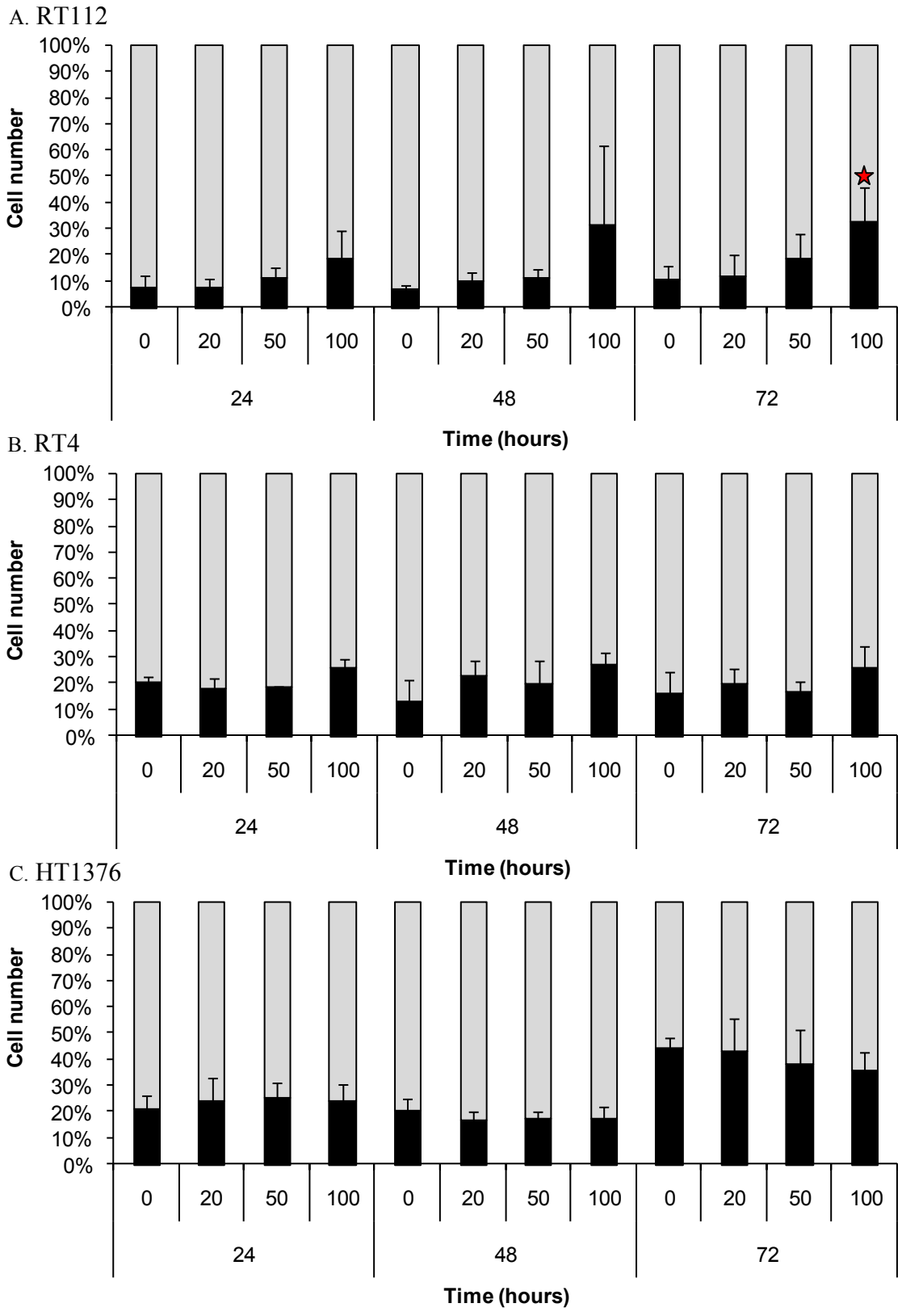


Figure 5.2-5 Mirtoselect TMRE Mitochondrial Membrane Potential Evaluation
Bladder cancer cell lines RT112 (A), RT4 (B) and HT1376 (C) were treated with a single continuous dose of mirtoselect. Measures of the mitochondrial membrane potential using TMRE exclusion as a marker of apoptosis were taken over a 72 hour period (alive □, apoptotic cells ■). Results are the mean of three independent experiments (n=3) ±SD, $p \leq 0.05 =$ ★.

5.2.1.2.b Combined Studies

Combined mirtoselect and MMC studies were performed, with cell treatments being identical to those used in the above annexin V/PI apoptosis study. MMC-induced apoptosis was measurable in all three cell lines, with a time dependent increase in the number of apoptotic cells in RT112 and RT4 cells (Figure 5.2-6A and Figure 5.2-6B). The HT1376 cells contained very high levels of apoptosis in response to all treatments at 48 hours (~70%), the highest of the three cell lines (Figure 5.2-6C), however there was no increase at 72 hours. RT112 cells exhibited a mirtoselect dose dependent increase in apoptosis at 48 and 72 hours, but this was not significantly higher than the MMC alone treated control. RT4 cells also showed a mirtoselect dose dependent increase in apoptotic cell numbers at 48 hours but by 72 hours levels were similar amongst all treatment combinations. Importantly in none of the cell lines investigated did mirtoselect treatment cause the number of apoptotic cells to be lower than in the MMC alone treated samples.

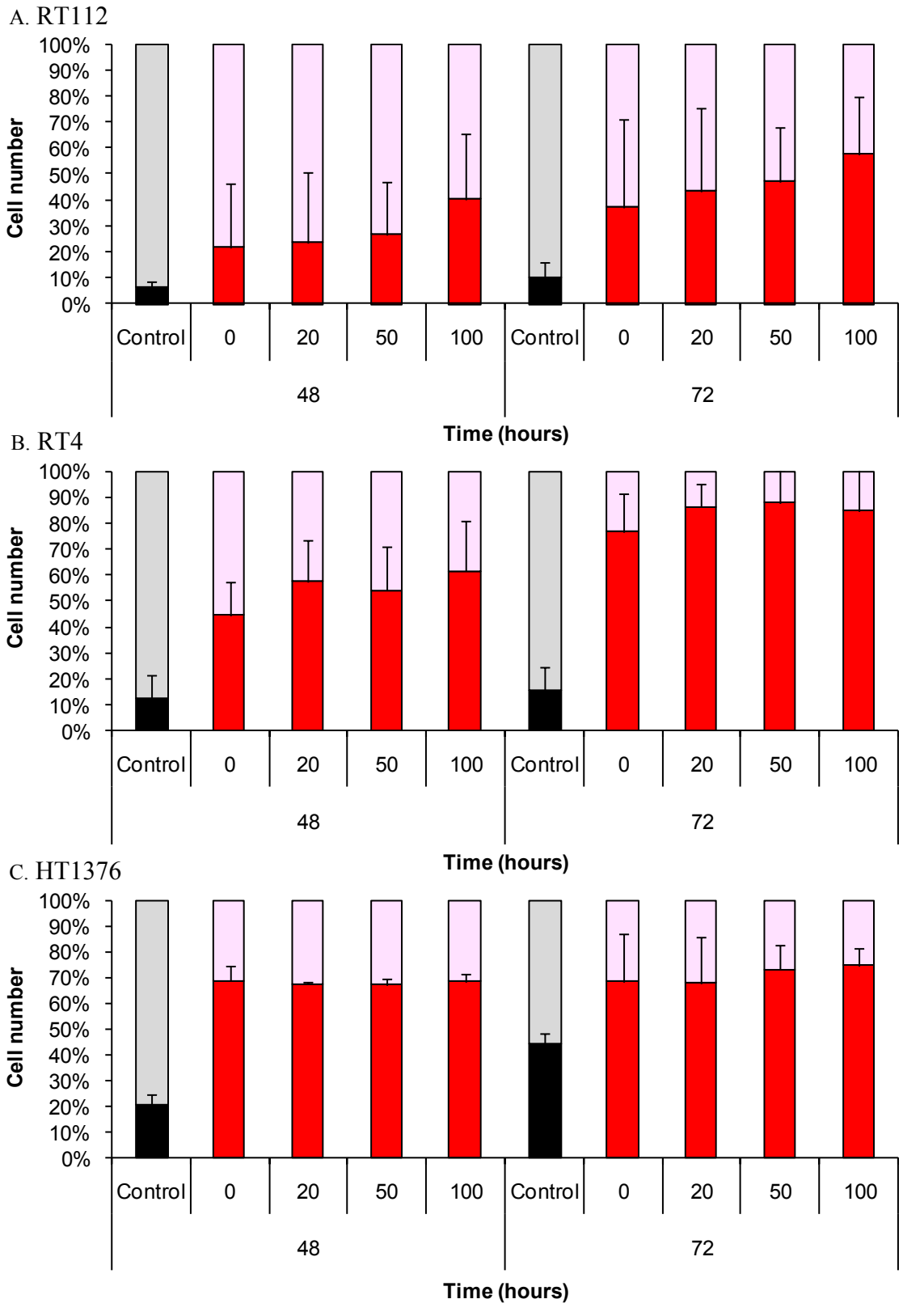


Figure 5.2-6 Combined TMRE Mitochondrial Membrane Potential Results
Bladder cancer cell lines RT112 (A), RT4 (B) and HT1376 (C) were treated with a single dose of mirtoselect followed by 10µM MMC. Measures of the mitochondrial membrane potential using TMRE exclusion on a FACS machine were taken over a 72 hour period post mirtoselect exposure (alive ▩ □, apoptotic cells ■ ■). Results are the mean of three independent experiments (n=3) ±SD.

5.2.2 Cell Cycle Changes

Cell cycle distribution changes were monitored initially in cells treated with mirtoselect alone, exposing cells to a single continuous dose of mirtoselect, and analysed using flow cytometry every 24 hours. In combined experiments, cells were treated for 24 hours with varying does of mirtoselect followed by a one hour treatment with 1µM MMC. Cells were then left in drug free media and were analysed at 48 and 72 hours post mirtoselect treatment. A lower concentration of MMC was necessary for cell cycle experiments, compared to those carried out to assess cell death, as a dose of 10µM caused a complete block in S phase, in RT112 and HT1376 cells, which ModFit software was unable to model and therefore meant the effects of mirtoselect could not be quantified. Figure 5.2-7 shows representative DNA histograms produced using CellQuest software in cells treated with MMC at varying time points. A noticeable change in cell cycle distribution can be seen in the RT112 cells over time in response to treatment with MMC (Figure 5.2-7C and D) as compared to their respective controls (Figure 5.2-7A and B). DNA histograms were subsequently analysed, using ModFit software, to estimate the percentage of cells in each phase of the cell cycle.

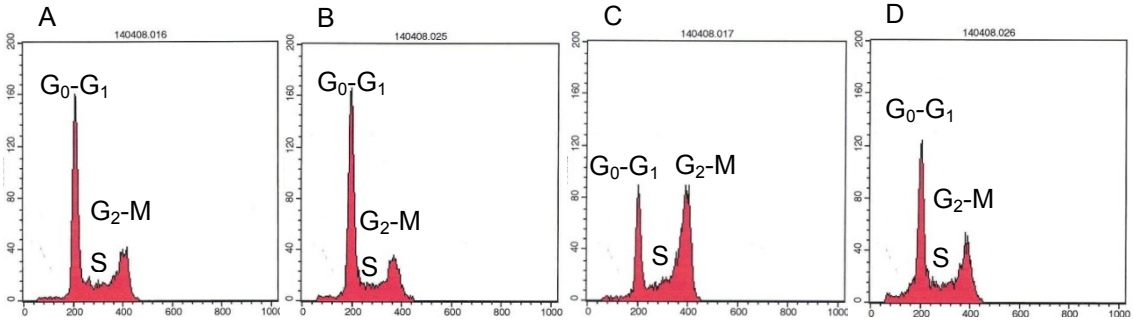


Figure 5.2-7 Cell Cycle Profiles
Flow cytometry analysis of the MMC-induced variation in cell cycle distribution. Representative profiles of RT112 cells at different incubation periods in drug free media, as determined by CellQuest software. (A-B) Control cells at 48 and 72 hours respectively, (C-D) 1µM MMC treated cells at 48 and 72 hours respectively.

5.2.2.1 Effect of Mirtoselect Treatment on Cell Cycle Distribution

In non drug treated control cells the RT112 cell line had a distribution of cells typical of an asynchronous population: 43% in G_0/G_1, 36% in S and 21% in G_2/M phase, and under normal growth conditions there was an accumulation of cells in the G_0/G_1 phase of the cell cycle over time (Figure 5.2-8A). In response to mirtoselect treatment there were no substantial changes in cell cycle distribution at any concentration of mirtoselect, as these also accumulated in G_0/G_1 over time, similar to the control. At 72 hours, the distribution of cells in control and 100µg/ml treated cells were almost indistinguishable with ~60% in G_0/G_1, 30% in S and 10% in G_2/M phase. Compared to the RT112 cell line, RT4 non drug treated control cells had a greater proportion of cells in G_0/G_1 phase of the cell cycle and consequently less in the S and G_2/M phases (Figure 5.2-8B). Under normal growth conditions there were only very minor changes in the distribution profiles of RT4 cells. In response to mirtoselect an initial decrease in the number of cells in the G_0/G_1 phase of the cell cycle was observed, at 24 hours, in a dose dependent manner, with a consequential increase in cells in S phase. At 48 and 72 hours, however, any differences in cell cycle distribution had been lost and profiles, as a result of mirtoselect treatment, were indistinguishable from control. HT1376 non drug treated control samples exhibited a similar profile of cell distribution at 24 hours to the RT112 cell line with: 36% in G_0/G_1, 36% in S and 28% in G_2/M phase, and under normal growth conditions this cell line also had an accumulation of cells in the G_0/G_1 phase of the cell cycle over time (Figure 5.2-8C). In response to mirtoselect treatment there was a small effect at 24 hours with a dose dependent increase in the number of cells in S phase of the cell cycle and relative decrease in those in G_2/M phase. At 48 and 72 hours, however, there was no clear mirtoselect dose dependent effect.

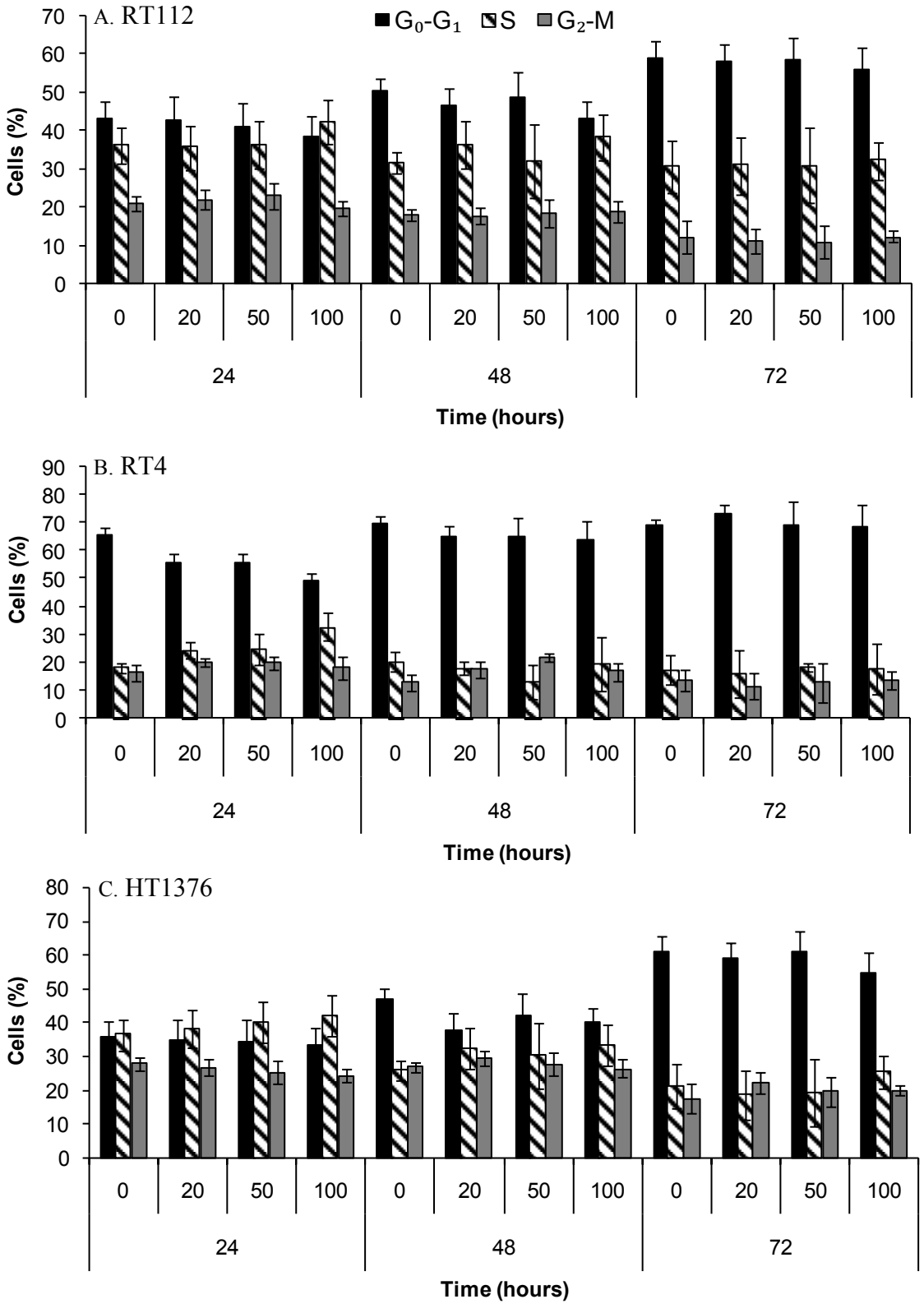


Figure 5.2-8 Cell Cycle Distribution in Response to Mirtoselect
Bladder cancer cell lines RT112 (A), RT4 (B) and HT1376 (C) were treated with a single dose of mirtoselect which was left on the cells for the duration of the experiment. Distribution of cells in the phases of the cell cycle: G_0/G_1 ■, S ◪ and G_2/M □, was assessed using ModFit software. Results are the mean of three independent experiments (n=3) ±SD.

5.2.2.2 Effect of Combined Mirtoselect and MMC Treatment on Cell Cycle Distribution

Analysis of combined mirtoselect and MMC treated cells was performed at 48 and 72 hours, having been given a 24 hour exposure to mirtoselect followed by 1 hour exposure to mirtoselect and MMC combined and then analysed after 24 and 48 hours in drug free media. Control samples were non drug treated. RT112 and RT4 cell lines exhibited normal distribution of cells in the control samples (Figure 5.2-9A and B). HT1376 cells, however, had a slightly different distribution of cells at 48 hours, with a higher proportion of cells in the G_2/M phase of the cell cycle (Figure 5.2-9C) but at 72 hours cellular profiles resembled a more typical distribution of cells.

In all three cell lines treatment with MMC alone caused a G_2/M block at 48 hours, with the addition of an increase in cells in S phase in the HT1376 cell line (Figure 5.2-9). At 72 hours the cells exited the block and returned to a distribution similar to that in the non drug treated controls at 48 hours. Treatment with mirtoselect prior to MMC did not, in any of the cells lines at any concentration of mirtoselect, cause any significant variation in the induced MMC block at 48 hours or subsequent exit from the block at 72 hours.

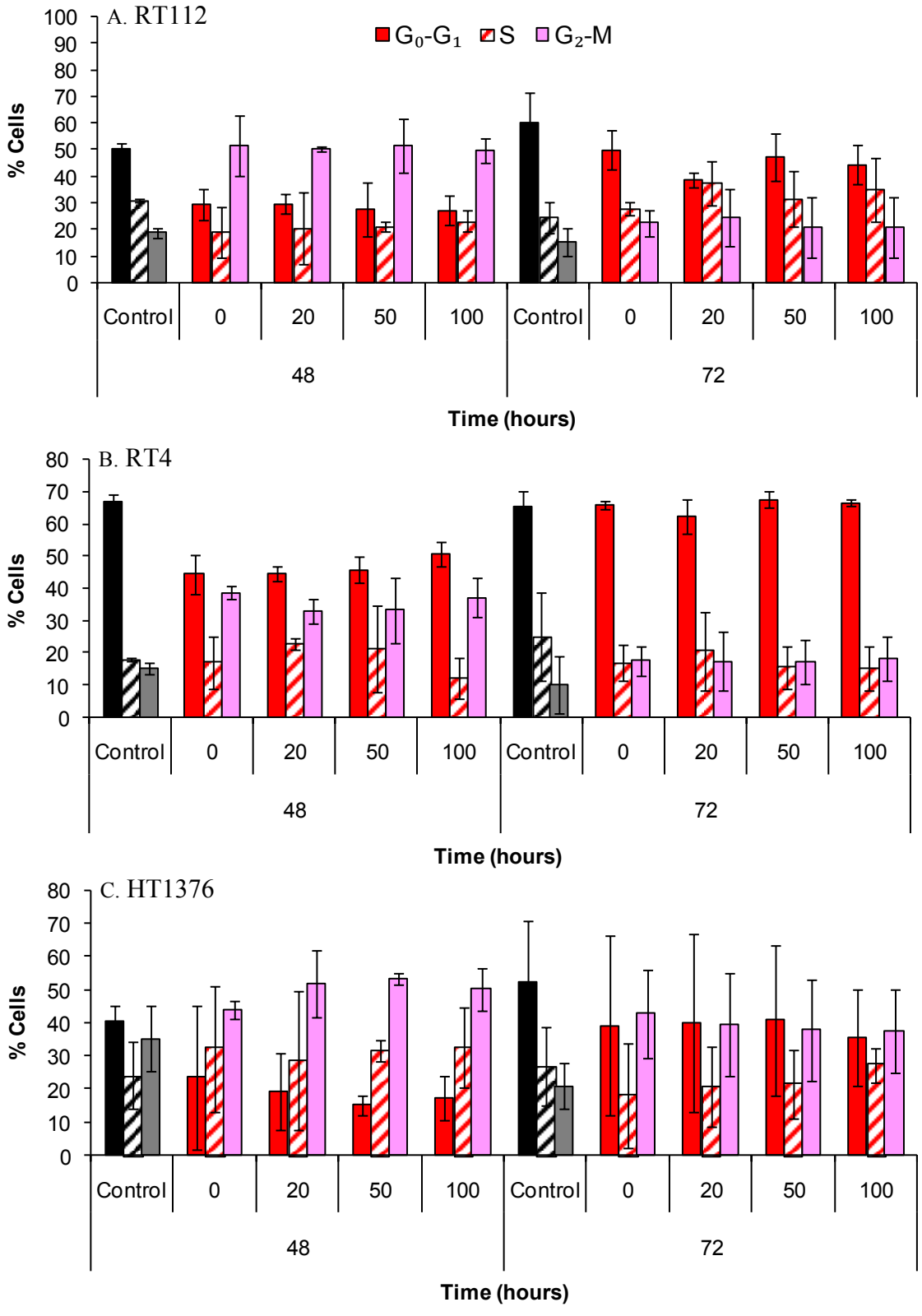


Figure 5.2-9 Cell Cycle Distribution in Response to Mirtoselect and MMC Combined
Bladder cancer cell lines RT112 (A), RT4 (B) and HT1376 (C) were treated with mirtoselect at varying concentrations for 24 hours followed by a 1 hour exposure to 1μM MMC and then left in drug free media prior to analysis. Distribution of cells in the phases of the cell cycle: G_0/G_1 ■, S ◪ and G_2/M ■, in control non drug treated cells and G_0/G_1 ■, S ◪ and G_2/M □, in mirtoselect and MMC combined treated cells, were assessed using ModFit software. Results are the mean of three independent experiments (n=3) ±SD.

5.3 Discussion

Analysis of cell death and cell cycle distribution were carried out to confirm that combined treatment did not adversely interfere with the activity of MMC and to also further tie together previous mirtoselect findings when studying growth, clonogenic survival and comet assay crosslink formation.

5.3.1 Mirtoselect-Induced Cell Death

Two methods were employed to detect apoptosis; Annexin V-FITC and TMRE staining. Using both techniques apoptosis was measurable in cells treated with MMC and there was agreement between methods, with RT4 showing the highest level of MMC-induced apoptosis. This is in agreement with MMC survival data as determined by growth (Section 3.2.1.2) and clonogenic studies (Section 3.2.2).

In mirtoselect alone studies only minor levels of apoptosis, <25% of all cells analysed, were detected using the annexin V-FITC assay. In RT112 cells, for example, a significant level of apoptosis was measurable at 48 hours in 100µg/ml treated cells, yet this was only 15% of the analysed population, a figure that does not equate to or account for the significant decrease seen in the growth studies. Although levels of apoptosis were low, a sensitivity ranking to mirtoselect could be distinguished: RT4 > RT112 > HT1376. This rank order of sensitivity correlates with survival data (clonogenic assay, Section 3.2.2), with the most sensitive cell line, RT4, showing the most significant induction of apoptosis.

TMRE analysis was performed in this study as it was hypothesised that anthocyans could potentially, through their ability to reduce cellular ROS, inhibit the induction of

apoptosis through the intrinsic mitochondrial pathway. Previous studies have shown that anthocyans act as antioxidants (Galvano *et al*. 2004, Zheng and Wang, 2003) reducing ROS activated events, such as the opening of mitochondrial megachannels which ultimately results in the loss of mitochondrial membrane potential. There was, therefore, a potential risk that pre-treatment with mirtoselect could block any MMC-induced apoptosis, which was generated through the activation of the intrinsic mitochondrial pathway. In measures of $\Delta\Psi m$, RT112 cells were the only cell line to exhibit any significant dose dependent effect due to mirtoselect treatment with an increase in the amount of apoptosis being measured at 72 hours; indicating in this cell line, at least, that mirtoselect was not protecting against the activation of apoptosis. Other researchers have also concluded that the induction of apoptosis by different chemopreventive agents is via the induction of ROS (Hou *et al*. 2005, Jeong *et al*. 2010, Hail, 2008) and could be measured through the loss of mitochondrial membrane potential. In HT1376 cells, however, an opposite effect was observed with a trend towards mirtoselect protecting cells against the loss of mitochondrial membrane potential. In previous measures of growth and cell survival, HT1376 cells had shown to be the least sensitive to the effects of mirtoselect, evidence from these experiments suggests a trend towards mirtoselect acting in this cell line to protect against mitochondrial mediated apoptosis.

5.3.2 Mirtoselect-Induced Cell Cycle Distribution Changes

Cell cycle distribution changes were also investigated in this chapter as it has been previously shown that MMC is able to induce cell cycle arrest. Also in light of the significant anti-proliferative effect seen in RT112 and RT4 cell lines in response to

mirtoselect treatment it was important to determine whether mirtoselect itself caused any blocks, or, in combination with MMC, altered the pattern of cell cycle distribution.

In mirtoselect treated RT4 and HT1376 cells a small effect was observed within the first 24 hours, however, none of the cell lines showed any prolonged changes in the distribution of cells as a result of mirtoselect treatment. Mirtoselect is made up of a mixture of 15 different anthocyanins. The effect of anthocyans on cell cycle distribution changes has been carried out in range of cell types with a number of different anthocyanins and anthocyanidins with different results being reported. Chen *et al*. (2005) reported that exposure of breast cancer cell line HS578T for 48 hours to cyanidin- and peonidin-3-glucoside caused a significant increase in G_2/M cells with a subsequent decrease in S-phase cells, whereas Shih *et al*. (2005) showed that gastric cancers cells exposed to malvidin for 24 hours accumulated in G_0/G_1. In a recent study by Lazze *et al*. (2004) the effect of two anthocyanins, delphinidin and cyanidin, were studied for their effects on two cancer cell lines (colon and uterine). In analysis of cell cycle distribution, delphinidin was able to induce a significant decrease in cells in the G_0/G_1 phase of the cell cycle with an accompanied appearance of a sub-G_1 peak, representing the induction of apoptosis. Cyanidin, however, did not cause any significant effect on either of the two cancer cell lines. In another study also investigating the effects of anthocyanins on colon cancers cells, an extract from chokeberry, containing mainly cyanidin anthocyanins, was shown to induce a block in the G_0/G_1 phase of the cell cycle at 24 hours (Malik *et al*. 2003). These varying results suggest anthocyanins do not evoke a standard response in cancer cells and that the composition of anthocyanins present, as well as the cell line being studied, have an impact on the effects seen in cell cycle distribution studies (Koide *et al*. 1997).

Although delphinidin is the predominant anthocyanidin in mirtoselect (42%) there is also a significant amount of cyanidin (29%) present in the mixture (Teller *et al*. 2009). Potentially these two anthocyanins could be acting on the bladder cancer cell lines with differing actions, therefore accounting for why, in this study, we did not see the same significant effects on cell cycle distribution as previously seen in studies using single agents.

5.3.3 Analysis of Apoptosis and Cell Cycle Distribution Changes in Combined Mirtoselect and MMC Treated Cells

In combined studies, cell cycle analysis and measures of apoptosis, using the annexin V-FITC assay and TMRE staining, revealed that pre-treatment with mirtoselect did not adversely interfere with the induction of MMC cell cycle blocks or MMC-induced apoptosis.

In annexin V-FITC experiments in was shown that combined treatments were in general additive and in some instances; RT112 and HT1376 100µg/ml mirtoselect/MMC, were more than additive. Although in this study the apoptotic enhancement by mirtoselect was non-significant, other chemopreventive agents have been shown to have significant effects in combination with cytotoxic cancer therapy agents (Sharma *et al*. 2004, Kuhar *et al*. 2007). In studies using TMRE to analyse apoptosis, MMC-induced apoptosis was measureable in all three cell lines and in HT1376 cells it was confirmed that although mirtoselect alone caused a dose dependent decrease in $\Delta\Psi m$, in combination with MMC there was no protective effect by mirtoselect seen in combined studies and apoptosis levels were identical. This once more demonstrates that combined treatment of mirtoselect, up to 100µg/ml, does not have any adverse effect on the actions of MMC

which is still able to induce an apoptotic response in all three of the bladder cancer cell lines investigated.

In studies of cell cycle distribution MMC treatment caused all three cell lines to arrest in the G_2-M phase of cell cycle; combination with mirtoselect did not affect the accumulation of cells in the G_2-M phase or the release and recovery of cells from this block. As previous experiments using mirtoselect alone had shown no substantial effects, the lack of cell cycle distribution changes in response to combined treatments was not to be unexpected. MMC is not considered to be cell cycle specific in its actions (Badalament and Farah, 1997) and therefore any minor alterations in cell cycle distribution by mirtoselect would not account for the increase in crosslink formation seen, however, the fact that mirtoselect does not interfere with the recovery of cells from the MMC-induced block could account for the similar rates of repair of crosslinks seen in comet assay experiments (Chapter Four). Many studies into interstrand crosslink (ICL) repair have historically been carried out in Fanconi anemia (FA) cells due to their increased sensitivity to crosslinking agents (Ahmad *et al*. 2002, D'Andrea and Grompe, 2003). FA cells, treated with MMC, arrest in the cell cycle at or near G_2 (Kaiser *et al*. 1982). Different ICL agents are known to trigger different cell cycle responses (Dronkert and Kanaar, 2001). Akkari *et al*. (2000) demonstrated that low dose exposure to an ICL agent resulted in a G_2/M arrest whereas high doses caused an S phase arrest (Akkari *et al*. 2000). Investigators using low dose cisplatin concluded that the arrest seen in the G_2 phase of the cell cycle is a consequence of cell synchronisation in late S phase, resulting in increased numbers of cells entering G_2 at the same time, rather than an actual block (Vaisman *et al*. 1997). This synchronisation/arrest in S phase is more than likely due to the recognition of the crosslink and its subsequent

repair, as seen in the treatment of HeLa cells with MMC (Mladenov *et al*. 2007, Mladenova and Russev, 2006). In this study treatment with MMC did cause a dominant G_2/M effect, but a slight increase in the percentage of cells in S phase at 48 hours in HT1376 cells was observed and at high doses of MMC (10μM) a dominant S phase arrest was indeed observed in RT112 and HT1376 cells (Appendix B) indicating that MMC is causing an S phase arrest to allow for repair of the ICLs. Since at 72 hours the profiles of the mirtoselect/MMC combined treated cells and the MMC alone treated cells were identical we can presume that the cells repair response to the crosslinks is similar, despite more crosslinks being measured in combination samples, which is in agreement with the response seen in comet assay studies of repair (Section 4.2.5.2). This once again demonstrates that mirtoselect pre-treatment of cells does not adversely interfere with the activity of MMC.

5.3.4 Conclusion

In this study the levels of apoptosis measured were, in general, not significant in mirtoselect alone studies and showed little enhancement in combined studies. This, therefore, suggests that the enhanced decreases of cellular growth seen in combined studies (Section 3.2.1.3) were not due to an increase in apoptosis.

It has previously been mentioned that the growth assay used in this project is unable to distinguish between cytotoxic events, cells dying, or cytostatic events, reduced cell growth, when cell numbers do not fall below initial seeding numbers. Results from this chapter aimed to address this by measuring cell death directly, yet it is still unclear as to what effects mirtoselect is having on the bladder cancer cell lines to bring about such a significant anti-proliferative effect. One potential hypothesis, in light of the lack of

mirtoselect-induced cell cycle blocks, is that there is a general slowing of the cell cycle which coupled with the low levels of apoptosis measured could account for the anti-proliferative effect seen. In the following chapter these issues are directly addressed and the hypotheses tested; also further investigations will be undertaken into the DNA damaging sensitizing effects of mirtoselect.

Chapter Six

Further Investigations into the Anti-Proliferative, Apoptotic and DNA Damage Enhancing Effects of Mirtoselect on Bladder Cancer Cell Lines

6.1 Introduction

The overall aim of this project was to determine the suitability of mirtoselect to be used as a chemopreventative agent in combination with current chemotherapeutic regimens. Whilst investigating this, however, questions were raised as to the mechanism of action of mirtoselect itself as, although it was proven to have significant anti-proliferative effects (Chapter Three), there was no substantial increase in levels of apoptosis or prominent cell cycle changes (Chapter Five) to provide supportive evidence of any phenotypic consequence, to its mode of action. In this chapter a number of different techniques were employed to further investigate the effects and actions of mirtoselect alone, on the bladder cancer cell lines.

6.1.1 CellTrace CFSE Proliferation Assay

The CellTrace CFSE proliferation assay provides a method for assessing individual cellular division. Cells are stained with carboxyfluorescein diacetate succinimidyl ester (CFDASE) which is membrane permeable so readily enters cells. CFDASE is non-fluorescent until the acetate groups are cleaved by intracellular esterases yielding carboxyfluorescein succinimidyl ester (CFSE) which is highly fluorescent. CFSE is far less membrane permeable and so accumulates inside of cells, where the succinimidyl ester groups react with intracellular amines, forming fluorescent conjugates that are highly retained. Any un-conjugated CFSE reagent can be removed, with repeated washing of the cells, as it passively diffuses into the extracellular medium. The dye-protein adducts formed by CFSE are well retained and consequently cells can be traced *in vivo,* making CFSE labelling a useful tool in studies of cell migration (Weston and Parish, 1990). Although CFSE was not originally developed for the analysis of cellular

proliferation, it's even distribution between daughter cells after cellular division and lack of transfer to adjacent cells within a population, makes it an ideal dye for assessing cellular division (Lyons and Parish, 1994). With each round of cell division there is a halving of the fluorescent intensity. In experiments using lymphocytes it is possible to see distinct fluorescent intensity peaks made up of cells in subsequent cell divisions from the undivided progenitor cells (Lyons and Parish, 1994). In cell populations with heterogeneous amounts of proteins, as is the case in most cancer cell lines, distinct peaks for each cellular generation are not often seen. Using labelling techniques and mathematical models of analysis, however, proliferating cancer cells, *in vitro* and *in vivo,* can be analysed by flow cytometry to track proliferation of the labelled cell samples (Matera *et al*. 2004) (Figure 6.1-1).

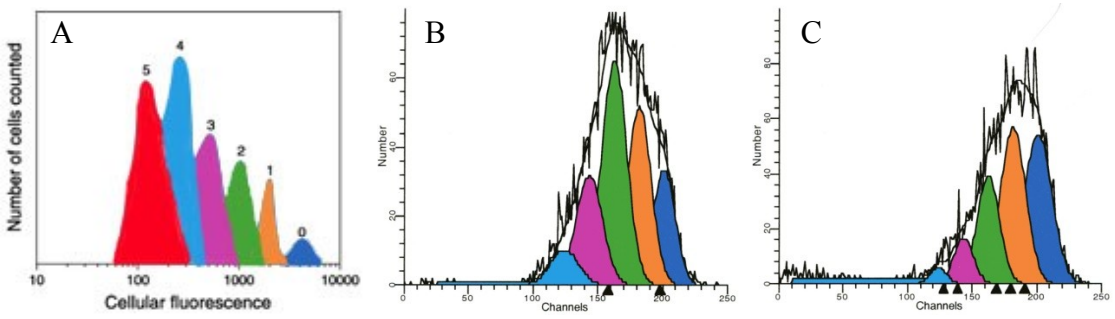


Figure 6.1-1 CellTrace CFSE Profiles
Tracking of an asynchronous cell population stained using CFSE. After each successive cell division there is a halving of the CFSE fluorescence resulting in a cellular fluorescence histogram in which the peaks represent successive generations 0 ■, 1 ■, 2 ■, 3 ■, 4 ■, 5 ■ (A) (image adapted from Invitrogen). The effect of treatment with mirtoselect can then be assessed by growing cells in culture in normal growth media (B, RT4 cells grown in control media at 24 hours) and in the presence of mirtoselect (C, RT4 cells grown in 100µg/ml mirtoselect media at 24 hours) and comparing the number of cellular division the cells have undergone.

In this project the CFSE reagent will be used to label bladder cancer cells before they are left to proliferate in the presence and absence of mirtoselect. The cells will then be analysed at varying time points to assess the percentage of cells that have undergone successive rounds of cellular division. Using this approach we will aim to address some

of the questions raised during Chapter Five with respect to the discussion of the cell cycle data and whether mirtoselect, although not causing substantial cell cycle blocks, could be causing cells to progress through the cell cycle at a slower rate compared to untreated controls.

6.1.2 ApoTox-Glo Triplex Assay

Apoptosis, cellular proliferation and cytotoxicity have all been measured in this project with respect to mirtoselect treatments using a number of techniques but they had not been carried out simultaneously. The ApoTox-Glo Triplex assay combines three assays in one to assess viability, cytotoxicity and caspase activation within a single well to aid determination of the mechanism of cytotoxicity of an agent. This triplex method provides an advantage over other assays which measure the same markers in separate wells, as when results are to be co-analysed the data can be normalised using the internal controls present which relate to the same population of cells. Within each well the ratio of live to dead cells is independent of cell number, providing a means for comparison of the data acquired from different wells, plates and days (Niles *et al*. 2009, Niles *et al*. 2007).

The first part of the assay simultaneously measures two protease activities; one being a marker of cell viability and the other a marker of cytotoxicity. The live cell protease activity is restricted to intact cells and is measured using a fluorescent, cell permeable, peptide substrate (glycylphenylalanyl-aminofluorocoumarin [GF-AFC]). The substrate enters intact cells where it is cleaved by the live cell protease activity to generate a fluorescent signal (AFC) proportional to the number of living cells. This live cell protease becomes inactive upon loss of cell membrane integrity and leakage into the

surrounding culture medium. A second, fluorogenic cell-impermeable peptide substrate (bis-alanylalanyl-rhodamine 110 [bis-AAF-R110]) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity (Figure 6.1-2). Because bis-AAF-R110 is not cell permeable, essentially no signal from this substrate is generated by intact live cells. The live and dead cell proteases produce different products, AFC and R110 respectively, which have different excitation and emission spectra, allowing them to be detected simultaneously.

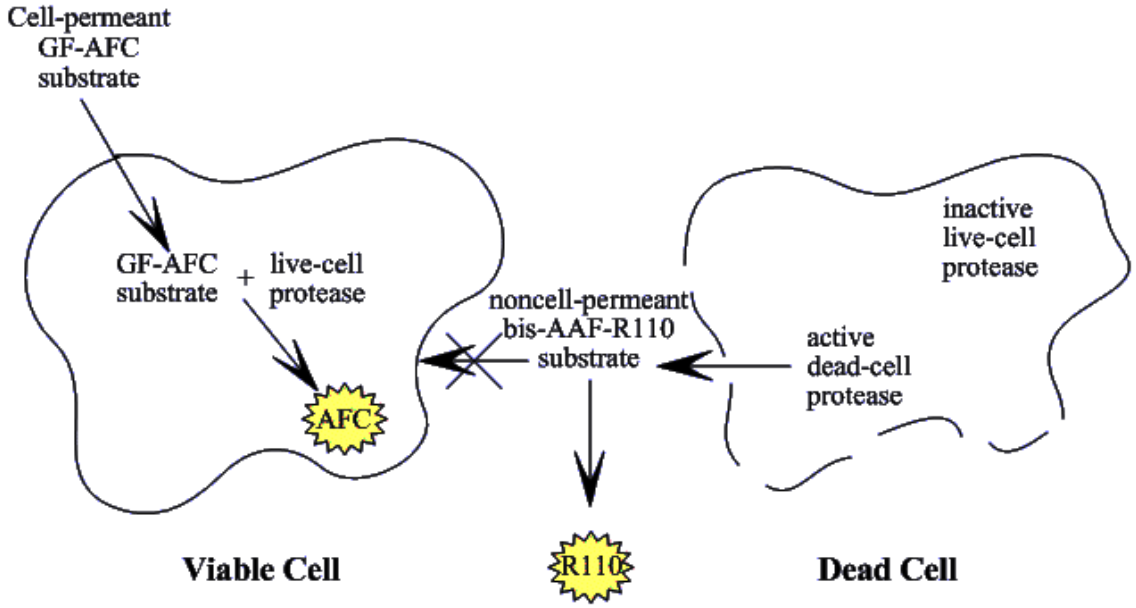


Figure 6.1-2 The Biology of the Viability/Cytotoxicity Assay
The GF-AFC substrate can enter cells where it is cleaved by the live-cell protease to release AFC. The bis-AAF-R110 cannot enter live cells but instead can be cleaved by the dead-cell protease to release R110.

The second part of the assay uses Caspase-Glo assay technology to measure apoptosis. Caspase-3 and 7 are members of the aspartate-specific cysteine protease family, members of which play keys roles in the apoptotic pathway in mammalian cells. Caspases exist as procaspases, to avoid cell death occurring as a consequence of unscheduled caspase activation, and signal in a two-step cascade. 'Initiator caspases',

such as caspase-8 and -9, undergo auto-activation which, once activated, activate 'executioner caspases' which go on to cleave cellular substrates. Caspase-3 and -7 both belong to the executioner group of caspases (Lamkanfi *et al*. 2002). Two independent apoptotic signalling cascades exist; the extrinsic and intrinsic pathways (Figure 6.1-3) (Lamkanfi and Kanneganti, 2010). Caspase-3 and -7 are involved in both of the pathways and bring about the biochemical and morphological hallmarks, such as phosphatidylserine exposure, nuclear condensation and genomic DNA fragmentation, distinguishing apoptosis from other forms of cell death.

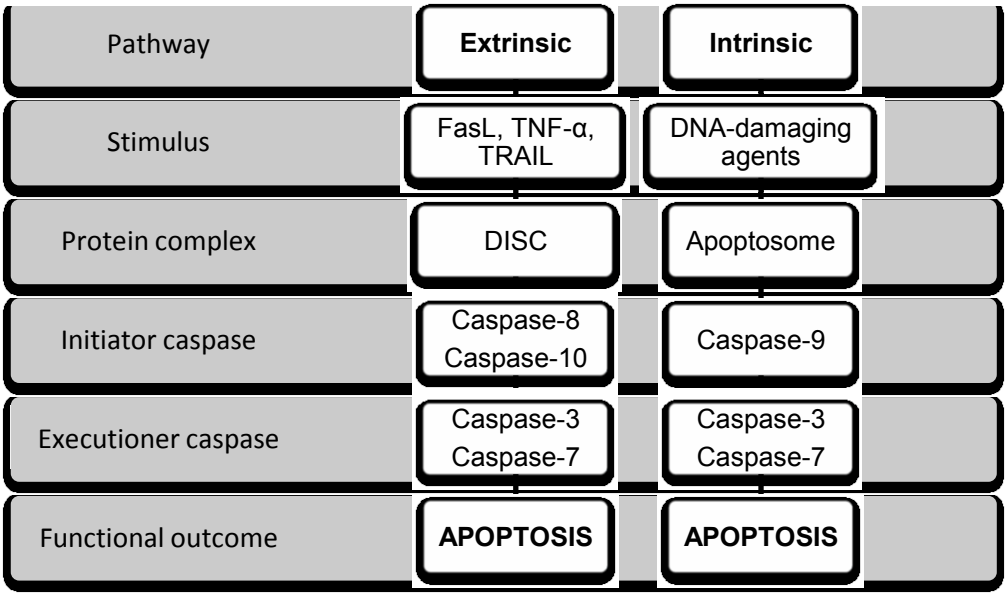


Figure 6.1-3 Flowchart Indicating the Different Mechanisms of Caspase Activation
Apoptosis signalling can occur via two independent cascades; the extrinsic and intrinsic pathways. In each case, however, initiator caspases (caspase-8, -9 and -10) are first activated bringing about the cleavage and activation of the executioner caspases (caspase-3, -7) which result in the biochemical and morphological hallmarks of apoptosis. (Adapted from Lamkanfi and Kanneganti, 2010).

Measurement of caspase-3 and -7, by means of the target amino acid sequence, DEVD, attached to a luciferase substrate, therefore provides a means of assessing early signalling events in the apoptotic pathway of cells committed to undergo cell death. In this assay the luminogenic caspase-3/7 substrate, which contains the tetrapeptide

sequence DEVD, is supplied in a reagent buffer optimized for caspase activity, luciferase activity and cell lysis. The caspase-Glo 3/7 reagent is added, after fluorescent readings (for viability and cytotoxicity) have been acquired, resulting in cell lysis, followed by caspase cleavage of the substrate and the generation of a 'glow-type' luminescent signal produced by luciferase (Figure 6.1-4). Luminescence is proportional to the amount of caspase activity present.

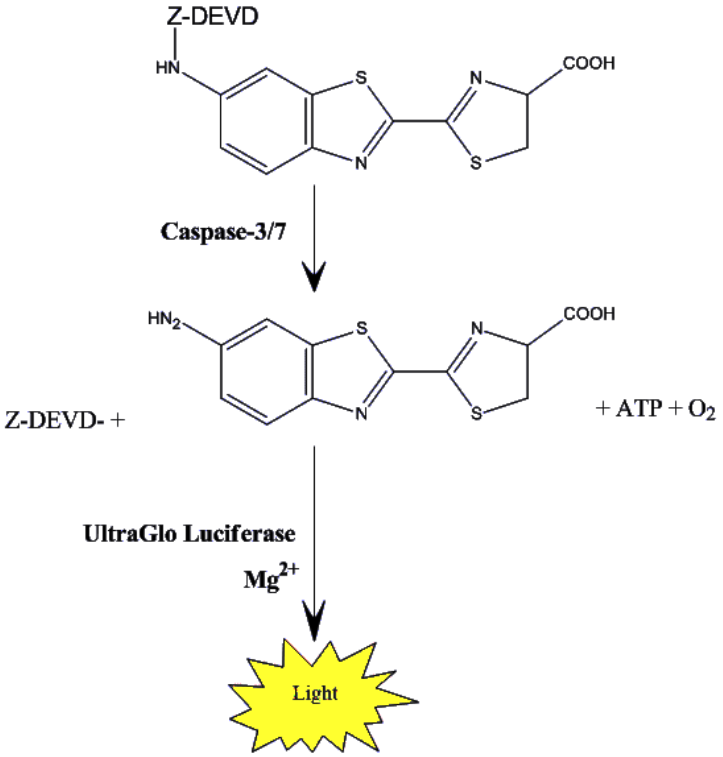


Figure 6.1-4 Caspase-3/7 Cleavage of Luminogenic Substrate Containing the DEVD Sequence
Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and production of light.

6.1.3 DNA Damage Sensitivity

DNA is a very important cellular target in cancer therapy. High levels of DNA damage can be lethal to a cell and many cancer treatments rely on inducing substantial levels of damage to cause cell death. In measures of DNA damage using a modified version of

the standard comet assay (Chapter Four) mirtoselect was shown to be able to enhance levels of DNA damage caused by MMC. To further investigate the role of mirtoselect in enhancing the actions of directly acting DNA damaging agents, an approach was required to investigate the direct DNA damaging effects of mirtoselect.

Radiation is an effective DNA damaging agent, with the majority of its associated biological effects being a consequence of modifications to DNA, where it acts both directly and indirectly to bring about DNA damage. Irradiation of DNA produces a number of different lesions, including double and single strand breaks as well as DNA base damage. The induction of DNA damage from ionising radiation resulting in strand breaks can be measured easily using the comet assay. During the work carried out in Chapter Four, it was established that mirtoselect did not affect the induced levels of radiation damage needed for the measurement of crosslinks. For the purposes of the modified comet assay, for measurement of crosslinks, this was an important factor. Under that protocol, however, the mirtoselect was not present during the irradiation, having already been washed away, along with the MMC, prior to exposure to X-rays. In all previous experiments, where interactions between mirtoselect and MMC had been noted, the cell lines were pre-treated with mirtoselect and then exposed briefly to MMC, however, the mirtoselect and MMC were always co-administered. In this chapter radiation will be used as a probe to determine if exposure to mirtoselect during irradiation has any effect on the damage sensitivity of the cell lines which might help explain previous results.

As covered in Chapter One (Section 1.2) the majority of bladder cancers present as non muscle invasive tumours, yet a significant number, 30%, are muscle invasive. For muscle invasive bladder cancer patients, radiotherapy (RT) can offer tumour control

whilst still retaining bladder function. To be considered as a primary treatment option for patients, however, RT is often used in conjunction with other treatments, such as chemotherapy, in order to achieve disease free survival. Chemopreventive agents occurring naturally in the diet are therefore appealing candidates as possible radiosensitizers, especially as their toxicity profiles are low (Javvadi *et al*. 2008). If mirtoselect is, therefore, able to sensitise the DNA of the bladder cell lines to radiation then there is also potential for its use as a radiosensitizer for the treatment of muscle invasive bladder cancer, hopefully improving the success rate of radiotherapy and sparing the patient from having to undergo both primary or salvage cystectomy.

6.1.4 Aims of this Chapter

The aim of this chapter is to probe further into the biological effects of mirtoselect; to further evaluate whether it is safe to be used as a chemopreventative agent in conjunction with current chemotherapeutic treatment, MMC, and to determine if it can sensitize DNA to the effects of radiation. Assays will be used to analyse cellular division using a cell tracking dye (cellTrace CFSE proliferation assay), assess viability, cytotoxicity and apoptosis in a single well (Apo-Tox-Glo triplex assay) and assess radiation-induced DNA damage (comet assay).

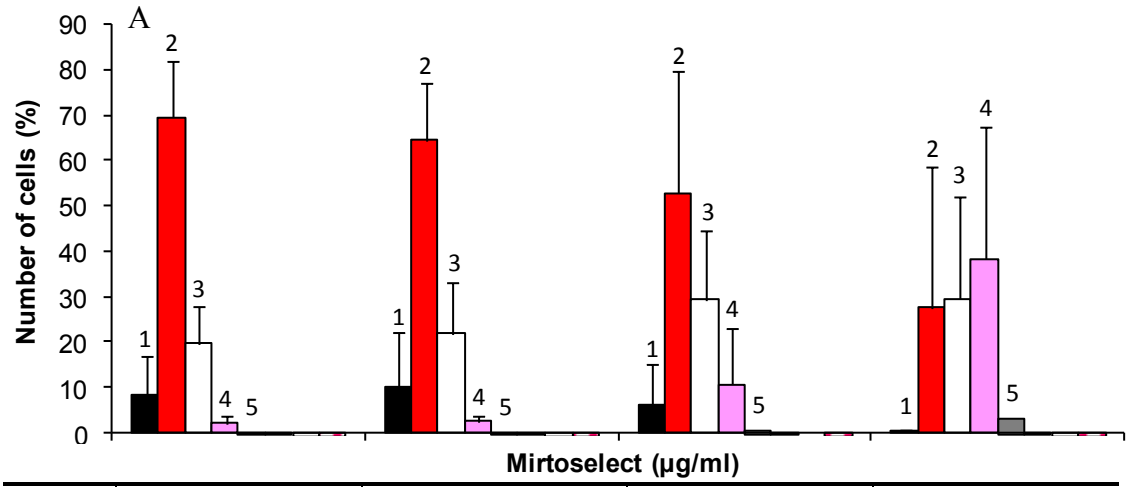
6.2 Results

6.2.1 CellTrace CFSE Proliferation Assay

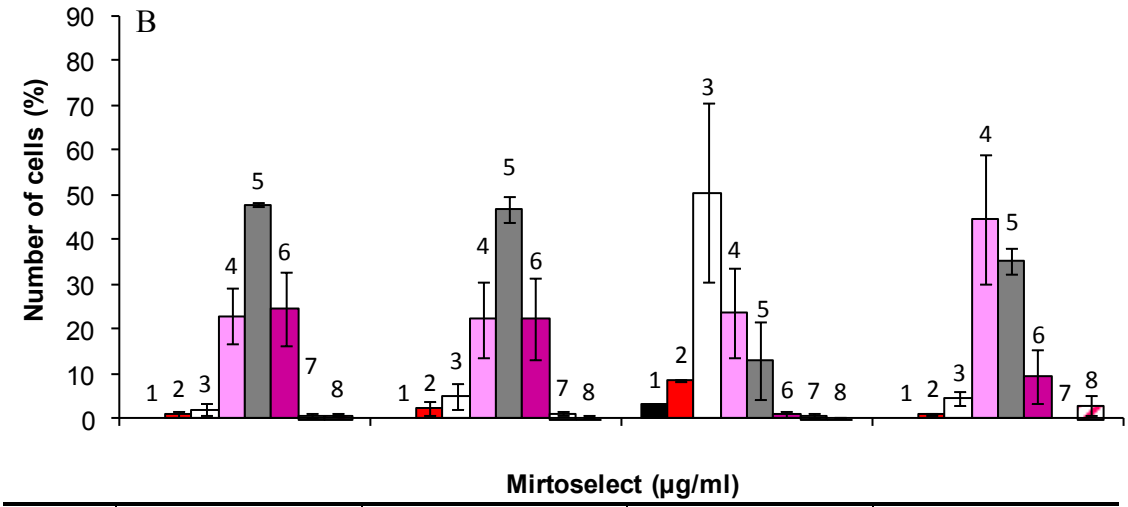
All three cell lines were stained with CFSE, then left to attach for 24 hours prior to being treated with mirtoselect. A single dose of mirtoselect was left on the cells for the duration of the experiment. After 24 and 72 hours treatment with mirtoselect cells were harvested and analysed using flow cytometry. Figure 6.2-1 to Figure 6.2-3 present the

data in a graphical format, based on the percentage of cells per cellular generation as assessed using the proliferation peak analysis function within Modfit. Proliferation indices (ProI) were also generated by Modfit, these are an approximation of the average number of cells that the original stained cells became.

RT112 cells exhibited the most significant anti-proliferative effect as a result of treatment with mirtoselect, as measured by the CyQuant proliferation assay (Section 3.2.1.1) and also cell survival was significantly decreased, as measured using the clonogenic assay (Section 3.2.2). After 24 hours treatment with mirtoselect, profiles of the number of cells in each successive cellular generation were similar amongst control and 20µg/ml treated cells (Figure 6.2-1A). In 50µg/ml treated cells, although not substantial, there was a trend towards treatment causing cells to undergo more cellular divisions than control with a proliferation index for 50µg/ml cells of 2.53 compared to 2.10 for control. In 100µg/ml treated cells the effects of mirtoselect on cellular division were more pronounced with the majority of cells being of the 4^{th} successive generation compared to control which were only the 2^{nd}, indicating that treatment with high doses of mirtoselect did cause cells to undergo more cellular divisions within the first 24 hours of treatment. Proliferation indices also reflected this, with 100µg/ml treated cells having an index of 4.30. At 72 hours, control cells had undergone a number of cellular divisions (ProI 13.78) and the majority of cells (48%) were found to be the 5^{th} successive generation (Figure 6.2-1B). Treatment with 20µg/ml mirtoselect caused no differences in the number of successive generations undergone but there was a trend towards cells proliferating slower with a ProI of 12.87. A definite decrease in cellular divisions was seen in 50µg/ml mirtoselect, with the majority of cells (50%) being the 3^{rd} generation (ProI 8.69). In the 50µg/ml treated cells it appears that the majority of cells



	0	20	50	100
ProI	2.10	2.13	2.53	4.30



	0	20	50	100
ProI	13.78	12.87	8.69	10.52

Figure 6.2-1 RT112 CellTrace CFSE Proliferation Assay Graphs
RT112 cells were stained with CFSE and grown in the presence of varying concentrations of mirtoselect. The bars represent successive cellular generations (1 ■, 2 ■, 3□, 4 □, 5 ■, 6 ■, 7 □, 8 ▨). (A) Cells analysed at 24 hours and (B) 72 hours post mirtoselect treatment. Proliferation indices (ProI) are an approximation of the average number of cells that an initial cell became as calculated by Modfit software. Results are the mean of three independent experiments (n=3) ±SD.

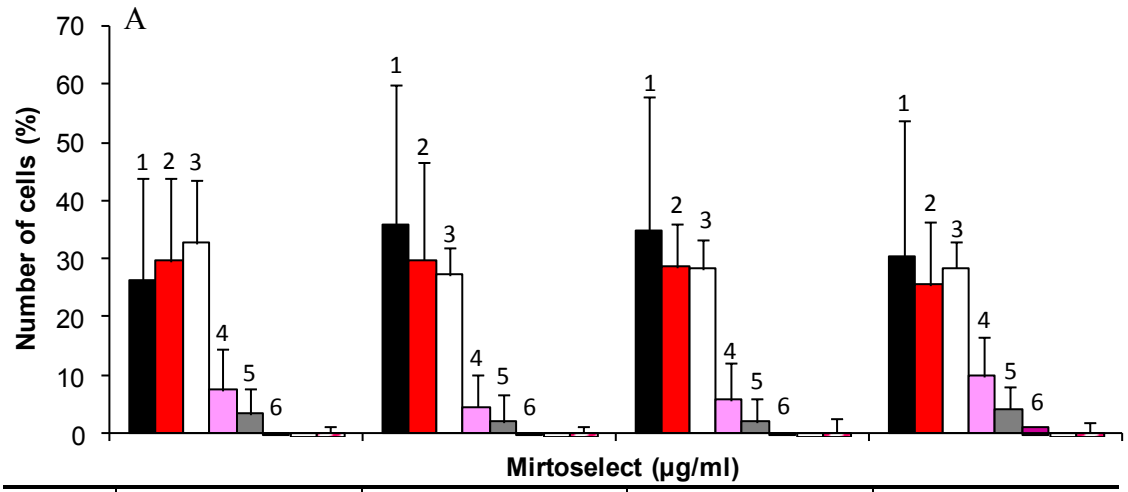
had only undergone one round of replication within the 48 hour time period, compared to control, which had undergone between three and four rounds. In the 100µg/ml treated cells the majority of cells (45%) analysed were of the 4th generation with the remaining cells being predominantly of the 5th generation (35%) (ProI 10.52). In

comparison to the profile seen in the 100µg/ml treated cells at 24 hours and the control at 72 hours, continued treatment with 100µg/ml caused a slowing of proliferation.

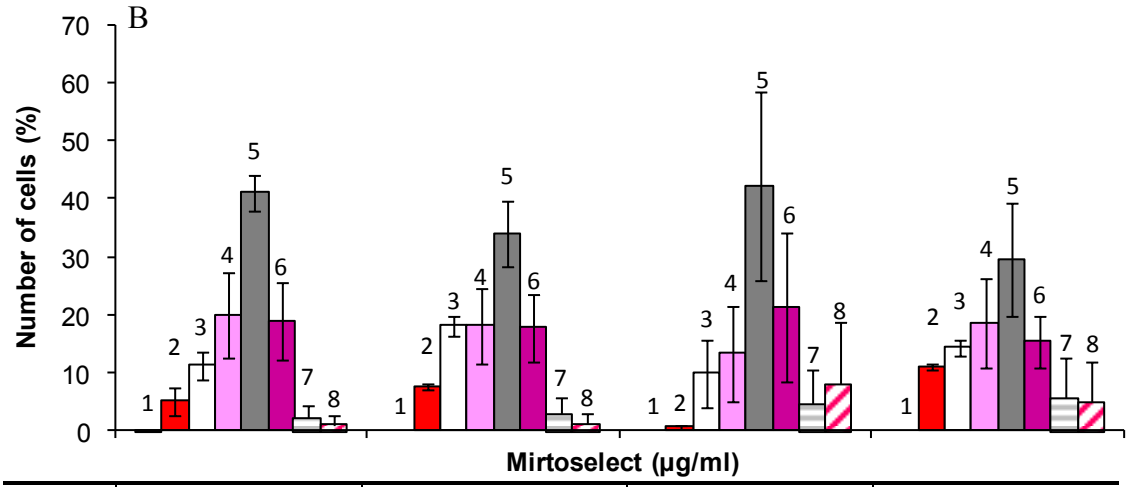
In CFSE stained RT4 cells, treatment with 20 and 50µg/ml mirtoselect caused little effect on the cellular division of cells with similar profiles for the proportion of cells per cellular generation and similar proliferation indices in both the treated and control cells at 24 and 72 hours (Figure 6.2-2A and B). In 100µg/ml treated cells, profiles were similar to control at 24 hours, however, at 72 hours a slight difference was observed with the profile of the treated cells indicating a possible slowing of cellular proliferation (ProI 7.21 compared to 8.98).

CFSE staining revealed very little effect of mirtoselect on cellular division in HT1376 cells. At 24 hours, control and mirtoselect treated cells were identical in the proportion of cells in each successive generation (Figure 6.2-3A). Analysis at 72 hours of control cells showed that the majority were the 4^{th} or 5^{th} generation, this was also the situation in cells treated with mirtoselect (Figure 6.2-3B). Analysis of the proliferation indices for the 72 hours samples revealed a trend towards treatment causing a slowing of cellular division (control ProI 9.02, 100µg/ml 7.02).

In all three cell lines, analysis at 72 hours indicated that none of the cells were of the original cellular generation and that all cells, irrespective of treatment, had undergone at least one or two cellular divisions since CFSE staining had taken place.

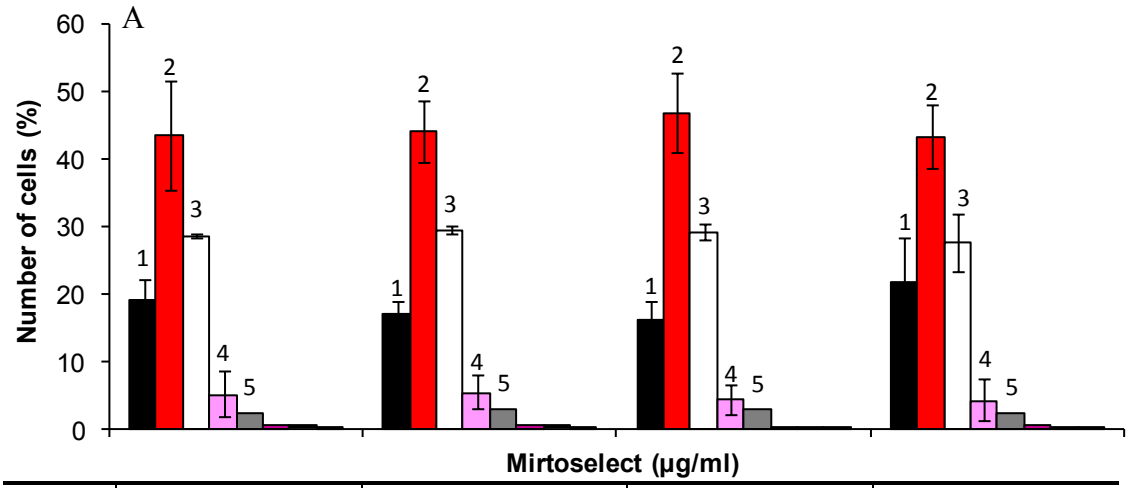


	0	20	50	100
ProI	2.00	1.73	1.77	2.03

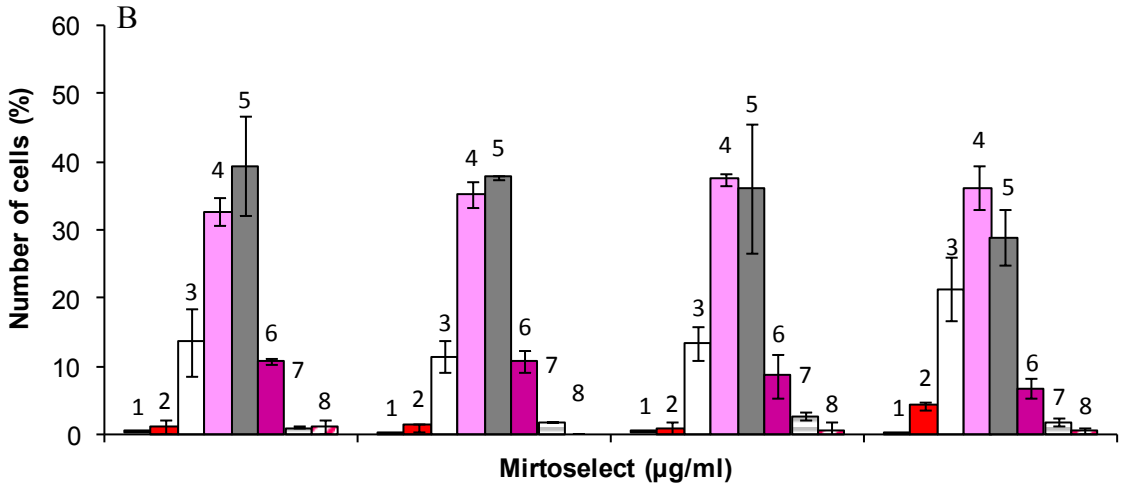


	0	20	50	100
ProI	8.98	7.52	9.26	7.21

Figure 6.2-2 RT4 CellTrace CFSE Proliferation Assay Graphs
RT4 cells were stained with CFSE and grown in the presence of varying concentrations of mirtoselect. The bars represent successive cellular generations (1 ■, 2 ■, 3□, 4 □, 5 ■, 6 ■, 7 □, 8 ▨). (A) Cells analysed at 24 hours and (B) 72 hours post mirtoselect treatment. Proliferation indices (ProI) are an approximation of the average number of cells that an initial cell became as calculated by Modfit software. Results are the mean of three independent experiments (n=3) ±SD.



	0	20	50	100
ProI	2.05	2.11	2.10	1.97



	0	20	50	100
ProI	9.02	9.25	8.85	7.02

Figure 6.2-3 HT1376 CellTrace CFSE Proliferation Assay Graphs
HT1376 cells were stained with CFSE and grown in the presence of varying concentrations of mirtoselect. The bars represent successive cellular generations (1 ■, 2 ■, 3□, 4 □, 5 ■, 6 ■, 7 □, 8 ▨). (A) Cells analysed at 24 hours and (B) 72 hours post mirtoselect treatment. Proliferation indices (ProI) are an approximation of the average number of cells that an initial cell became as calculated by Modfit software. Results are the mean of three independent experiments (n=3) ±SD.

6.2.2 ApoTox-Glo Triplex Assay

All three cells lines were plated in 96 well plates and exposed to varying concentrations of mirtoselect for up to 72 hours applying a single continuous dose of mirtoselect.

Analysis of cellular viability, cytotoxicity and apoptosis were all measured in a single well at 16, 24, 48 and 72 hours post mirtoselect administration.

6.2.2.1 Viability, Cytotoxicity and Apoptosis

Data is represented in the charts below as relative fluorescent units (RFU) for viability and cytotoxicity data and relative luminescent units (RLU) for caspase activity.

In the RT112 cell line, even at the earliest time point, 16 hours, there was a dose dependent decrease in cell viability as a consequence of mirtoselect treatment. By 72 hours, 100µg/ml mirtoselect treated cells had 66% viability compared to control (Appendix C). At none of the time points measured for RT112 cells did measures of cytotoxicity rise above that of the control. In measures of caspase activity increases in apoptosis were not measurable until 48 and 72 hours. At 48 hours there was a small dose dependent increase in measures of caspase activity and by 72 hours 100µg/ml treated cells had 17% more measureable luminescence compared to control.

In RT4 cells there was also a small dose dependent decrease in cell viability as early on as 16 hours, this was then more dramatic at 72 hours with ~69% viability compared to control (Appendix C). Cytotoxicity did not increase as a consequence of mirtoselect treatment over the first 48 hours of analysis, however, at 72 hours higher levels of cytotoxicity were measurable in the 100µg/ml treated cells compared to control. Increases in apoptosis, as a consequence of mirtoselect treatment, were not measured until 48 hours when there was a small dose dependent increase in the levels of caspase activity. At 72 hours, measures of luminescence in 100µg/ml treated cells were 2.4 times higher than in control cells.

The HT1376 cell line showed little effect of mirtoselect on the cells in terms of cellular viability using this assay. At 72 hours viability was lower in 100µg/ml treated cells compared to control, yet there was no effect in cells exposed to 20 or 50µg/ml (Appendix C). Measures of cytotoxicity did not increase as a result of mirtoselect treatment and readings were consistently lower in treated cells compared to control. In measures of apoptosis, levels of caspase activity were not increased at any of the time points analysed. At 72 hours, caspase activity was actually lower in mirtoselect treated cells compared to control, with around 13% less luminescence in 100µg/ml treated cells compared to control.

6.2.2.2 Normalisation of the Data

The major advantage to the ApoTox-Glo assay compared to other methods assessing viability and apoptosis is that all three measures were carried out in a single well. This means that data can be normalised and data between different plates and experiments can be analysed irrespective of cell number (Section 6.1.2). Readings for cytotoxicity and caspase activity were corrected to account for any increases in cell number based on the linear relationship between fluorescence and cell number seen using this assay (Niles *et al*. 2007). A ratio of the number of live cells (fluorescent units) to dead cells (fluorescent [cytotoxic] or luminescent [apoptotic] units) was generated for each time point, and mirtoselect dose, and plotted in Figure 6.2-4 and Figure 6.2-5. Figure 6.2-4 therefore represents the normalised fluorescent reading as a relative value per cell and Figure 6.2-5 the normalised luminescent reading per cell. No increases in mirtoselect-induced cytotoxicity was observed in RT112 and HT1376 cell lines (Figure 6.2-4A and C); but normalisation of the RT4 data revealed a 3-fold increase in cytotoxicity at 72 hours in 100µg/ml treated cells compared to control (Figure 6.2-4B). In normalised

RT112 apoptosis data, control cells exhibited a gradual increase in caspase activity over the 72 hours analysed (Figure 6.2-5A). At the early time points, 16 and 24 hours, there was no difference in activity between treatments but by 48 hours there was a visible mirtoselect dose dependent increase in caspase activity and by 72 hours there was a significant difference in 100µg/ml treated cells compared to control, with a 1.7-fold increase in caspase activity. In RT4 cells there was a visible, but non-significant, mirtoselect dose dependent increase in caspase activity at early time points and by 72 hours there was a significant difference in 100µg/ml treated cells compared to control, with a 2.7-fold increase in caspase activity. In HT1376 cells there was an increase in caspase activity in control cells over time but there was no mirtoselect dependent increase in activity above control with no significant differences between treatment and control at any time point (Figure 6.2-5C).

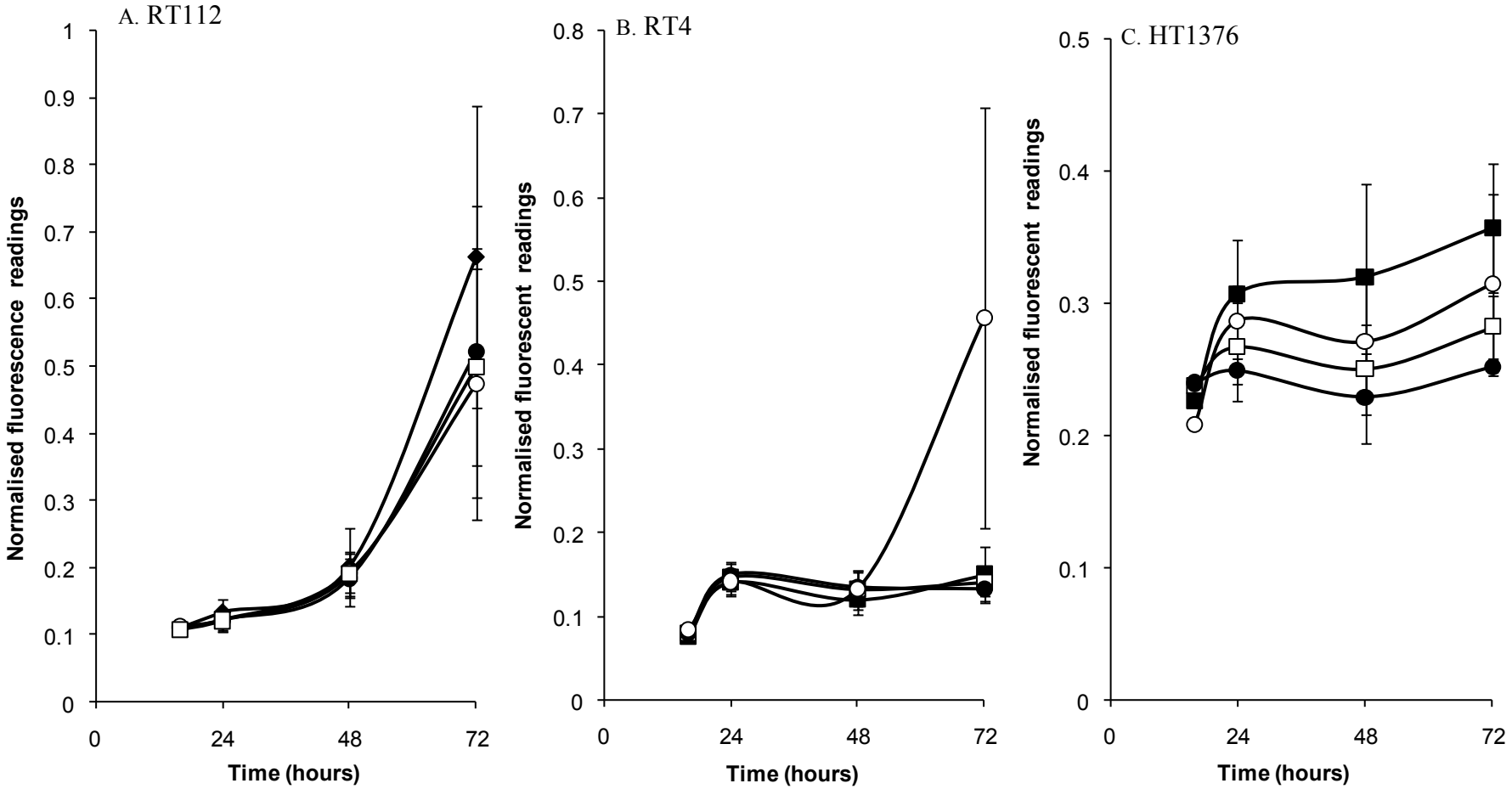


Figure 6.2-4 Normalisation of Measures of Cytotoxicity Against Measures of Viability
Normalisation of cytotoxicity data from ApoTox-Glo experiments in which RT112, RT4 and HT1376 cells were treated with 0 ■, 50 □, 75 ●, and 100µg/ml ○ mirtoselect for up to 72 hours. Data was normalised by calculating the relative fluorescence (cytotoxic) per cell basis by using viability data as a measure of cell number.

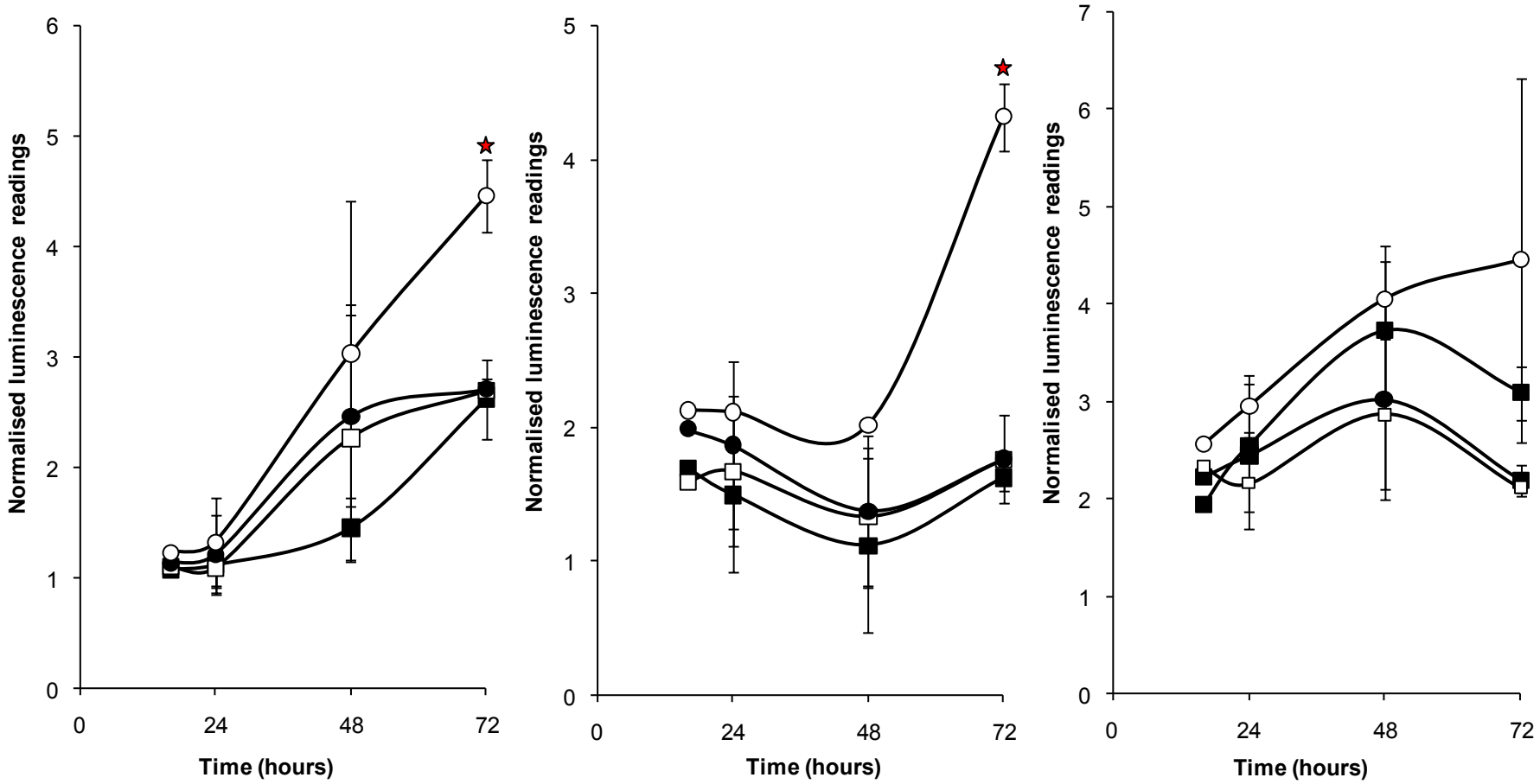


Figure 6.2-5 Normalisation of Caspase Activity Against Measures of Viability
Normalisation of caspase activity data from ApoTox-Glo experiments in which RT112, RT4 and HT1376 cells were treated with 0 ■, 50 □, 75 ●, and 100µg/ml ○ mirtoselect for up to 72 hours. Data was normalised by calculating the relative luminescence (apoptosis) per cell basis by using viability data as a measure of cell number, $p \leq 0.05 = ★$.

6.2.3 Measures of DNA Damage using the Comet Assay

In order to test whether mirtoselect was able to sensitise DNA to directly acting DNA damaging agents, radiation was employed as a damage probe and the standard comet assay, measuring single strand DNA damage, as the test procedure. RT112 cells were used in these experiments as these had exhibited the biggest mirtoselect anti-proliferative effect. Furthermore clonogenic studies with these cells revealed a mirtoselect enhancement effect on MMC-induced cell survival and there was a significant enhancement of MMC-induced DNA damage in the presence of 100µg/ml mirtoselect.

6.2.3.1 Repeat Treatment of Cells with Mirtoselect Followed by Irradiation in PBS ± Mirtoselect

In order to confirm the results seen in comet assay experiments involving mirtoselect pre-treatment and MMC, where mirtoselect treatment had not caused an increase in radiation-induced DNA damage, cells were treated with mirtoselect then irradiated in PBS, containing no mirtoselect, at varying doses of radiation (Figure 6.2-6). Although a small dose dependent increase in DNA damage was observed at 0Gy there was no significant mirtoselect dependent increase in radiation-induced DNA damage at any of the doses of radiation used or indeed at 0Gy.

As differences in radiation sensitivity, assessed by means of DNA damage response, were once again not observed between mirtoselect pre-treated cells, experiments were carried in order to mimic combined experiments where the DNA damage response of

cells was enhanced, in which mirtoselect was present in the media of cells during treatment with MMC (Section 4.2.4).

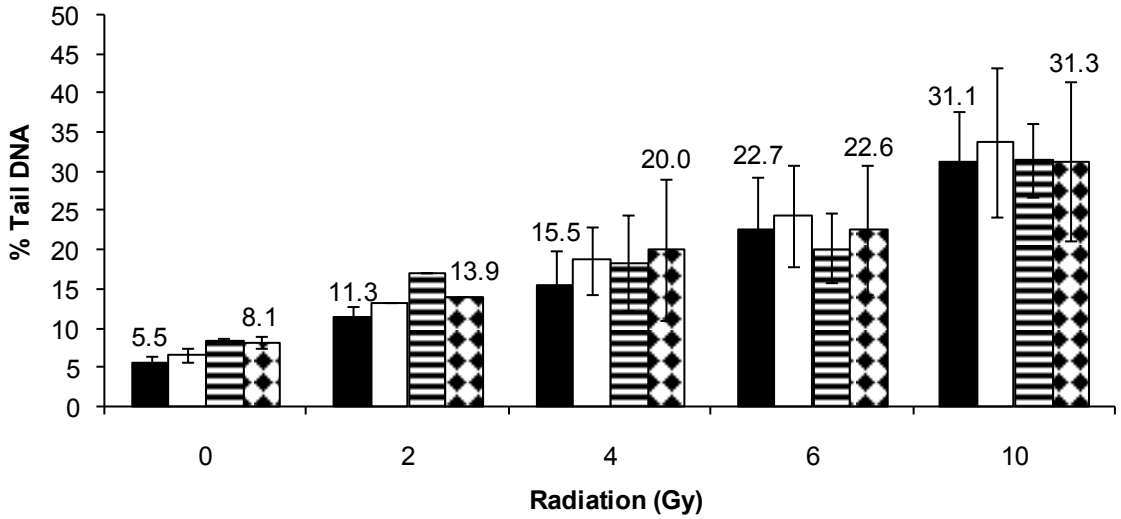


Figure 6.2-6 DNA Damage Levels in RT112 Cells Pre-Treated with Mirtoselect which was Removed Before Irradiation in PBS
Bladder cancer cell line RT112 was pre-treated with repeat doses of mirtoselect at 0 ■, 20 □, 50 ▤, and 100µg/ml ◪ then irradiated at varying doses of radiation in PBS. Cells were immediately processed for the comet assay and DNA damage levels were measured using the alkaline version of the assay. Results are the mean of three independent experiments (n=3) ±SD.

It was hypothesised that mirtoselect needed to be present at the same time as the DNA damaging agent in order for any enhancement to be seen. As processing of the samples for the comet assay involved washing in PBS it was felt that mirtoselect could have been washed out of the cells prior to irradiation, so experiments were carried out pre-treating cells with mirtoselect but also performing the irradiations in PBS containing mirtoselect (Figure 6.2-7). A radiation dose dependent increase in DNA damage was seen for each mirtoselect treatment, indicating mirtoselect did not inhibit the effects of radiation-induced DNA damage. Moreover, there was a mirtoselect dependent increase in DNA damage at each radiation dose. At the lowest and clinically relevant dose of 2Gy there was an increase (~9%) in %TD measured in 100µg/ml treated cells compared to non-mirtoselect treated cells. The largest mirtoselect-induced DNA damage

enhancement occurred at 4Gy, where a significantly higher amount (~15%) of DNA damage was measured in 100µg/ml treated cells compared to non-mirtoselect treated cells.

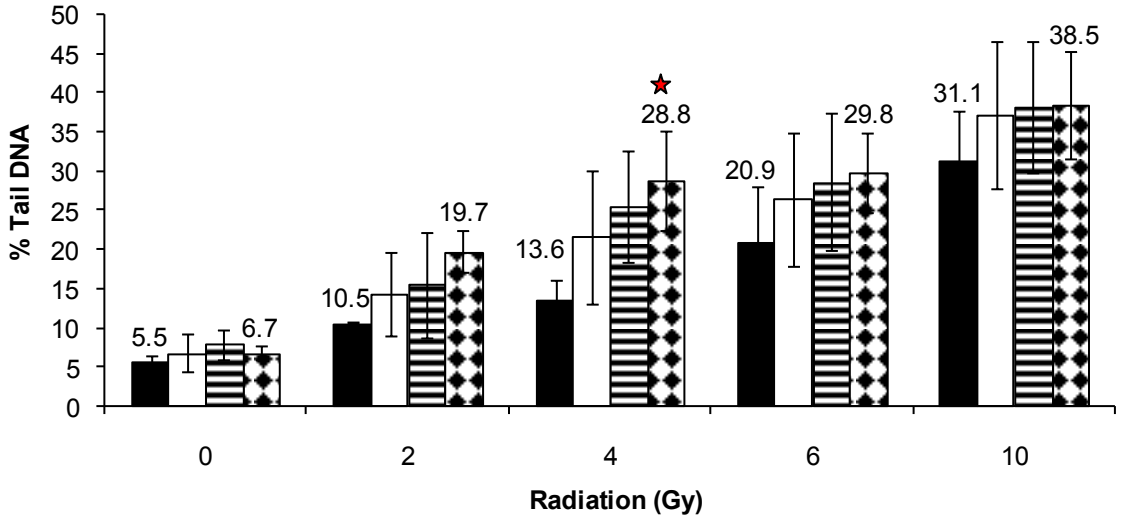


Figure 6.2-7 DNA Damage Levels in RT112 Cells Pre-Treated with Mirtoselect and Irradiated in the Presence of Mirtoselect
Bladder cancer cell line RT112 was pre-treated with repeat doses of mirtoselect at 0 ■, 20 □, 50 ▭, and 100µg/ml ◪ then irradiated at varying doses of radiation in PBS containing the corresponding concentration of mirtoselect. Cells were immediately processed for the comet assay and DNA damage levels were measured using the alkaline version of the assay. Results are the mean of three independent experiments (n=3) ±SD, $p \leq 0.05$ = ★.

6.2.3.2 Irradiation in PBS Containing Mirtoselect with No Pre-treatment of Cells

As a means of determining the contribution of the pre-treatment of cells with mirtoselect on induced radiation DNA damage, RT112 cells were not pre-treated with mirtoselect but irradiated in the PBS containing mirtoselect. As per the previous experiment where mirtoselect was present during irradiation, a radiation dependent increase in DNA damage was measured in each of the samples and there was also a mirtoselect dependent increase in DNA damage (Figure 6.2-8). Although in this experiment the largest enhancement effect of mirtoselect on radiation-induced DNA

damage was seen at 10Gy, with ~15% more DNA damage in cells irradiated in 100µg/ml PBS compared to control, the only significant enhancement was at 4Gy.

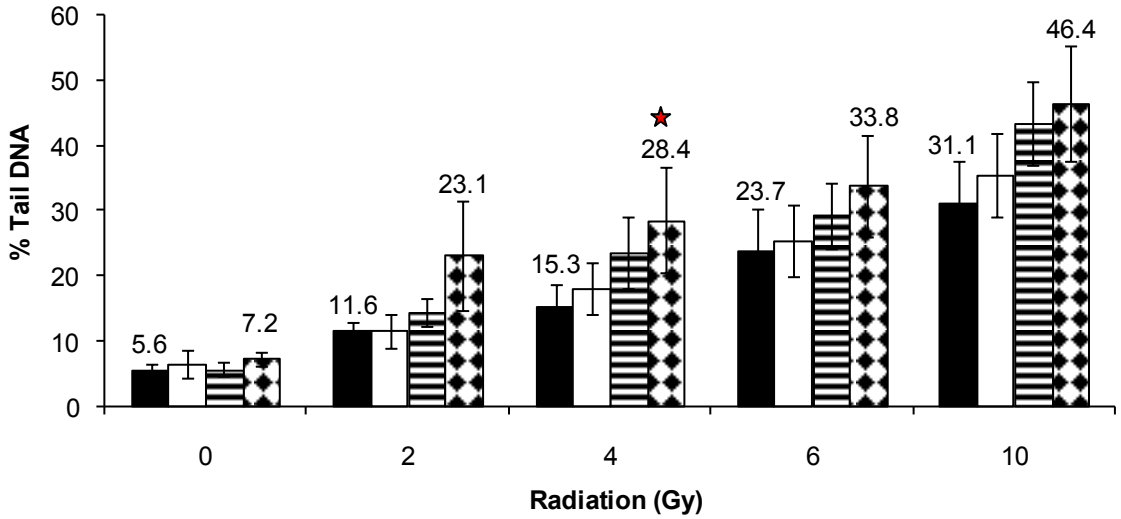


Figure 6.2-8 DNA Damage Levels in RT112 Cells Irradiated in the Presence of Mirtoselect with No Pre-Treatment
Bladder cancer cell line RT112 was irradiated at varying doses of radiation in PBS containing mirtoselect at 0 ■, 20 □, 50 ▬, and 100µg/ml ▫. Cells were immediately processed for the comet assay and DNA damage levels were measured using the alkaline version of the assay. Results are the mean of three independent experiments (n=3) ±SD, $p \leq 0.05$ =★.

6.3 Discussion

6.3.1 CFSE staining experiments

In previous analysis of cellular growth (Chapter Three), RT112 and RT4 both exhibited significant anti-proliferative responses to mirtoselect treatment, yet there was no effect on cell cycle distribution and although apoptosis was measured in mirtoselect treated cells, it was not sufficient to account for the biological response seen. It was therefore hypothesised that as well as cells undergoing apoptosis, treatment with mirtoselect might be causing cells to progresses through the cell cycle more slowly, generating an anti-proliferative effect. CFSE staining was therefore performed on the three cell lines, to determine cellular proliferation status in response to mirtoselect treatment by

measuring the number of cellular divisions the cells underwent within a given time frame in order to provide evidence to support this hypothesis.

In RT4 and HT1376 cell lines no substantial differences between control and mirtoselect treated cells were seen in CFSE staining experiments, however, there was a trend in both cases towards mirtoselect caused a slowing of proliferation at 72 hours. In the RT112 cell line, however, significant differences were observed between control and 50 and 100µg/ml treated cells.

Previously, RT112 were the cells most significantly affected by mirtoselect in measures of cellular growth, with a dose dependent decrease in cell number seen as early as 24 hours and a significant difference in cell number at 48 and 72 hours in 75 and 100µg/ml mirtoselect treated cells (Figures 3.2-1 and 3.2-2). Significant levels of apoptosis were only observed in cells at 48 hours in annexin V-FITC studies (Figure 5.2-2) and at 72 hours in TMRE studies (Figure 5.2-5), therefore it was hypothesised that differences in rates of cellular division could account for the visible dose dependent decrease in cell number observed. In mirtoselect treated samples there was a trend at 24 hours towards treatment causing cells to undergo more cellular divisions, compared to control, and in 100µg/ml mirtoselect treated cells there was a definite shift in the proportion of cells being later cellular generations, indicating more cellular divisions had occurred opposed to the hypothesised slowing of the cell cycle. This data does not therefore fit together with the response seen in growth studies.

In solid tumours *in vivo* one of the causes of treatment failure is the repopulation of the tumour by surviving cancer cells (Kim and Tannock, 2005). In cancer therapy repopulation of the tumour occurs during the gaps in fractionated radiotherapy or

chemotherapy which are present to allow for normal tissue recovery. Accelerated repopulation of the tumour occurs during these treatment gaps and current research suggests these proliferating cells to be cancer initiating cells with stem-like properties, i.e. cells with the capacity to regenerate the tumour and that are resistant to therapy, that are in G0 phase of the cell cycle during the period when treatment is administered (Sell and Glinsky, 2010). After an initial treatment insult the cancer stem-like cells may be activated to produce more cells so that regrowth of the cancer occurs more rapidly between treatment gaps. RT112 cells had the most significant anti-proliferative response to mirtoselect yet were found be to less sensitive in studies of clonogenic survival than RT4 cells. This resistance to mirtoselect in studies of long term survival could be attributable to the accelerated repopulation of the cancer cells seen within the first 24 hours of mirtoselect treatment.

CFSE staining was also analysed after 72 hours treatment with mirtoselect. At this time point 50 and 100µg/ml mirtoselect treated RT112 cells had undergone fewer cellular divisions than control. Low dose mirtoselect, 50µg/ml, caused a slowing of cellular division, as predicted. The fact that 50µg/ml mirtoselect can induce a biological effect on RT112 cells is note worthy as in other measures of biological response to mirtoselect alone; growth and apoptosis studies, 50µg/ml did not, in general, cause a significant effect apart from in clonogenic cell survival studies where a significant decrease in the surviving fraction of cells was observed. These data appear to suggest that low dose mirtoselect is indeed able to affect the division of RT112 cells.

6.3.2 ApoToxGlo experiments

Although apoptosis had been previously measured (Section 5.2.1) using alternative methods, these were based on the detection of morphological hallmarks of apoptosis rather than the initiating cascade of protein activation. The triplex assay provided a means of measuring caspase activity, cell viability and cytotoxicity within a single well, making all the results comparable. In general, assays involving the measurement of caspase activity are normalised to the control, however, cell number differences between control and treated samples can influence results masking increases in caspase activity. In this assay results can be normalised to the internal control that exists due to fact that viability data is collected from the same well, removing the influence of cell number on results.

The only cell line to exhibit an increase in cytotoxicity was the RT4 cell line and this was at 72 hours where a concurrent increase in caspase activity was also observed. This data indicates that mirtoselect does not primarily cause cell killing via necrosis. Increases in caspase activity were measured in two of the three cell lines, RT112 and RT4. These findings are in agreement with annexin V-FITC and TMRE results. Caspase activation is one of the earliest measureable events in apoptosis yet in mirtoselect alone treated cells caspase activity did not increase significantly above control until 72 hours and only in 100µg/ml treated cells. Since it is the activation of executioner caspases, which cleave cellular substrates to bring about the biochemical and morphological hallmarks measured in the annexin V-FITC and TMRE assays, it is possible that the time points chosen in these assays were too early to fully detect any mirtoselect-induced, caspase mediated, apoptosis.

6.3.3 Radiation/Mirtoselect comet assay experiments

In previous comet assay experiments mirtoselect had been shown to enhance the formation of crosslinks in cells treated with MMC. During the development of these experiments it was proved that mirtoselect treatment did not interfere with the radiation-induced DNA damage needed in order to measure crosslinks. Although for the purposes of the modified comet assay this was ideal, it highlighted an anomaly in the method by which mirtoselect was able to enhance the effects of DNA damaging agents. As the DNA damaging effects of radiation are well documented and can been measured using the comet assay, experiments using radiation, as a source of DNA damage induction, were carried to test whether mirtoselect had to be present during the induction of DNA damage in order to have any enhancement effect.

Experiments proved once again that pre-treatment with mirtoselect alone did not significantly alter radiation-induced DNA damage, yet in experiments in which mirtoselect was present during irradiation there was a significant increase in DNA damage. In comparing data from experiments where cells had been pre-treated with mirtoselect and irradiated in the presence of mirtoselect and those simply irradiated in the presence of mirtoselect with no pre-treatment, there was no significant difference between the levels of DNA damage measured in the comparable samples, indicating that pre-treatment has no effect on radiation-induced DNA damage but that presence of mirtoselect during irradiation does significantly enhance the DNA damaging effect of radiation.

Clinical trials have investigated the potential of chemopreventive agents to lessen side effects associated with radiation treatment. The majority of these trials only give the

agents during the period of treatment. Mixed results have been reported, with some trials showing no effect by the agents (Halperin *et al*. 1993), some showing a decrease in side effects with no adverse affect on tumour control or survival (Mills, 1988), whilst others have shown that overall survival can be reduced amongst patients taking the agents compared to the placebo (Ferreira *et al*. 2004). A few studies have also been carried out to determine if chemopreventive agents are suitable adjuvant or concomitant therapies. *Bairati et al.* (2005) carried out a clinical study to determine whether vitamin supplementation could reduce the risk of secondary head and neck cancers in patients undergoing radiation therapy (Bairati *et al*. 2005b). In the original study design patients were assigned to receive both α-tocopherol and β-carotene during radiation and for three years thereafter, however, β-carotene supplementation was halted due to results from another trial showing supplementation was associated with an increase in lung cancer incidence. In patients who received α-tocopherol alone follow-up over the first 3.5 years indicated that supplementation significantly increased the risk of second primary cancers compared to placebo, however, during the period after supplementation was withdrawn, patients who had received α-tocopherol had lower rates of second primary cancers than patients who had received the placebo. In the few patients who took both α-tocopherol and β-carotene supplementations the rate of second primary cancers was still higher than in the placebo arm, however, was lower than patients in the α-tocopherol arm. Nayak *et al.* (2010) investigated the effect of curcumin on prostate cells for potential radiosensitizing effects, demonstrating that combined treatment had a synergistic effect on clonogenic cell death at the therapeutic dose of 2Gy (Nayak *et al*. 2010).

In the treatment of muscle invasive bladder cancer, regimens most commonly use 2-3Gy fractions of radiation, ultimately exposing the cancer to a total of 60-66Gy over the course of 6.5 weeks or 55Gy over 4 weeks (Petrovich *et al*. 2001). This study has shown that *in vitro,* mirtoselect is able to enhance the levels of DNA damage induced, as measured with the comet assay, at doses of radiation that are clinically relevant. In these experiments exposure of cells to 2Gy of radiation in the presence of 100µg/ml mirtoselect-induced levels of DNA damage equivalent to 6Gy radiation. At present, radiation dose is limited because of normal tissue toxicities. Concurrent chemoradiotherapy has shown potential in bladder-preserving programmes (Sherwood *et al*. 2005), however, the use of chemotherapy as a concurrent therapy potentially increases drug-related normal tissue toxicity. If the enhancement seen in mirtoselect/radiation experiments was confirmed to occur exclusively in tumour cells, with normal tissue radiosensitivity unaffected, then patients would not only have to undergo fewer radiation fractions to achieve the same tumour cell kill, limiting normal tissue exposure, but intrinsic drug-related normal tissue toxicity may not be an issue as mirtoselect has been shown to be well tolerated in patients (Thomasset *et al*. 2009). Whilst a differential therapeutic gain between tumour tissue and a normal tissue equivalent (Southgate *et al*. 1994) must be established for mirtoselect to be considered a true radiosensitizing agent, presently we demonstrate, at a proof-of-principle level, that mirtoselect can radiosensitize bladder cancer cells.

Chapter Seven

General Discussion

7.1 Overview of the Results in this Thesis

Previous studies have shown that anthocyanins and their parents compounds (anthocyanidins), can inhibit the growth of various cancer cell lines (Cooke *et al*. 2005), however, these agents, to our knowledge, had not been investigated in bladder cancer cell lines. It was therefore the aim of this project to determine the effects of mirtoselect, a mixture of 15 different anthocyanins, on the three bladder cancer cell lines, most representative of non-muscle invasive bladder cancer, and determine if any of these in any way interfered with the known cytotoxic actions of MMC.

In Chapter Three, cell growth and survival were investigated. A varying response to mirtoselect was observed in the cell lines utilised proving that mirtoselect was indeed anti-proliferative in bladder cancer cell lines. The results obtained, using the two different techniques, however, did not entirely correlate. Both methods found the HT1376 cell line to be the least sensitive to mirtoselect, whereas RT112 was the most sensitive in growth experiments (Figures 3.2-1 and 3.2-2) and RT4 the most sensitive in clonogenic experiments (Figure 3.2-6). This discrepancy in the sensitivity ranking of the cell lines to mirtoselect was attributed to the fact that the clonogenic assay measures not only the immediate anti-proliferative effects of an agent but also the effects on long-term survival and can be considered a more accurate/valid assessment of cell sensitivity. Clonogenic results showed that mirtoselect itself was cytotoxic at concentrations ≥20μg/ml in both RT112 and RT4 cells with significant decreases in surviving cell numbers. This was result is rather unexpected since anthocyanins have been shown to be antioxidants and protect cells against DNA damage and yet here we find that an anthocyanin rich mixture is able to cause cytotoxicity. In combined mirtoselect and MMC studies, mirtoselect pre-treatment caused an enhancement of the cytotoxic effects

of MMC in all three cell lines (Figures 3.2-4 and 3.2-6). In growth experiments this was most evident at lower/intermediate concentrations of mirtoselect (2-50µg/ml) and suggested a possible synergistic relationship between the two agents (Figure 3.2-5). In clonogenic experiments this was only evident in RT112 cells in 30µg/ml pre-treated cells (Figure 3.2-7), however this was not a significant enhancement. Overall the conclusion from both measures of cell growth and survival was that mirtoselect pre-treatment of cells did not appear to hinder the cell-killing effect of MMC and indeed in some instances, appeared to actually enhance MMC cytotoxicity whilst itself being cytotoxic.

Measures of MMC-induced crosslink formation and repair, obtained using a modified version of the comet assay (Chapter Four) revealed that pre-treatment of cells with mirtoselect, caused a dose dependent increase in crosslinks in RT112 and RT4 cell lines (Figure 4.2-8). Significant differences in DNA damage levels were observed in RT112 cells (Figure 4.2-7) and in measures of crosslink numbers in RT4 cells (Figure 4.2-9), however these were not consistent. The comet assay is a fast, inexpensive and sensitive way of measuring DNA damage and repair in single cells, however, experimental variation is a problem and standard deviations can be rather large. Many of the steps involved in the assay affect reproducibility including slide preparation, cell lysis and electrophoresis conditions which is why the introduction of 'internal' standards have been investigated to minimise intra- and inter- experimental variation (Zainol *et al.* 2009). Although internal standards are still under development, their use here would help to tighten up the data and minimise experimental variation. Differences in measures of crosslinks were still evident after 72 hours in drug free media (Figure 4.2-9) and in terms of the extent of crosslink repair, mirtoselect pre-treatment did not alter

the repair capacity of the cell lines i.e. the induction of greater numbers of crosslinks did not cause repair to be altered (Table 4.2-1).

The induction of apoptosis by mirtoselect and MMC was assessed using two different flow cytometric techniques (annexin V-FITC assay and TMRE assay) in each of the bladder cell lines (Chapter Five). Mirtoselect treatment caused only a small induction of apoptosis in treated cells, however, a rank order of sensitivity to mirtoselect could be determined: RT4 > RT112 > HT1376, which was consistent with clonogenic studies. In TMRE studies, HT1376 cells exhibited a trend towards mirtoselect protecting cells against the loss of mitochondrial membrane potential, suggesting that mirtoselect could be acting in this cell line to protect against mitochondrial mediated apoptosis (Figure 5.2-5). In combined experiments MMC-induced apoptosis was measurable in all three cell lines using both techniques (Figures 5.2-3 and 5.2-6). In experiments using the annexin V-FITC assay mirtoselect pre-treatment of cells prior to MMC caused a trend towards an increase in apoptosis (Figure 5.2-3) and in some instances there was a more than additive response (Tables 5.2-1 and 5.2-2). In TMRE experiments the potential protective effect of mirtoselect against mitochondrial mediated apoptosis in HT1376 cell line was not observed in combined mirtoselect and MMC studies, with similar levels of apoptosis observed between treatment combinations (Figure 5.2-6).

MMC-induced cell cycle distribution changes were detected in each of the three bladder cancer cell lines (Chapter Five). In each of the cell lines a G_2/M block was observed 24 hours after MMC administration (Figure 5.2-9). Mirtoselect had no sustained cell cycle effects on its own (Figure 5.2-8) and in combined studies there was no substantial or consistent mirtoselect effect on MMC-induced cell cycle blocks or the subsequent recovery of the cells from those blocks (Figure 5.2-9).

In Chapter Six, CFSE staining experiments revealed that mirtoselect was able to affect the rate of cell growth in the bladder cancer cell lines. This was not as predicted, i.e. the cell cycle slowing, but instead there was an increase in the number of cellular divisions undergone in RT112 cells after 24 hours treatment in mirtoselect, however, at 72 hours, cells had slowed in comparison to the control (Figure 6.2-1). RT4 and HT1376 cell lines both showed no effect at 24 hours but with a trend towards a slowing of cell growth at 72 hours (Figures 6.2-2 and 6.2-3). Although this method has been successfully used in the analysis of lymphocytes its use in cancer cell lines is still relatively new and may not be appropriate. Cancer cells have a heterogeneous amount of proteins and therefore distinct peaks for each cellular generation are not often seen. This method is therefore somewhat limited in cancer cell lines and relies on the accuracy of computer software to mathematically model the appropriate cellular generations and estimate the percentage of cells were peak. This data would therefore benefit from being verified using other methods for the measurement of cellular proliferation such as BrdU labelling. Apoptosis was again assessed in this chapter as part of a triplex assay to measure cell viability, cytotoxicity and caspase activity. A decrease in cell viability was measured in all three cell lines in response to mirtoselect treatment (Figure 6.2-4). In analysis of cytotoxicity, RT4 was the only cell line to exhibit any increase above control, with a 3-fold increase in measures at 72 hours in 100µg/ml treated cells (Figure 6.2-5). Apoptosis was assessed via measurement of caspase activity. In RT112 and RT4 cell lines there was a significant increase in caspase activity (1.7- and 2.7 fold increase respectively) at 72 hours in 100µg/ml treated cells compared to control (Figure 6.2-6). Finally in Chapter six the enhanced DNA damaging activity of MMC in mirtoselect pre-treated cells was investigated, using radiation as a probe for the induction of DNA damage and the standard comet assay as a

means of measuring that damage. Mirtoselect was shown to only increase DNA damage in cells that were irradiated in the presence of mirtoselect (Figures 6.2-7 and 6.2-8) irrespective of pre-treatment (Figure 6.2-9). A concentration dependent enhancement by mirtoselect was seen at each radiation dose, with a significant enhancement in cells co-exposed to 100µg/ml mirtoselect and 4Gy radiation. Although a statistically significant increase in DNA damage was observed in 100µg/ml / 4Gy samples in experiments irradiated in the presence of mirtoselect once again this was only observed in samples where the standard deviation of the control was small and calls for a way of reducing the degree of variation between experiments. At 2Gy, the clinically relevant dose for muscle invasive bladder cancer, there was a 2-fold increase in DNA damage in cells co-exposed to 100µg/ml mirtoselect and 2Gy radiation compared to radiation alone, with DNA damage levels (%TD) being equivalent to that seen for cells exposed to 6Gy radiation alone.

7.1.1 Determining the Effects of Mirtoselect in Bladder Cancer Cell Lines

Although this study was not focused on determining the status of mirtoselect as a chemopreventive agent, in order to assess its combined effects with MMC it was necessary to firstly determine the response of the cell lines under investigation to mirtoselect alone. Mirtoselect has previously been shown to decrease tumour number and burden in APC^{Min} mice, a model of human familial adenomatous polyposis, and decrease urinary levels of the pyrimidopurinone adduct (M_1dG) of deoxyguanosine (Cooke *et al*. 2006). We have shown that cell growth and cell survival is significantly decreased in bladder cancer cells exposed to mirtoselect. For many anthocyanins their anti-proliferative effects have been attributed to concomitant cell cycle changes (Shih *et al*. 2005, Malik *et al*. 2003, Chen *et al*. 2005); in two of the cell lines investigated there

was a trend at 24 hours towards mirtoselect increasing the percentage of cells in S phase, however, at the concentrations used here, these alterations were not sustained. It appears, therefore, that at the concentrations of mirtoselect used in this study, the anti-proliferative effects are not due to alterations in the cell cycle via regulation of cell cycle proteins. Correlations between certain cell cycle proteins and an increased risk of bladder cancer recurrence and progression have been identified as potential predictors of tumour development (Cote and Datar, 2003). The three main proteins identified; p53, p21 and pRb, are all associated with the recognition of DNA damage and induce arrest at the G_1/S boundary. In patients with loss of function (i.e. tumour suppressor genes) or ubiquitously active (i.e oncogenes) copies of these proteins, the G_1/S checkpoint is overridden and cells progress into S phase. Of the three cell lines studied RT4 was the only cell line to have wild type (WT) copies of p53, p21 and pRb (Bilim *et al*. 2000, Welcome trust sanger institute, 2010). RT112 and HT1376 both have p53 mutations (Bilim *et al*. 2000, Warenius *et al*. 2000) and HT1376 also has a mutation in pRb (Welcome trust sanger institute,2010). Interestingly it was RT4 cells which were the most affected by mirtoselect in cell cycle studies (Chapter Five) potentially indicating that mirtoselect acts on low stage bladder cancers, with intact p53, p21 and pRb, as an inhibitor of cancer promotion rather than on high stage cancers to inhibit progression. RT4 does, however, have a mutation in CDKN2A which codes for $p16^{INK4A}$ a member of the INK4 family which inhibits cdk4 and cdk6 and $p14^{ARF}$. Both p16 and p14 are involved in cell cycle control at the G1 checkpoint stabilising p53 so promoting apoptosis if DNA damage is detected. CDKN2A is an important tumor suppressor gene and the mutations documented in RT4 cells could therefore explain the lack of cell cycle arrest observed in mirtoselect treated cells and lack of measurable apoptosis. Previous studies have shown that anthocyans and indeed mirtoselect are able

to inhibit the EGFR and its downstream signalling pathways (Meires *et al.* 2001, Fridrich *et al.* 2008, Teller *et al.* 2009). Nutt *et al.* (2004) used a FACS based method to quantify the number of EGF receptors in two of the three bladder cancer cell lines investigated in this thesis. They showed that RT4 cells had ~twice the number of receptors (1.9×10^4) compared to RT112 cells (9×10^3) (Nutt *et al.* 2004), however no data was available for HT1376 cells. Despite having more receptors RT4 cells were found to be more sensitive to EGFR inhibition however a clear relationship between receptor number and sensitivity to inhibitors of EGFR is not clear (Nutt *et al.* 2004, Dominguez-Escrig *et al*. 2004) suggesting that there is no direct correlation between receptor numbers and growth inhibition.

From the analysis performed assessing apoptosis (annexin V-FITC, TMRE and caspase activity) it appears mirtoselect exhibits some anti-proliferative effects through the induction of apoptosis. Mirtoselect sensitive cell lines, RT112 and RT4, had a significant increase in apoptosis compared to control, whereas the HT1376 cell line, that showed little response to mirtoselect in growth and cell survival assays, had little or no induction of cell death using any of the markers of apoptosis. Apoptosis provides cells with a mechanism for dealing with DNA damage in instances where repair is insufficient. These data once again suggest that mirtoselect is able to target the low stage bladder cancer cell lines inducing apoptosis as a means inhibiting the promotion of carcinogenesis. Measures of apoptosis were concluded to be too low, however, to account for the full anti-proliferative effects seen in these cell lines. Furthermore, as the cell cycle changes observed were also only minor, experiments were conducted (CFSE) to assess the growth rate of cells, as it was thought that a slowing of cellular growth coupled with an induction of apoptosis, might account for the anti-proliferative effects

observed. In RT4 and HT1376 cell lines this hypothesis appeared to hold with analysis at 72 hours showing a trend towards a slowing of cell division (Chapter Six). This effect alone could be enough to account for the small decrease in cell numbers seen in HT1376 cells. RT4 cells exhibited the largest apoptotic response, which was confirmed by analysis of caspase activity, and were also found to exhibit some cytotoxic response to the highest dose of mirtoselect. These findings coupled with the small decrease in rate of cell division observed could also account for the response seen in this cell line. Investigations with EGCG and curcumin have observed similar findings, with retinal pigment epithelial cells showing anti-proliferative effects in response to the agents but with varying degrees of inhibition of cell division and apoptosis/necrosis (Alex *et al*. 2010). In RT112 cells a dose dependent increase in cell division was initially observed, which was unexpected considering that total cell numbers measured in growth studies showed a clear anti-proliferative effect. CFSE staining suggested that RT112 cells were being stimulated by mirtoselect to proliferate faster. This was hypothesised (Chapter Six) to be a result of accelerated repopulation, an occurrence associated with treatment failure in clinic (Kim and Tannock, 2005). Although this may appear to suggest a negative response to mirtoselect, this may actually be another differential response which could be attributed to p53 status. Treatment of colon cancer cell lines with cisplatin produces a differential response in p53-deficient compared to p53-proficient cells (Ubezio *et al*. 2007). In the p53-proficient cells a large cell cycle block was observed following treatment with highest dose of 100μM cisplatin, with CFSE staining revealing release from the block was slow with the majority of cells being only 1st or 2nd generation 72 hours post treatment. In the p53-deficient cell line, however, the block was more quickly overcome and cells passed into the third, fourth and fifth generations. Based on outcomes seen in the clinic, this would predict the p53-deficient

cells to be the more resistant to cisplatin, however, in other studies, p53-deficient cell lines have shown a greater sensitivity to cisplatin (Almeida *et al*. 2008, Cote *et al*. 1997). Regarding mirtoselect, the ability to cause an initial increase in cell division in bladder cancer cell lines deserves greater investigation to truly understand how it may be acting in this cell line to cause its anti-proliferative effects. One possibility still could be related to cell death. In the aforementioned cisplatin study, Ubezio *et al*. (2007) also investigated cell lethality, in the p53-deficient and -proficient colon cancer cell lines. Treatment with 100μM cisplatin-induced a large amount of cell death in p53-proficient cells, measured using the TUNNEL assay, yet in p53-deficient cells there was a lower percentage of positive cells. Once again this would suggest a more resistant phenotype, however, the investigators noted a large number of disrupted cells (negative staining cells that had a DNA content lower than G_1 cells), indicating cell loss (Ubezio *et al*. 2007). Sub-G_1 analysis was not performed as part of this study, yet may hold some clues as to the actions of mirtoselect.

7.1.2 Influence of Mirtoselect on Markers of MMC Cytotoxicity

In light of the significant anti-proliferative effects seen with mirtoselect, it was possible that this might affect the cytotoxicity induced by MMC as this activity requires that cells are proliferating and entering S phase for the crosslinks to trigger DNA damage checkpoints and for apoptosis to be initiated. In measures of cell growth, cell survival, apoptosis, cell cycle distribution and DNA damage this did not hold true and mirtoselect pre-treatment seemed to act in harmony with MMC. In cell survival experiments RT112 cells were the least sensitive to MMC, with 23% survival in 2μM MMC treated cells (Chapter Three). This cell line also exhibited the most proficient repair of crosslinks (80% at 72 hours), as measured using the modified comet assay (Chapter

four). In combination studies, mirtoselect enhanced the cytotoxic effects of MMC resulting in a substantial increase in crosslink numbers. The biological significance of this was evident as RT112 exhibited the largest mirtoselect enhancement of MMC cell killing in the clonogenic assay and also a more than additive response in measures of apoptosis.

In measures of crosslinks using a modified version of the comet assay (Chapter four) mirtoselect pre-treatment enhanced the formation of crosslinks in a dose dependent manner. This was most noticeable at the highest MMC concentration, which is promising for clinical applications, which uses concentrations of 2mM when administered intravesically (Malmstrom, 2003). As previously mentioned mirtoselect has been shown to suppress the tyrosine activity of EGFR which could account for its growth inhibitory effects (Teller *et al*. 2004). In studies using gefitinib, a EGFR-specific tyrosine kinase inhibitor the authors found a synergistic effect on the inhibition of cell proliferation in combination with cisplatin in MCF-7 cells (Friedmann *et al.* 2004). Friedmann *et al.* (2004) also measured cisplatin-induced crosslink formation and repair in gefininb treated cells and although the authors did not measure any increases in the quantity of initial crosslinks measured, they did notice an inhibition of crosslink repair in combined treated cells with no evidence of apoptosis. Although this data does not correlate with result obtained here with MMC (increase in crosslinks with no effect on the extent of repair) it does highlight the involvement of the EGFR receptor in the enhancement of crosslink cytotoxicity. It also important to mention that MMC is a interstrand crosslinks whereas cisplatin forms both inter-and intrastrand crosslinks and therefore the two agents could have a different mechanism of crosslink formation.

The ability of mirtoselect to enhance levels of induced DNA damage was confirmed using radiation as an alternative DNA damaging agent. The results suggest that mirtoselect may potentially enhance the efficacy of chemotherapeutic drugs or radiotherapy, without itself being DNA damaging, as assessed via measures of ssDNA breaks using the comet assay. In a parallel study by a PhD student in this group, mirtoselect was found to protect against DNA damage, both ssDNA breaks and base lesions, induced by hydrogen peroxide but was unable to protect against the effects of radiation DNA damage (Zainol *et al*. unpublished data). This suggests that mirtoselect is able to differentially protect against indirectly acting agents whilst not affecting directly acting agents, such as chemotherapy drugs and radiation. Previous reviews on the limited data of combination therapies have attributed the ability of chemopreventive agents to potentiate cancer therapies to molecular mechanisms mainly involving genes involved in multidrug resistance (Sarkar and Li, 2006). Here we have shown that mirtoselect is able to enhance the radiosensitivity of RT112 cells simply by being present during irradiation. In clonogenic assays involving radiation and gefitinib combination was found to significantly enhance the cytotoxic effect of 4Gy irradiation whereas the number of colonies formed at 2Gy was not significantly different from radiation alone (Dominguez-Escrig *et al*. 2004). Data in this thesis suggests a potential mechanism for mirtoselect to alter the chromosomal configuration of DNA into a more damage sensitive state for directly acting agents such as MMC and radiation. The Ribosomal S6 kinase (RSK) family are also activated by the MAPK pathway and have been shown to be involved in chromatin remodelling via phophoryaltion of histone H3 (Baker and Reddy, 2004). If mirtoselect is therefore able to inhibit EGFR it could potentially be altering the activation of the MAPK pathway and altering chromatin remodelling. An example of agents that can modify chromatin structure and so

potentially alter DNA damage sensitivity are the histone deacetylase (HDAC) inhibitors (Karagiannis and El-Osta, 2006). Inhibitors such as trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), M344 (an analogue of hydroxamic acid) and depsipeptide (FR90228) have all been shown to enhance radiation sensitivity in human squamous carcinoma lines (SQ-20B and SCC-35) (Zhang *et al*. 2004). TSA and SAHA have also been shown to enhance the cytotoxic effects of DNA targeting chemotherapeutic drugs (e.g. doxorubicin and cisplatin) in various cell lines (e.g. human glioblastoma and breast cancer cell lines) (Kim *et al*. 2003). In an *in vivo* study it was shown that sulforaphane (SFN), a constituent of cruciferous vegetables, was able to inhibit HDAC activity in mice fed SFN compared to controls (Myzak *et al*. 2007). Myzak *et al.* (2007) also showed that the consumption of broccoli sprouts (68 g BroccoSprouts broccoli sprouts [approximiately 105 mg SFN; equivalent to approximately 570 g of mature broccoli]), by healthy volunteers, was also able to inhibit HDAC activity in peripheral mononuclear cells collected from the volunteers between 3-6 hours after consumption. In another clinical trial healthy volunteers, including smokers and non-smokers, were asked to follow a controlled diet for 10 days and a broccoli diet for 10 days, containing a daily portion of 200g of broccoli, blood samples were collected at the beginning and end of each dietary period (Riso *et al*. 2009). The investigators found that consumption of the broccoli diet significantly decreased lymphocyte DNA damage in both smokers and non-smokers as measured using the comet assay, both groups also had a decrease in FPG-sensitive sites, although this was only significant in smokers; however, supplementation did not significantly affect HDAC activity in either group (Riso *et al*. 2009). Riso *et al*. (2009) attributed the differences observed in the ability of broccoli to inhibit HDAC, to the variation in relative broccoli consumed by the volunteers (500g compared to 200g) and length of

intervention (acute ingestion verses regular consumption), potentially highlighting different mechanisms between low and high concentrations.

The most noticeable effects of the mirtoselect/MMC combination were seen at low concentrations of mirtoselect. In growth studies, low concentrations of mirtoselect, which individually caused no significant anti-proliferative effect, in combination with MMC were able to bring about a dose dependent decrease in cell number. It was also observed that cells treated with low concentrations of mirtoselect prior to MMC, had higher measures of crosslinks than MMC controls suggesting low concentrations of mirtoselect could indeed have a biological effect. Although CFSE staining experiments were not performed for combined mirtoselect and MMC incubations, data from mirtoselect experiments showed cells did respond to low concentrations. Evidence therefore indicates that low concentrations of mirtoselect can influence cellular division and bring about biological effects which could be beneficial in the treatment of bladder cancer. Until the exact mechanism by which high concentrations of mirtoselect cause their anti-proliferative effects can be determined, low concentrations of mirtoselect should be considered for *in vivo* and/or clinical investigation.

7.2 Bioavailability of Mirtoselect

Bioavailability can defined in many ways but for dietary derived agents, which are normally administered orally, bioavailability generally refers to the quantity of the ingested dose that is absorbed (Heaney, 2001). The bioavailability of anthocyanins is often measured by plasma concentrations or urinary excretion (McGhie and Walton, 2007). Obviously, for the purposes of this study, urinary excretion is of most interest. Dietary consumption of anthocyanins was originally believed to be of the magnitude of

180-255mg/day (Kuhnau, 1976), however, more recent studies now suggest that anthocyanin consumption is much lower and report values of 12.5mg/day in the United States (Wu *et al*. 2006). A linear relationship has been observed between oral doses of fresh fruit and anthocyanins in the urine of volunteers consuming dietary relevant amounts of strawberries (Hollands *et al*. 2008). The authors found a mean urinary excretion of strawberry anthocyanins ranging from 0.70-2.35% of the original anthocyanin dose. Based on these findings a dietary intake of 12.5g/day could achieve a urinary concentration of 87-294µg anthocyanins. A few studies have investigated the bioavailability of anthocyanins from bilberries *in vivo* and also in clinical trials. In a study using male C57BL/6 mice, a single oral dose of bilberry extract (500mg/kg body weight) (anthocyanin concentration 67.3µmol/100mg extract) was given and urine collected over a 24 hour time period post administration (Sakakibara *et al*. 2009). The authors found that the average total anthocyanin excreted, 1.88% of total anthocyanin ingested (range of 0.62-3.62%), were of the same magnitude as that seen by Hollands *et al*. (2008). In another study this time using APC^{Min} mice, animals were fed a diet containing 0.3% mirtoselect (~9mg per mouse or 450mg/kg each day) for 12 weeks (Cooke *et al*. 2006). Urinary concentrations of anthocyanins reached 12.3µg/ml. As mirtoselect is made up of 36% anthocyanins a urinary concentration of 12.3µg/ml anthocyanins is equivalent to a urinary mirtoselect concentration of 35µg/ml. The authors extrapolated that in order to achieve an equivalent dose in humans to that of 0.3% supplementation in mice, a dose of ~2.6g (of mirtoselect)/80 kg human would be required (Cooke *et al*. 2006). It is therefore feasible that the low concentrations of mirtoselect used in this study could be achieved with dietary supplementation; however, based on the assumption of a direct relationship between dose and urinary excretion, a dose of 7.4g would be needed to achieve a urinary concentration of 100µg/ml

mirtoselect, which is unlikely to be achievable clinically, due to the large number of capsules that would be needed to provide this dose.

Mirtoselect has also been investigated clinically. In a small pilot study pharmacodynamic and pharmacokinetic information were gathered for mirtoselect to aid in the design of future studies investigating mirtoselect as a potential chemopreventive agent for colorectal cancer (Thomasset *et al*. 2009). Patients were given an oral formulation of mirtoselect, at daily doses of 1.4, 2.8 or 5.6g, for 7 days with the final dose being administered the morning before patients went for surgery. Urine was collected 1 hour and ~11.5 hours after the penultimate mirtoselect dose. Similar to Hollands *et al*. (2008) a relationship was seen between total anthocyanin levels and mirtoselect dose (Thomasset *et al*. 2009). In patients receiving 2.8g of mirtoselect, a dose equivalent to the extrapolated human dose calculated by Cooke *et al*. (2006), ~240µg anthocyanin/mmol creatinine was measured. In another pilot study with mirtoselect two patients with colorectal liver metastases who were about to undergo surgical resection received a single dose of 1.88g mirtoselect via a naso-gastric or –jejunal tube (Cai *et al*. 2010). Total concentrations of anthocyanins in the urine peaked in urinary fraction collected 2-6 hours post dose and were 760µg/ml for nasogastric administration and 32µg/ml for nasojejunal administration. The investigators suggested, therefore, that absorption of anthocyanins is much more efficient in the stomach of humans compared to the jejunum (Hong *et al*. 1990). The absorption and excretion of different anthocyanins has been shown to vary (McGhie *et al*. 2003) with the type of sugar moiety attached to the anthocyanin playing an important role in absorption (He *et al*. 2006), yet intact anthocyanins have been detected in the urine of mice (Cooke *et al*. 2006) and humans (Thomasset *et al*. 2009). It is possible

that mirtoselect could be administered intravesically during bladder cancer surgery (TURBT) therefore negating any effects absorption may cause and so achieving the higher concentrations of mirtoselect studied in this project.

7.3 Can Mirtoselect Impart a Therapeutic Advantage

In this study it has been demonstrated that mirtoselect can effect growth and induce apoptosis and in combination can enhance the cytotoxic actions of MMC. Although this is encouraging, since some combinations of antioxidants and chemotherapeutic agents have shown adverse effects (Lamson and Brignall, 1999), the clinical use of such a combination would only be adventitious if normal cells were not also sensitised to MMC by mirtoselect. A few studies have investigated the ability of anthocyanins to cause differential effects in normal and cancerous cell lines derived from the same tissue. Malik *et al*. (2003) demonstrated that an ARE from chokeberries selectively inhibited the growth of colon cancer cell line HT29 (65% inhibition) compared to the normal colon epithelial cell line NCM460 (<10% inhibition) at concentrations of 50µg anthocyanin/ml after 24 hour exposure (Malik *et al*. 2003). In a similar study Zhao *et al*. (2004) showed that AREs from bilberry and chokeberry at concentrations ranging from 10-75µg anthocyanin/ml were able to inhibit HT29 cells to a greater extent than NCM460 cells, with a significant inhibition in NCM460 cells only occurring at higher concentrations and longer time points (Zhao *et al*. 2004). This in part could be due to differences in the antioxidant effects of anthocyanins, as demonstrated by Feng *et al.* (2007) who showed that cyanidin-3-rutinoside was able to reduce intracellular levels of hydrogen peroxide in normal peripheral blood mononuclear cells *in vitro* yet caused the accumulation of ROS in leukaemic cells, which lead to an apoptotic response (Feng *et al*. 2007).

The use of cell lines derived from normal urothelium, such as HCV29, HU609, HS0767, as models of normal cells should be used with caution, however, as their ability to grow indefinitely in culture is regarded as an abnormal feature of normal human urothelium (Masters *et al*. 1986). Southgate *et al*. (1994) have described the successful isolation and use of normal human urothelial (NHU) for *in vitro* study. NHU can be isolated from surgical specimens and grown in low calcium and serum-free medium (Southgate *et al*. 1994); the cell cultures can only be propagated a finite number of times, going through about 20 population doublings before undergoing spontaneous replicative senescence (Crallan *et al*. 2006). Authors investigating these cultures have described them as having a basal/intermediate urothelial cell phenotype with the potential for both stratification (Southgate *et al*. 1994) and differentiation (Varley *et al*. 2004b, Varley *et al*. 2004a, Cross *et al*. 2005). In conclusion, these cultures would make ideal candidates for the investigation of mirtoselect as a chemopreventive agent and to further elucidate whether the effects seen in this study are specific to bladder cancer cell lines, therefore imparting a therapeutic advantage in non-muscle invasive bladder cancer treatment.

7.4 Future Work

- As mirtoselect has shown to have a significant cytotoxic effect in cancer cell lines it is of utmost importance that this effect is not seen in normal cells as well if mirtoselect it going to be used as a chemopreventative agent. It would therefore be interesting to investigate the effects mirtoselect has in normal immortalised cell lines as well bladder cancer cells isolated from normal epithelium.

- Perform western blotting for the expression of RTKs in the three bladder cancer cell lines to determine why HT1376 cells were not as responsive to mirtoselect compared to the other two cell lines and investigate any gene expression changes in relation to mirtoselect treatment in the responsive (RT112 and RT4) cell lines compared to the unresponsive cell line (HT1376) to see if any biomarkers could be identified.
- Utilise another method for the detection of MMC-induced crosslinks (^{32}P-postlabelling assay) and compare it with the results obtained by the comet assay to determine if mirtoselect is increasing the numbers of crosslinks formed.
- Undertake a clinical trial to determine the pharmacokinetics and absorption of mirtoselect, measuring its appearance in the urine and also uterine tissue levels by collecting tissue samples from patients undergoing radical cystectomy for muscle invasive disease.
- Investigate further the effects of mirtoselect on radiation induced DNA damage by carrying out clonogenic studies to see if an enhancement in radiation cytotoxicity is achieved as a result of the increased DNA damage observed.

Appendix A

Assessment of Cellular Growth using the Trypan Blue Exclusion Assay in Mirtoselect Treated RT112 Bladder Cancer Cells

In order to confirm that the anti-proliferative effect seen in the treatment of cells with mirtoselect, using the CyQUANT NF cell proliferation assay, was a true representation of the action of mirtoselect and not as a result of mirtoselect interfering with the assay conditions, comparative experiments were carried out using the trypan blue exclusion assay (Section 2.3) as a reference technique for cell counting. RT112 cells were treated with varying concentrations of mirtoselect for up to 72 hours; for each concentration of mirtoselect and time point analysed two replicate wells were treated and counted. Data is the average of two experiments, one experiment having been carried out by a fellow PhD student (Zainol, M.); cell number is expressed as a percentage of relative cell number compared to the untreated control at 0 hours.

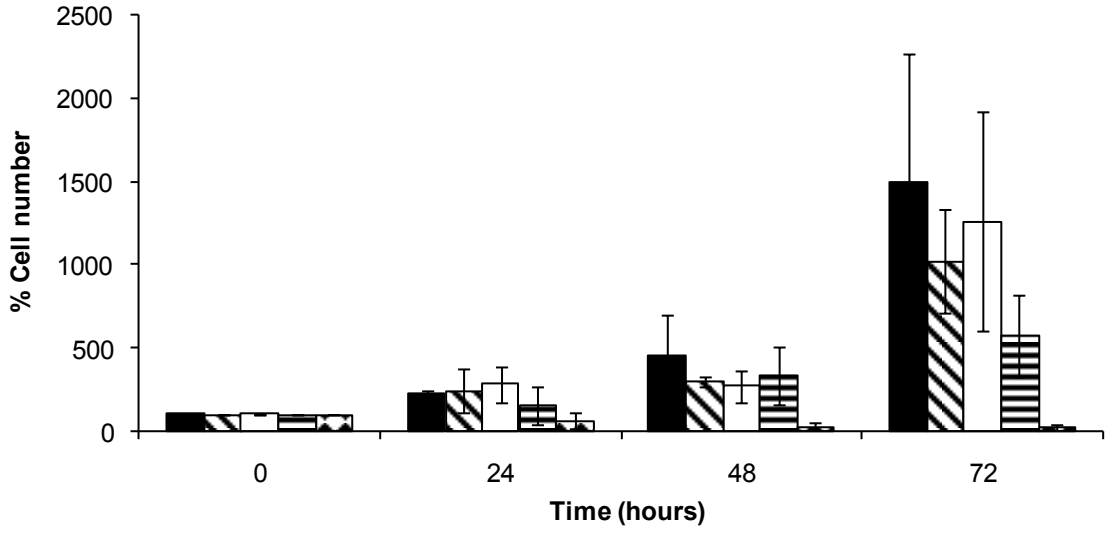


Figure A.1-1 Cell Counts in RT112 Cells Treated with Mirtoselect
RT112 cells were treated over 72 hours with fresh mirtoselect being applied every 24 hours at 0 ■, 2 ▨ 20 □, 50 ▬ and 100µg/ml ◧. Results are the mean of 2 independent experiments (n=4) ±SD. Results are expressed as percentage cell number relative to the number of cells at 0 hours.

Similar to the results obtained using the CyQUANT NF cell proliferation assay, mirtoselect caused a concentration dependent decrease in cell number. In both methods 100µg/ml treated cells caused a time dependent decrease in cell number as early as 24

hours, using the trypan blue exclusion assay cell numbers were only 56% of the starting number of cells, a figure comparable to that obtained in growth experiments in Chapter Three. It was therefore concluded that the CyQUANT NF cell proliferation assay (Chapter Three) was an accurate, higher throughput method for assessing cell number and that mirtoselect itself did not interfere with the workings of the assay so that any decrease measured were true measures of its anti-proliferative properties.

Appendix B

Assessment of Cell Cycle Distribution Changes in Bladder Cancer Cell Lines Treated with 10μM MMC

During investigations into cell cycle distribution changes in MMC treated cells, two concetrations of MMC were evaluated for their ability to cause cell cycle arrest in the three bladder cancer cell lines. The bladder cancer cell lines were treated with varying concentrations of mirtoselect for 24 hours followed by either 1µM or 10µM MMC (Section 2.9). A full set of results for both 1µM MMC treated cell lines with and without mirtoselect are included in Chapter Five (Section 5.2.2). After initial evalutation the 10µM concentration of MMC was not used any futher as assessment using ModFit LT was not possible due to the scale and duration of the cell cycle block induced. Figures A.2-1 to A.2-6 show the profiles obtained using CellQuest software for untreated cells, cells treated with 1µM MMC alone, 10µM MMC alone, and cells pre-treated with 20-100µg/ml mirtoselect followed by 10µM MMC.

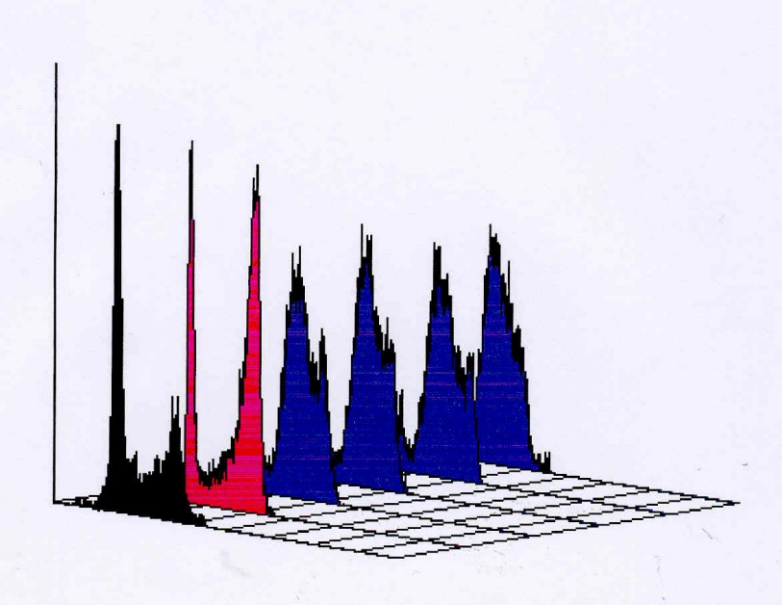


Figure A.2-1 RT112 Cell Cycle Profiles at 48 Hours
Flow cytometry analysis of the MMC-induced variation in cell cycle distribution. Representative profiles, as determined by CellQuest software, of RT112 cells treated with/without mirtoselect for 24 hours followed by 1 or 10µM MMC for 1 hour and 23 hours in drug free media. (Black profile) Control, untreated cells, (Red profile) 1µM MMC alone, (Blue profiles) cell pre-treated with 0-100µg/ml mirtoselect for 24 hours followed by 10µM.

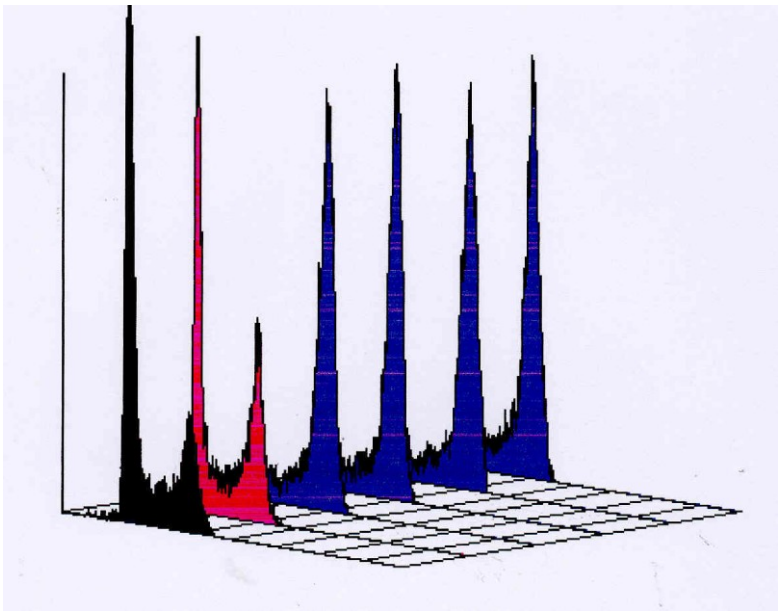


Figure A.2-2 RT112 Cell Cycle Profiles at 72 Hours
Flow cytometry analysis of the MMC-induced variation in cell cycle distribution. Representative profiles, as determined by CellQuest software, of RT112 cells treated with/without mirtoselect for 24 hours followed by 1 or 10µM MMC for 1 hour and 47 hours in drug free media. (Black profile) Control, untreated cells, (Red profile) 1µM MMC alone, (Blue profiles) cell pre-treated with 0-100µg/ml mirtoselect for 24 hours followed by 10µM.

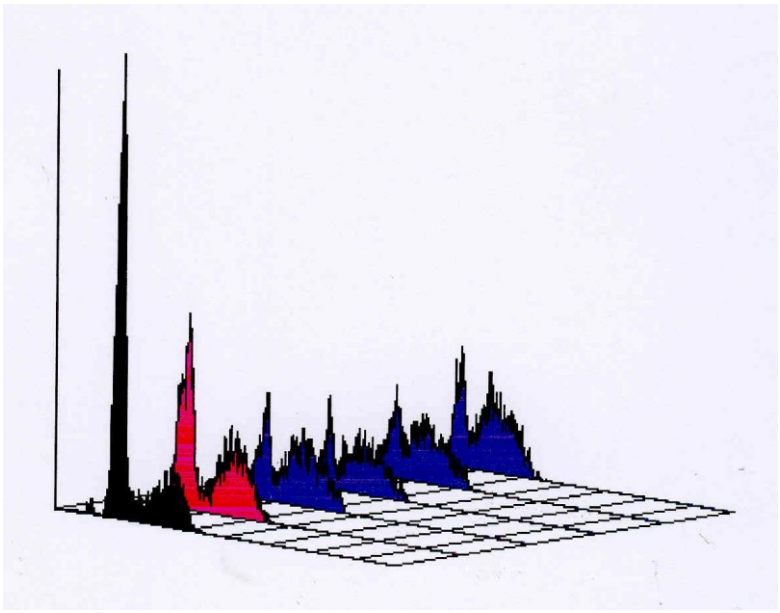


Figure A.2-3 RT4 Cell Cycle Profiles at 48 Hours
Flow cytometry analysis of the MMC-induced variation in cell cycle distribution. Representative profiles, as determined by CellQuest software, of RT4 cells treated with/without mirtoselect for 24 hours followed by 1 or 10µM MMC for 1 hour and 23 hours in drug free media. (Black profile) Control, untreated cells, (Red profile) 1µM MMC alone, (Blue profiles) cell pre-treated with 0-100µg/ml mirtoselect for 24 hours followed by 10µM.

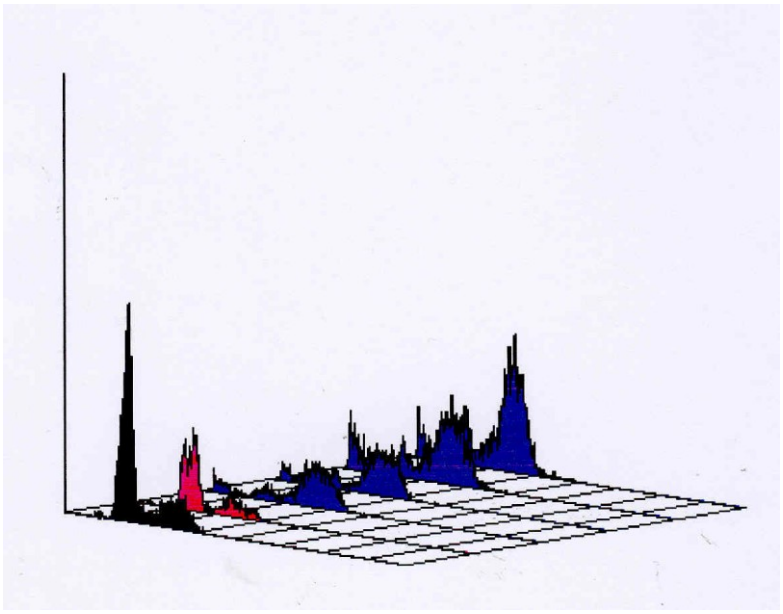


Figure A.2-4 RT4 Cell Cycle Profiles at 72 Hours
Flow cytometry analysis of the MMC-induced variation in cell cycle distribution. Representative profiles, as determined by CellQuest software, of RT4 cells treated with/without mirtoselect for 24 hours followed by 1 or 10µM MMC for 1 hour and 47 hours in drug free media. (Black profile) Control, untreated cells, (Red profile) 1µM MMC alone, (Blue profiles) cell pre-treated with 0-100µg/ml mirtoselect for 24 hours followed by 10µM.

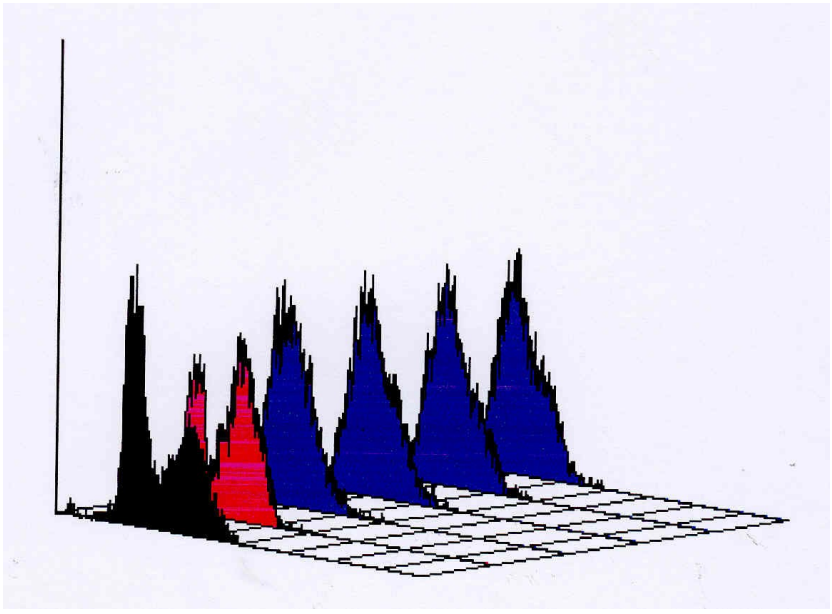


Figure A.2-5 HT1376 Cell Cycle Profiles at 48 Hours
Flow cytometry analysis of the MMC-induced variation in cell cycle distribution. Representative profiles, as determined by CellQuest software, of HT1376 cells treated with/without mirtoselect for 24 hours followed by 1 or 10µM MMC for 1 hour and 23 hours in drug free media. (Black profile) Control, untreated cells, (Red profile) 1µM MMC alone, (Blue profiles) cell pre-treated with 0-100µg/ml mirtoselect for 24 hours followed by 10µM.

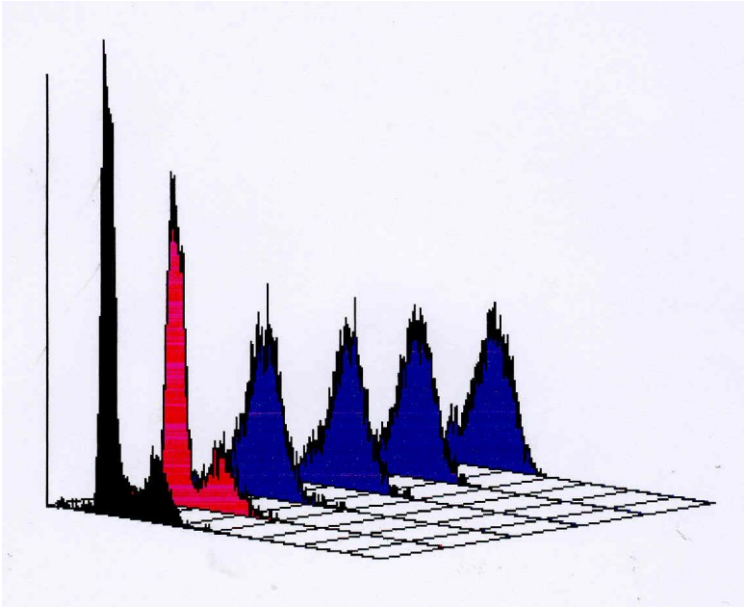


Figure A.2-6 HT1376 Cell Cycle Profiles at 72 Hours
Flow cytometry analysis of the MMC-induced variation in cell cycle distribution. Representative profiles, as determined by CellQuest software, of HT1376 cells treated with/without mirtoselect for 24 hours followed by 1 or 10µM MMC for 1 hour and 47 hours in drug free media. (Black profile) Control, untreated cells, (Red profile) 1µM MMC alone, (Blue profiles) cell pre-treated with 0-100µg/ml mirtoselect for 24 hours followed by 10µM.

Appendix C

ApoTox-Glo Triplex Assay Graphs

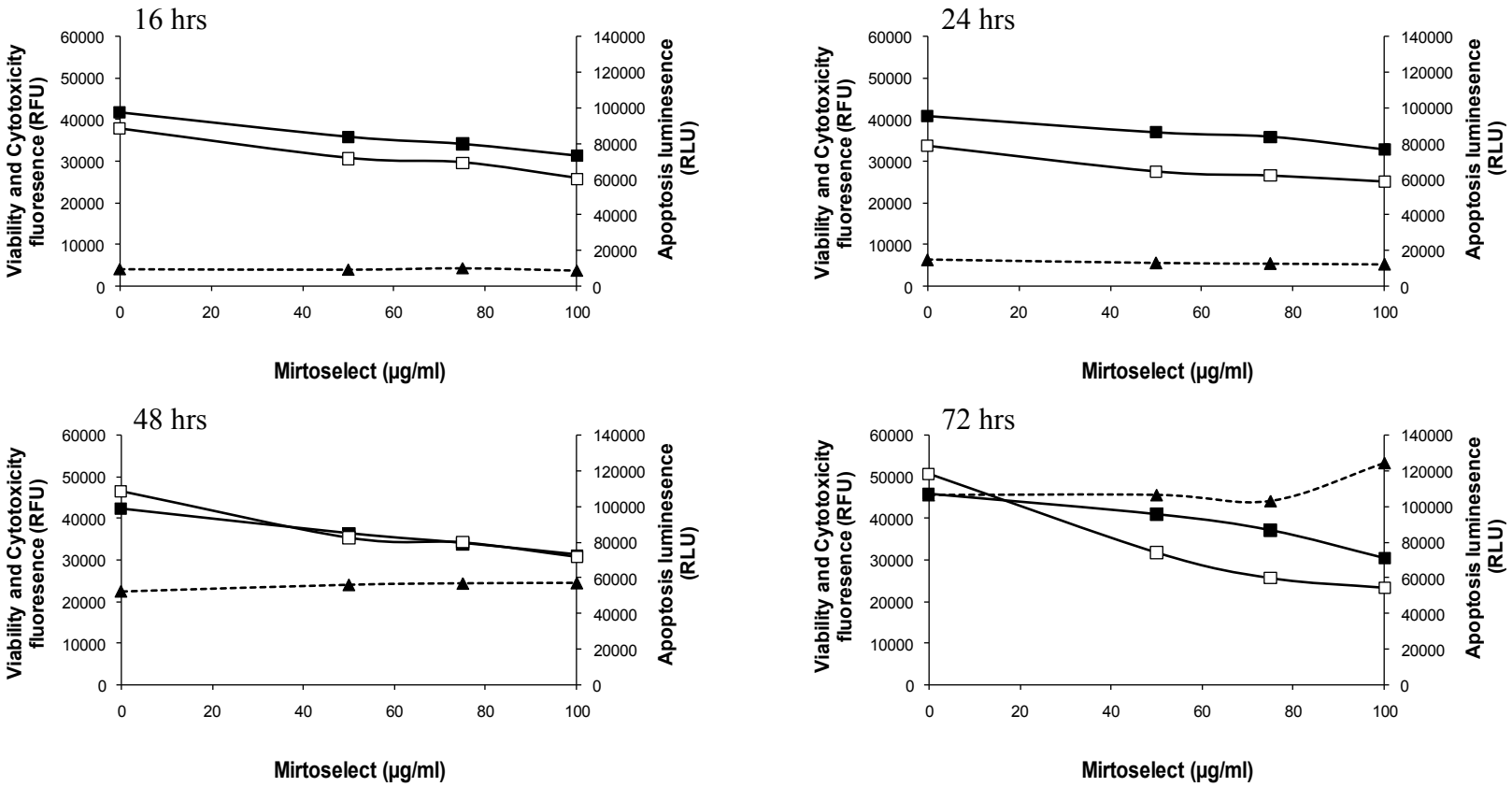


Figure A.3-1 RT112 ApoTox-Glo Triplex Assay Graphs
RT112 cells were treated with various doses of mirtoselect and analysed at 16, 24, 48 and 72 hours post treatment. Viability ■, cytotoxicity □, and apoptosis ▲were assessed using measures of fluorescence (viability and cytotoxicity markers, RFU) and luminescence (caspase activity, RLU). Results are the mean of three independent experiments.

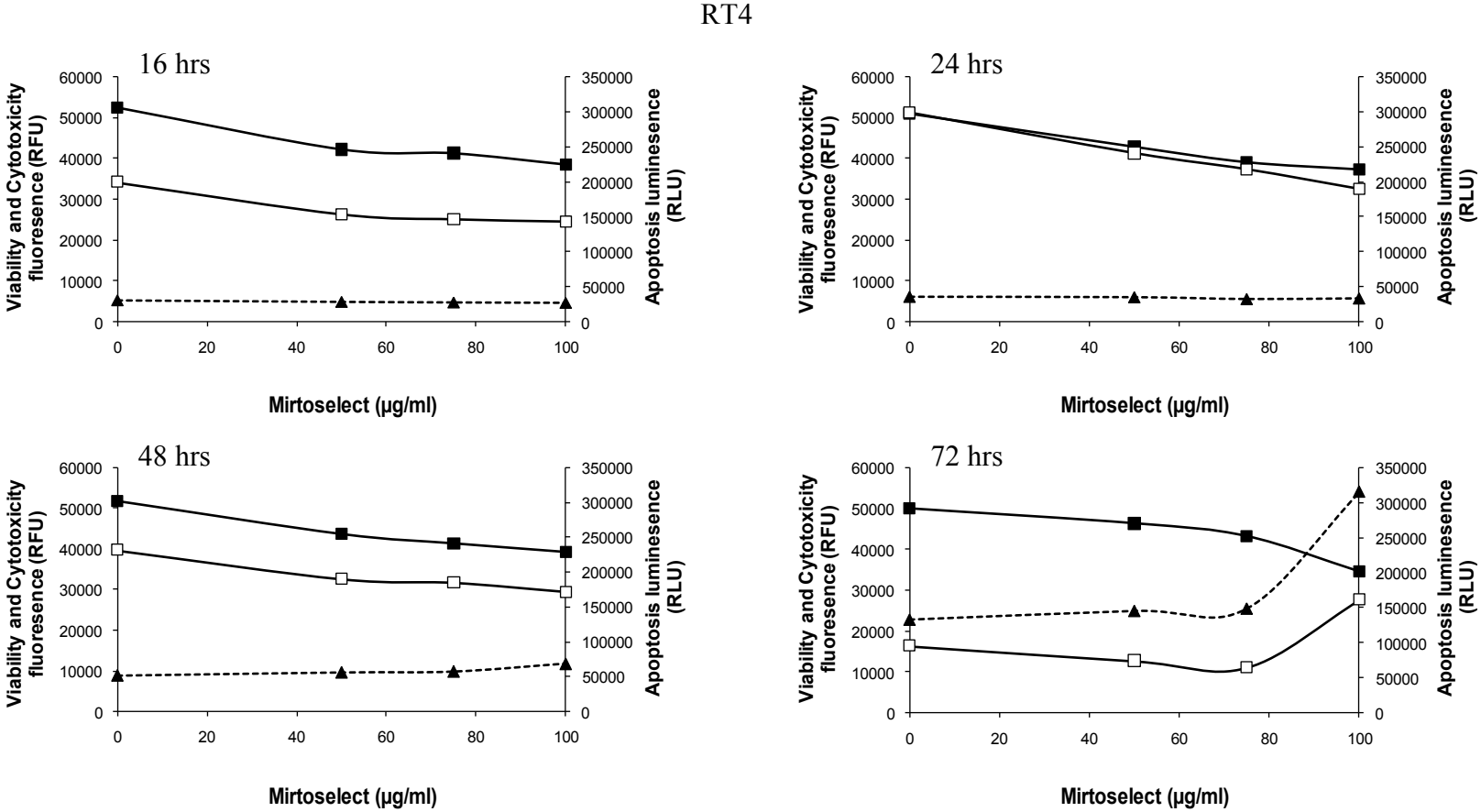


Figure A.3-2 RT4 ApoTox-Glo Triplex Assay Graphs

RT4 cells were treated with various doses of mirtoselect and analysed at 16, 24, 48 and 72 hours post treatment. Viability ■, cytotoxicity □, and apoptosis ▲were assessed using measures of fluorescence (viability and cytotoxicity markers, RFU) and luminescence (caspase activity, RLU). Results are the mean of three independent experiments

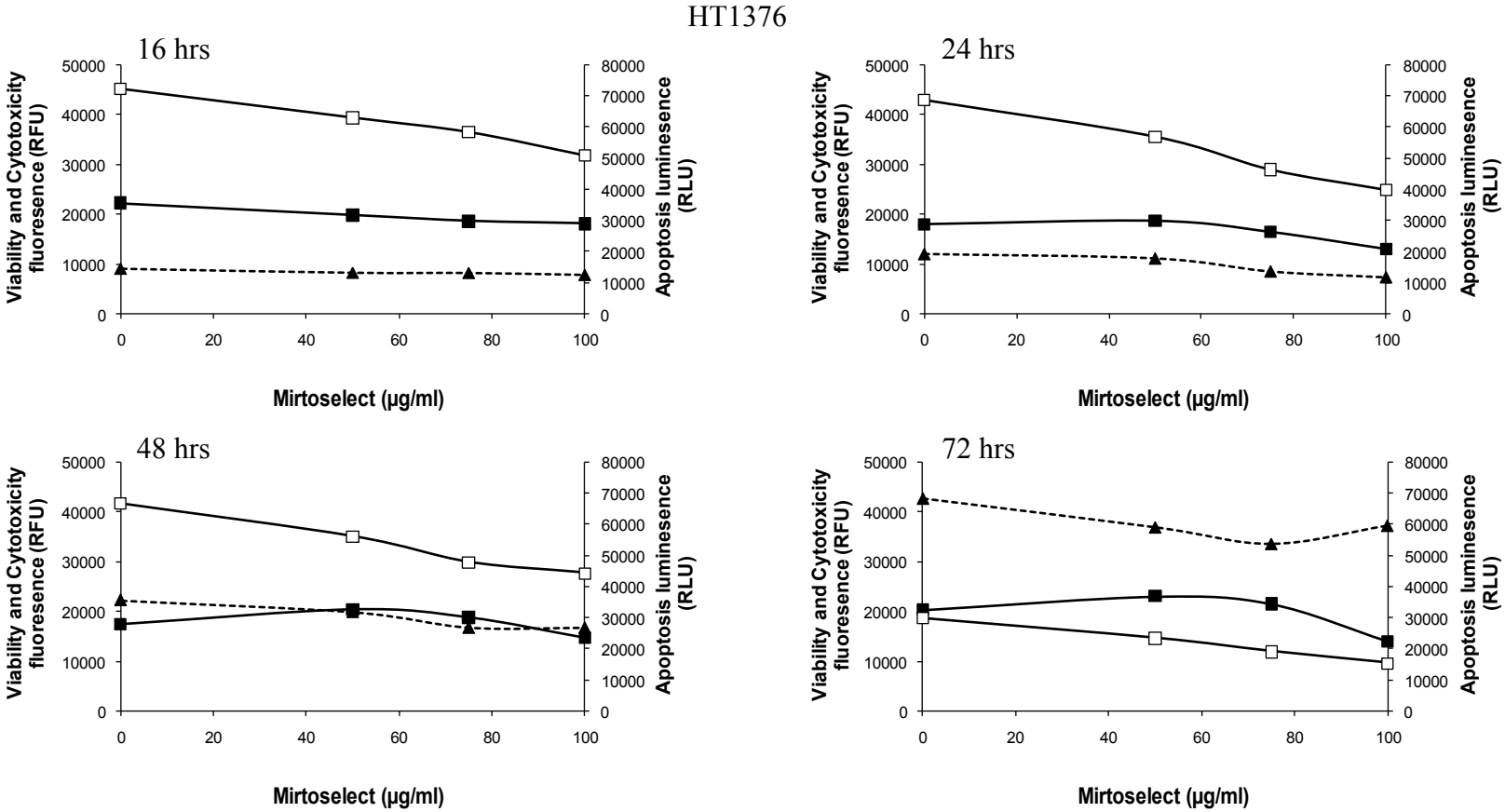


Figure A.3-3 HT1376 ApoTox-Glo Triplex Assay Graphs

HT1376 cells were treated with various doses of mirtoselect and analysed at 16, 24, 48 and 72 hours post treatment. Viability ■, cytotoxicity □, and apoptosis ▲were assessed using measures of fluorescence (viability and cytotoxicity markers, RFU) and luminescence (caspase activity, RLU). Results are the mean of three independent experiments

Appendix D

List of Publications and Communications in Scientific Meetings

- Higgins, J.A, Steward, W.P., Brown, K., and Jones, G.D., (**2009**). Can dietary agents be safely used as chemopreventive agents in bladder cancer? Presentation at the 32nd Annual Meeting of UKEMS, University of Leeds, Leeds, UK.

- Grigg, J., Tellabati, A., Rhead, S., Almeida, G.M., Higgins, J.A, Bowman, K.J., Jones, G.D., Howes, P.B,. (**2009**). DNA damage of macrophages at an air-tissue interface induced by metal nanoparticles. Nanotoxicology; 3(4): 348–354.

- Higgins, J.A, Steward, W.P., Brown, K., and Jones, G.D., (**2008**). The impact of chemoprevention on treatment regimes for non muscle invasive bladder cancer. Poster at the NCRI Cancer Conference, International Convention Centre, Birmingham, UK.

- Higgins, J.A, Steward, W.P., Mellon, J.K., Brown, K., and Jones, G.D., (**2008**). The impact of chemoprevention on treatment regimes for non muscle invasive bladder cancer. Poster at the UKEMs/NordEMS/MEG/IGG Joint Conference, University of Northumbria, Newcastle upon Tyne, UK.

- Higgins, J.A, Steward, W.P., Mellon, J.K., Brown, K., and Jones, G.D., (**2008**). You are what you eat - Fighting Cancer with Bilberries. The Impact of Chemoprevention on Treatment Regimes for Non Muscle Invasive Bladder Cancer. Poster at the Festival of Postgraduate Research, University of Leicester, Leicester, UK

- Higgins, J.A, Steward, W.P., Mellon, J.K., Brown, K., and Jones, G.D., (**2008**). The impact of chemoprevention on treatment regimes for non muscle invasive bladder cancer. Poster at the BAUS Section of Academic Urology Annual Scientific Meeting, UK

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