Assessment of Phytochemicals in Preventing Oxidatively Damaged DNA in Bladder Cancer

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by

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ABSTRACT

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Oxidatively damaged DNA is thought to be important in both the initiation and development of bladder carcinoma. Phytochemical compounds are thought to promote optimal health, partly via their antioxidant effects in protecting cellular components against damaging free radicals. The objective of this study is to assess the effect of a standardised bilberry extract, mirtoselect, on the level of endogenous and induced oxidatively damaged DNA in bladder cancer cells, as assessed by the Comet assay. The Comet assay, also known as single-cell gel electrophoresis, represents a simple method for measuring DNA strand break damage in eukaryotic cells. The sensitivity and specificity of the assay is greatly enhanced by the addition of bacterial repair endonucleases that recognise specific types of damage in the DNA and converts these lesions into additional DNA breaks.

Studies of mirtoselect against three bladder cancer cell lines (RT112, RT4 and HT1376) have shown significant antiproliferative activities against RT112 cells and against RT4 cells ($p<0.05$), but not against HT1376 cells. The treatment of all bladder cancer cell lines with mirtoselect (50 µg/ml) for a duration of seven days did not lower the level of endogenous oxidatively damaged DNA as detected by the modified endonuclease-alkaline Comet assay. However, a significant level of protection was observed when exogenous hydrogen peroxide was used to induce oxidatively damaged DNA in all the bladder cancer cell lines studied. Further studies revealed that mirtoselect may possibly mediate its antioxidant property through metal chelation, rather than free radical scavenging.

Our studies demonstrated that mirtoselect was potent enough to reduce levels of exogenously-induced oxidatively damaged DNA in the bladder cancer cell lines studied. Additionally, the ability of mirtoselect to reduce bladder cancer cells proliferation further highlights anthocyanins as promising future chemopreventive agents against bladder cancer.

ACKNOWLEDGMENT

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To the most important people in my life; my wife and children, this thesis is a special gift to them for their everlasting support. My wife always cheers me up when I felt miserable with my 'failed' experiments. My children lend me their laugh when things were not going well. The sacrifice they made has enabled me to complete my dream. I am also taking this opportunity to thank my parents for their prayers, endless support and encouragement during these last few years.

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I am most grateful to the Public Service Department of Malaysia and Institute for Medical Research, Malaysia for financial funding and support during this project.

Declaration of Interest

I, the undersigned, declare that the mirtoselect used in this study was kindly provided by Indena S.P.A Italy and that the company did not have any influence on the outcome of this study.

Murizal ZAINOL

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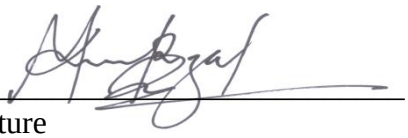

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Abbreviations

4-ABP	4-aminobiphenyl
8-oxoGua	7, 8-dihydro-8-oxoguanine
% TD	Percentage tail DNA
ALS	Alkaline labile site
AP site	Apurinic/Apyrimidinic site
ARE	Anthocyanin rich extract
C3G	Cyanidin-3-glucoside
CO_2	Carbon dioxide
COX2	Cyclo-oxygenase 2
CRUK	Cancer Research UK
ddH20	Double distilled water
DMEM	Dulbecco's modified eagle's medium
DPPH	2,2-diphenyl-1-picrylhydrazyl
FAP	Familial adenomatous polyposis
Fpg	Formamidopyrimidine DNA glycosylase
FRAP	Ferric Reducing Ability of Plasma
H_2O_2	Hydrogen peroxide
hOGG1	human 8-oxoguanine DNA glycosylase 1
HPLC	High Performance Liquid Chromatography
HPV	Human papilloma virus
LET	Low-linear energy transfer
LMP	Low melting point
MIBC	Muscle-invasive bladder cancer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MIBC	Muscle-invasive bladder cancer
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NEAA	Non-essential amino acid
NER	Nucleotide excision repair
NMIBC	Non muscle-invasive bladder cancer
NMP	Normal melting point
ORAC	Oxygen Radical Absorbance Capacity
OTM	Olive Tail Moment
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PI	Propidium iodide
RB	Retinoblastoma
ROS	Reactive oxygen species
RT	Radiotherapy
SCE	Sister chromatid exchange
SCGE	Single-cell gel electrophoresis
SD	Standard deviation
SEM	Standard error of the mean
SSB	Single strand break
TCC	Transitional cell carcinoma
TD	Tail DNA
TM	Tail moment
TNM	Tumour, nodes and metastasis
U/ml	International Unit/ml (enzyme concentrations)

WHO	World Health Organisation

Chapter 1.

General Introduction.

1.1 Cancer as a leading cause of death

According to the World Health Organization (WHO) approximately 13 % of all deaths occurring in the year 2008 were due to malignant neoplasms, with cardiovascular disease and infectious and parasitic diseases being responsible for ~ 30% and ~ 20%, respectively. This lead to an estimated 7.6 million cancer deaths that year (Ferlay et al., 2010; WHO, 2009). Malignant neoplasms are, therefore, one of the leading causes of death in the world, being the second major cause of death in developed countries after cardiovascular disease.

Lung cancer is the most lethal form of cancer worldwide, followed by stomach, liver, colorectal and oesophagus (Ferlay et al., 2010). In males, lung cancer was the most lethal cancer site in 2008, followed by liver, stomach and colon plus rectum cancer. Breast cancer was the leading cause of female cancer deaths in 2008 followed by lung, cervical and stomach cancer (Ferlay et al., 2010).

There are marked differences in the ranking of the leading cancer types causing death when comparing more developed regions with less developed ones, as seen in **Table 1.1** (Ferlay et al., 2010), and also between countries within these regions. In the majority of the developed countries, breast cancer ranks as number one for female cancer caused deaths. It is interesting to note, however, that even though breast cancer has the highest number of new cases every year, its mortality rate has been surpassed by lung cancer since the late eighties in the USA and from the early nineties in Canada. The same phenomenon is starting to occur in the UK where lung cancer is now deadlier than breast cancer (WHO, 2004). This trend probably reflects not only improvements in breast cancer screening and treatment but also an increase in lung cancer incidence.

Differences in cancer incidence are observed not only between different populations in distant regions of the globe, but also between neighbouring countries and

even within the same country. The fact that immigrants rapidly converge towards local cancer rates excludes a genetic explanation for these differences, for instance breast cancer incidence of Japanese immigrants in Hawaii (Ziegler et al., 1993). Therefore, by the 1960s, cancer epidemiologists concluded that most cancers are in principle preventable and many could be avoided by a suitable choice of lifestyle and environment. Many specific causes of cancer have now been identified, the most important being smoking, obesity and a few oncogenic viruses (Bianchini et al., 2002; Bosch et al., 1995; Hecht, 1999). However, a large proportion of global variation for common cancers remains unexplained (*e.g.* breast and prostate) (Peto, 2001).

Table 1.1 Rank orders of the leading types of cancer causing death in more (USA and European countries) and less developed (African countries) regions (Ferlay et al., 2010).

Rank Order	**More developed regions**		**Less developed regions**	
	Male	**Female**	**Male**	**Female**
1	Lung	Breast	Lung	Lung
2	Stomach	Lung	Liver	Breast
3	Liver	Colorectal	Stomach	Cervical
4	Colorectal	Cervical	Oesophagus	Stomach
5	Prostate	Stomach	Colorectal	Liver
6	Oesophagus	Liver	Prostate	Colorectal

Despite the fact that the number of cancer related deaths increases every year (as does the population), in the European Union (and similarly in the USA) there has been a decline in mortality cancer rates since the late 1980s. This is probably due to improved screening and early diagnostics, improvements in diet and downwards trends in tobacco smoking as well as to advances in cancer therapies (Levi et al., 2000).

1.2 Bladder cancer

1.2.1 Statistic and incidence

Bladder cancer is ranked as the ninth most common malignancy worldwide and has high gender variability in occurrence (Murta-Nascimento et al., 2007). There are approximately 380,000 new cases of bladder cancer diagnosed every year worldwide (Ferlay et al., 2010). Interestingly, the incidence of bladder cancer varies significantly among countries, with the highest rates being in the western world. Particularly high incidence rates are observed for Southern Europe, Northern Africa, North America and Western Europe, whereas its occurrence in Asian countries is relatively low (Wu et al., 2008). In the US, bladder cancer is the fourth most common cancer diagnosed in males, with an estimated 51,230 new cases and 9,950 deaths from bladder cancer in 2008 (Jemal et al., 2008).

Bladder cancer is also a common cancer in the UK, with 10,091 new cases diagnosed in 2007 (CRUK, 2010). It is the most frequently occurring tumour of the urinary system and accounts for around 1 in every 29 new cases of cancer each year in the UK. In the UK bladder cancer is the fourth most common cancer in males, with 7,284 new cases diagnosed in 2007. This compares to 2,807 female cases, giving a male:female ratio of 5:2. In females it is the eleventh most common cancer (CRUK, 2010).

1.2.2 Risk factor and aetiology

The two best established risk factors for bladder tumour are cigarette smoking and occupational exposures to urothelial carcinogens. Smokers have a 4-fold higher incidence of bladder cancer than the general population (Clavel et al., 1989). This risk correlates with the number of cigarettes smoked and duration of smoking (Augustine et

al., 1988). Exposure to benzidine, aromatic amines and ß-naphthylamine, typically found in the cigarette smoke plus the dye and rubber industries, is associated with a 30-fold increased risk of developing bladder cancer when compared with the general population (Sengupta et al., 2004).

Many cancers arise as a consequence of the accumulation of DNA mutations, with these serving to 'drive' the carcinogenesis process. Similar to many cancers, bladder cancer has long a latency period (approximately 20 years). The accumulation of DNA mutations due to the prolonged/continual exposure of bladder urothelial cells to carcinogens, leading to the inactivation of tumour suppressor genes and/or activation of oncogenes, will eventually promote the cancer initiation process (Knudson, 2001). **Figure 1.1** depicts the multilayer carcinogenesis process of many cancers. Bladder cancer has been shown to develop into *carcinoma in-situ* (CIS), also known as TIS (tumour *in situ*), after 20 years, with there then being less than five years until it becomes a fully developed cancer (O'Shaughnessy et al., 2002). However, the above description of bladder cancer initiation may not be entirely accurate. The non-muscle invasive bladder cancer (NMIBC) has two distinct morphologies (see **Section 1.2.2.2**) and it is suggested that these are driven by distinct genetic alterations (see **Section 1.2.2.2.a**).

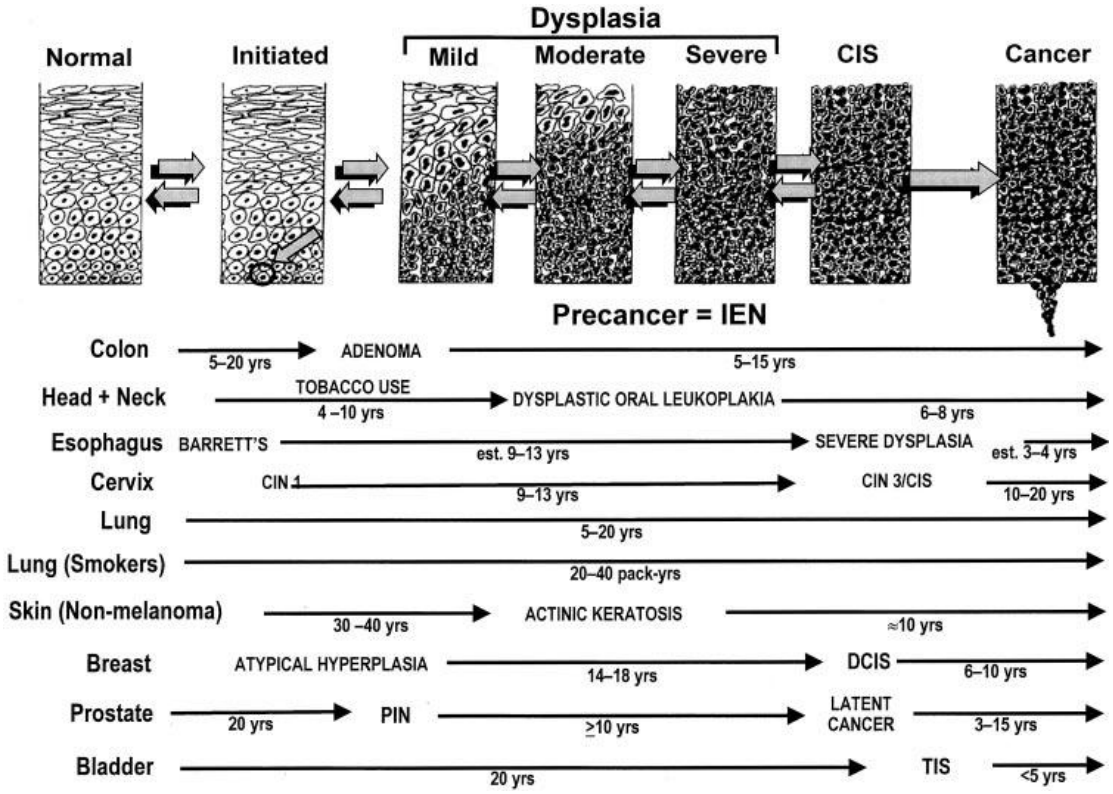


Figure 1.1: Multiyear process of human carcinogenesis (O Shaughnessy et al., 2002)

Epidemiologic studies suggest that the environmental contaminant 4-aminobiphenyl (4-ABP) is causally responsible for induction of human bladder cancer (Guerin MR, 1988.; Schulte PA, 1988; Vineis, 1994). Human exposure to 4-ABP and other related aromatic amines is common as they are present in cigarette smoke, fossil fuels, industrial chemicals and pollution and from other sources (Cole et al., 1972; Haugen et al., 1982; Melick et al., 1971; Steineck et al., 1990). The detection of haemoglobin- and DNA-4-ABP adducts in bladder cancer among the tobacco smokers provides strong evidence for 4-ABP's role as a bladder carcinogen (Airoldi et al., 2002; Talaska et al., 1991). The genotoxic and carcinogenic effects of 4-ABP became apparent when the proximate carcinogenic metabolites [*N*-hydroxy-4-acetylaminobiphenyl (*N*-OH-AABP) and *N*-acetoxy-4-acetylaminobiphenyl (*N*-OAc-AABP)] were studied via an *in vitro* system developed using human urothelial cells.

The human urothelial cells contained microsomal acetyl transferase, which has been shown to activate *N*-OH-AABP and *N*-OAc-AABP to reactive electrophilic species that bind to DNA. The presence of the acetyl transferase indicated that metabolic activation of the proximate metabolites of 4-ABP could occur directly in the human urothelial cells, thus exposing the cells towards 4-ABP induced bladder cancer (Swaminathan et al., 1994; Swaminathan S, 2000; Swaminathan and Reznikoff, 1992). Subsequently, through ^{32}P-postlabeling analysis of bladder cells exposed to N-OH-ABP or N-OH-AABP in culture, two primary DNA adducts were revealed. They were identified as *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) and *N*-(deoxyadenosin-8-yl)-4-aminobiphenyl (dA-C8-ABP) (Frederickson et al., 1992; Hatcher and Swaminathan, 1995a; Hatcher and Swaminathan, 1995b; Hatcher and Swaminathan, 2002). In addition, a minor adduct, 3-(deoxyguanosin-N^2-yl)-4-acetylamino-biphenyl (dG-N^2-AABP) was also identified from the horseradish peroxidase–mediated bioactivation of *N*-OH-AABP (Hatcher and Swaminathan, 1995a; Torino et al., 2001). Human urothelial cells also contain a number of peroxidases, including prostaglandin endoperoxide synthetase (Boyd and Eling, 1985; Wise et al., 1984). These peroxidises could convert *N*-OH-AABP to reactive electrophiles, the arylnitrenium and arylamidonium ions, with the latter possibly interacting with DNA bases to generate the covalent adducts containing the acetyl group. In addition, peroxidative metabolism may also generate redox active species that could interact with oxygen to produce superoxide anions. These superoxide anions ultimately generate hydroxyl radicals ($^{\bullet}$OH) and other associated reactive oxygen species (ROS) (Dizdaroglu, 1992; Marnett, 2000). Endogenous peroxidase–catalyzed activation of *N*-OH-AABP in the human cancer cell line (TCC 10) has been shown to produce ROS detected using a highly sensitive fluorescent assay (Burger et al., 2001). The ROS attack DNA bases, to produce

oxidatively modified bases such as 7, 8-dihydro-8-oxoguanine (8-oxoGua) (Ward et al., 1987). In fact, 8-oxoGua has been shown to cause miscoding by DNA polymerases *in vitro*, inducing G to T transversions. Such mutations are commonly seen in oncogenes and tumour suppressor genes following oxidative insult (Cheng et al., 1992; Hussain and Harris, 1998; Lepage et al., 1995; Moriya, 1993; Shibutani et al., 1991). The ROS, generated by endogenous peroxidases, will contribute to the DNA damage burden in urothelial cells and play a role in the initiation of bladder neoplasia (Burger et al., 2001).

The role of 4-ABP in bladder cancer is further supported by the uniqueness of mutational hotspots and DNA binding spectrum in human p53 gene in urothelial cells. For instance in lung cancer due to cigarette smoking, the p53 gene mutations are concentrated at several methylated CpG sites (codons 157, 158, 175, 245, 248, 273 and 282), whilst the p53 mutational hotspots in bladder cancer were evenly distributed along the p53 genes and not biased for CpG sites (codons 175, 248, 280 and 285). The same codons are also the preferential sites for 4-ABP adducts formation (Feng et al., 2002). 4-ABP generated from cigarette smoking and metabolically activated preferentially binds to codons 280 and 285 of the p53 gene but according to Lin and co-workers, mutations in these two codons rarely occur in lung cancer (Lin et al., 1994). These findings offer useful molecular evidence to the role of 4-ABP in bladder cancer.

Murata and co-workers also proposed similar mechanisms for the production of oxidatively damaged DNA in urothelial cells (**Figure 1.2**) induced by 4-ABP metabolites in the presence of NADH and the transition metal ion Cu (II) (Murata et al., 2001). It is suggested that 4-ABP is metabolised in the liver by CYP 1A2 to form *N*-hydroxy-4-aminobiphenyl (via the formation of a nitro derivative which is metabolised by nitroreductase to nitrosobyphenyl and finally yielding an *N*-hydroxy derivative).

Subsequently, the *N*-hydroxy derivative is esterified by *O*-acetyl tranferase to an N-acetoxy metabolite or glucuronidated by glucuronidase yielding an *N*-glucuronide, and transported into the blood and urine for excretion. However, whilst in the bladder, the *N*-glucuronide derivative can be hydrolysed by the acidic urine (Zenser et al., 1999) to form an active *N*-hydroxyamine derivative which can be transported into the urothelial cells.

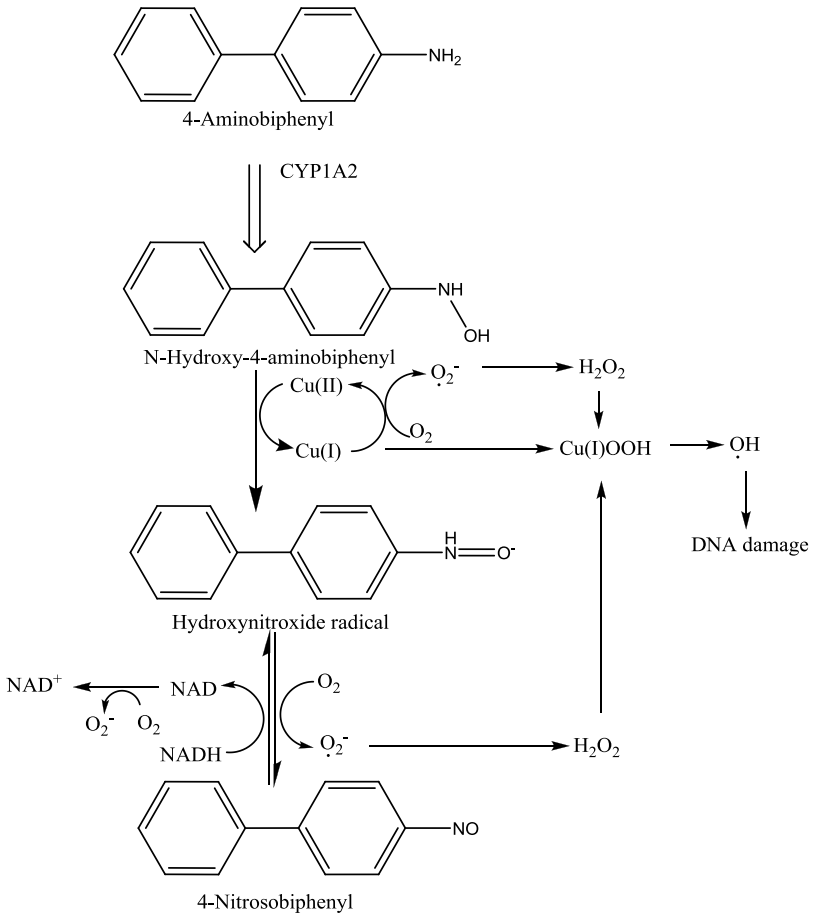


Figure 1.2 Proposed mechanism of oxidatively damage DNA induced by *N*-hydroxy-4-aminobiphenyl in the presence of NADH and Cu (II) (adapted from Murata et al., 2001).

The *N*-hydroxy derivatives of 4-ABP are proposed to autoxidise, an effect enhanced by the presence of Cu(II) (**Figure 1.2**), to the hydroxynitroxide radical. This is followed by further autoxidation, mediated by molecular oxygen, to the 4-nitrosobiphenyl species

(4-ABP(NO)). Addition of β-nicotinamide adenine dinucleotide disodium (NADH) leads to reduction of 4-ABP(NO) back to either the hydronitroxide radical or the *N*-hydroxy derivatives (4-ABP(NHOH)), after which autoxidation will again take place, suggesting that a redox cycle is formed in the presence of NADH (Murata *et al*., 2001). The copper mediated autoxidation of the *N*-hydroxy derivative and the following oxygen-mediated autoxidation of the hydroxynitroxide radical generate superoxide. The dismutation of superoxide produces H_2O_2 molecules that can react with copper and iron ions to form hydroxyl radicals (Murata et al., 2001).

1.2.2.1 Inflammation and Bladder Cancer (focused on Schistosomiasis)

Whilst carcinogens from the aromatic amines group are associated with the majority of bladder cancer carcinogenesis, there are certain parts of the world where bladder cancer is linked to an infection by a parasite called *Schistosoma haematobium*. This parasite is associated with the development of squamous cell carcinoma of the bladder and occurs commonly in areas where schistosomiasis (parasitic disease caused by trematode flatworms of the genus *Schistosoma*) is endemic (Shokeir, 2004).

Schistosomiasis-associated bladder cancer is one of the most common cancers in Egyptian males. It is also found in certain districts of Iraq, Yemen, Saudi Arabia, Sudan and several other African countries, though the incidence is less than in Egypt. This could be attributed to the lower degree of endemicity, intensity and persistence of schistosomiasis in these regions. Incidence of schistosome bladder cancer is higher in men than women (5:1) due to the fact that men are the main "breadwinner" for the family and the majority of them work as farmers in areas infested by these parasites (Elsebai, 1977).

Multiple factors have been suggested as causative agents in schistosome-associated bladder carcinogenesis. Of these, the *N*-nitroso compounds appear to be of particular importance since they are found at high levels in the urine of patients with schistosomiasis-associated bladder cancer (Mostafa et al., 1999). Studies conducted on schistosomiasis have demonstrated an increase in the activity of carcinogen-metabolising enzymes, for instance, *N*-nitrosodimethylamine-*N*-demethylase I (NDMA-dI) and cytochrome b5 soon after the infection, but this effect is reduced during later chronic stages of the disease (Badawi and Mostafa, 1993; Sheweita et al., 2003). This may contribute towards prolonged periods of exposure to activated *N*-nitrosamines, but also inflammatory cells, stimulated as a result of infection, may induce the endogenous synthesis of *N*-nitrosamines and ROS (Masuda et al., 2000; Ohshima and Bartsch, 1994). Free radicals may contribute towards higher levels of host cell DNA damage, and this seems to be further augmented by inefficiency of relevant enzymes to repair the damaged DNA (Mostafa et al., 1999).

It has been reported that schistosome-induced chronic inflammation and irritation in the urinary bladder are associated with increased cancer initiation at the site of inflammation (Rosin et al., 1994a; Rosin et al., 1994b). Inflammatory cells such as macrophages and neutrophils are important sources of endogenous oxygen radicals and contribute to the formation of carcinogenic *N*-nitrosamines (Reid and Loeb, 1992). Moreover, inflammatory cells induce genotoxic effects, such as DNA mutations (Reid and Loeb, 1992), sister chromatid exchanges (Weitberg, 1989), and DNA strand breaks (Shacter et al., 1988). These toxic effects may result from the formation and release of $^{\bullet}OH$ from the inflammatory cells (Dizdaroglu et al., 1993). Inflammatory cells also participate in the activation of procarcinogens, such as aromatic amines and polycyclic aromatic hydrocarbons, to their ultimate carcinogenic metabolites (i.e., the final reactive

form of the carcinogen) (Obrien, 1988). Since the aromatic amines are an important group of bladder carcinogens, an increased number of inflammatory cells in the urinary bladder of schistosomal patients may enhance the carcinogenic potential of these agents by increasing their rate of activation (Mostafa et al., 1999).

1.2.2.2 Molecular pathways of bladder cancer

Pathologists have long recognised the occurrence of two different type of bladder cancer when first diagnosed (Spruck et al., 1994). Molecular and cytogenetic data support the clinical impressions that low-grade and high-grade tumours may represent alterations in two distinct biochemical signalling pathways, resulting in the observed clinical behaviour, and leading to the progression of bladder cancer (Knowles, 2006; Mhawech-Fauceglia et al., 2006; Spruck et al., 1994; Wu, 2005). The first pathway is characterized by low-grade well-differentiated papillary tumours that infrequently invade the detrusor muscle. The second pathway is characterized by CIS and poorly differentiated tumours, including grade 3 non-muscle-invasive bladder cancer, with frequent recurrences and progression to detrusor muscle invasion (Ehdaie and Theodorescu, 2008; Spruck et al., 1994).

Low-grade papillary tumours frequently show a constitutive activation of the receptor tyrosine kinase–Ras pathway, exhibiting activating mutations in the *HRAS* and fibroblast growth factor receptor 3 (*FGFR3*) genes. Seventy percent of low-grade non-invasive papillary tumours have been shown to harbour *FGFR3* mutations compared with 10% to 20% of invasive tumours, which strongly suggests a role for the activated *FGFR3* gene in the genesis of low-grade papillary tumours (Bakkar et al., 2003; Rieger-Christ et al., 2003; van Rhijn et al., 2004). In contrast, CIS and invasive tumours frequently show alterations in the *TP53* and *RB* genes and pathways. Recognition of the

key molecular events in the alteration of these pathways is crucial, and an in-depth understanding of the epigenetic and genetic alterations involved can offer indications of key prognostic and therapeutic markers (reviewed by Mitra *et al*., 2006). The cell cycle is controlled by the p53 and RB pathways, with the cells receiving extracellular growth signals via the Ras–mitogen activated protein kinase (MAPK) pathway (**Figure 1.3**). Some of these molecules also show evidence of epigenetic modulation through aberrant promoter hypermethylation in invasive bladder cancer, and several have been demonstrated to be prognostically important (Mitra et al., 2006).

a) Ras-MAPK pathway

Most NMIBC show constitutive activation of the Ras-MAPK pathway, generally through the activation of FGFR3 (Billerey et al., 2001; van Rhijn et al., 2001). However, in invasive urothelial carcinoma, other receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) and ERBB2 (also called HER2) are overexpressed and associated with poor outcome (Colquhoun and Mellon, 2002; Kruger et al., 2002; Lipponen and Eskelinen, 1994; Ravery et al., 1995).

Binding of epidermal growth factor (EGF) causes activation of the already over expressed EGFR, leading to receptor dimerization and autophosphorylation. The activated receptor then recruits proteins that convert Ras to its activated state. This activated Ras protein can then transduce a mitogenic signal through the Ras-MAPK pathway by acting through the MAPK/ERK system (**Figure 1.3**). *RASSF1A* is a tumour-suppressor gene that encodes a Ras association domain family 1 protein that can inhibit the function of the activated Ras protein (Oxford and Theodorescu, 2003). This gene is generally highly methylated in bladder cancer (Catto et al.,

2005; Chan et al., 2003; Dulaimi et al., 2004; Lee et al., 2001; Maruyama et al., 2001; Yates et al., 2006), and increased methylation of the *RASSF1A* gene is associated with increasing tumour stage (Friedrich et al., 2004). The death-associated protein kinase (*DAPK*, an apoptosis promoter) prevents the translocation of extracellular-signal regulated kinase (ERK) protein from cytoplasm into the nucleus, thereby inhibiting signal transduction.

Translocation of ERK into the nucleus activates the mitogen-activated and stress-activated protein kinase 1 (MSK1; **Figure 1.3**), a histone H3 kinase that can relax chromatin, thus making it more transcriptionally accessible (Dunn et al., 2005). This alteration in the chromatin state induces *MYC*, (Hipfner and Cohen, 2004) a gene that encodes the c-Myc protein, a transcription factor that controls the cell cycle. No correlation has been found between the *MYC* methylation pattern and clinical stage of bladder cancer (Sardi et al., 1997) and studies have presented conflicting data on the significance of its protein expression levels with respect to prognosis (Lipponen, 1995; Masters et al., 1988). c-Myc signals downstream to promote expression of cyclins that complex with cyclin-dependent kinases (CDKs; **Figure 1.3**), which together regulate the RB pathway (Kerkhoff and Rapp, 1998; Mitra et al., 2005; Sears and Nevins, 2002).

b) p53 cell cycle regulation pathway

The p53 tumour suppressor protein is encoded by the *TP53* gene, critical in cell cycle control in bladder cancer (**Figure 1.3**). It is a central molecule in several important cellular programs related to cancer development, progression, and response to therapy, including cell-cycle regulation, angiogenesis, apoptosis, and DNA repair (Cote and Chatterjee, 1999). The p53 protein inhibits cell cycle

progression at the G1/S transition (Livingstone et al., 1992) and mediates its control through the transcriptional activation of *p21*$^{WAF1/CIP1}$ (Eldeiry et al., 1993). Mutations in the *TP53* gene are known to be a critical event in many cancers, including bladder cancer (Cote and Chatterjee, 1999). The mutations are generally missense point mutations, which result in altered protein that is resistant to normal regulatory degradation by the ubiquitin pathway (Dowell et al., 1994).

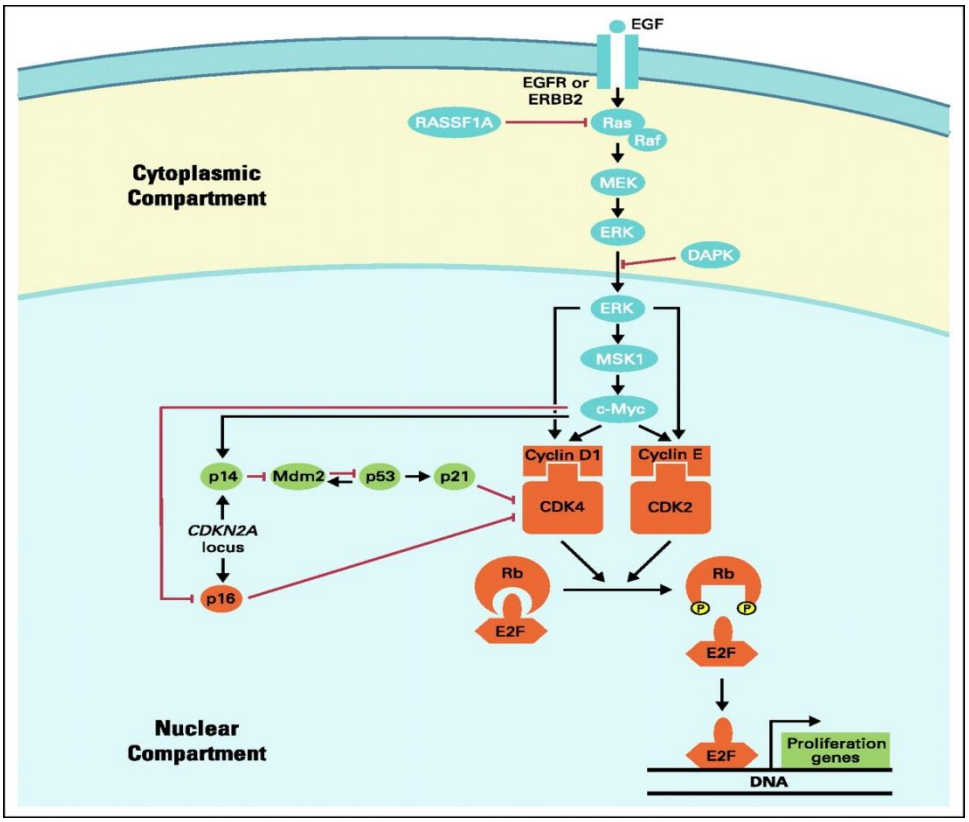


Figure 1.3: Interactions of the Ras-MAPK signal transduction (blue), p53 cell cycle regulation (green) and retinoblastoma (orange) pathways in invasive bladder cancer. Extracellular growth signals are transduced through the Ras-MAPK pathway to the nucleus, where cyclin/CDK complexes phosphorylate Rb and release E2F, causing transcription of genes that promote proliferation. This process is controlled by the p53 pathway. EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERBB2, erythroblastic leukemia viral oncogene homolog 2; Ras, GTPase H-ras; Raf, *RAF* proto-oncogene serine/threonine-protein kinase; RASSF1A, Ras association (RalGDS/AF-6) domain family 1, isoform A; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; DAPK, death-associated protein kinase; MSK1, mitogen-activated and stress-activated protein kinase 1; c-Myc, *MYC* proto-oncogene protein; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; CDK, cyclin-dependent kinase; Rb, retinoblastoma protein. (Adopted from Mitra *et al*., 2006)

This results in a more stable protein that can accumulate in the nucleus. In fact, nuclear accumulation of p53 as detected by immunohistochemistry (IHC) is highly correlated with mutations in the *TP53* gene (Cordoncardo et al., 1994; Esrig et al., 1993) although this is not always the case. It has been shown that p53 nuclear accumulation is predictive of outcome particularly for patients with invasive, organ-confined, node-negative (T1-2bN0) bladder cancer (Esrig et al., 1993; Mitra et al., 2006; Sarkis et al., 1993; Serth et al., 1995; Uchida et al., 1995). This has been recently confirmed by meta-analysis studies (Malats et al., 2005; Schmitz-Drager et al., 2000a; Schmitz-Drager et al., 2000b).

Although p53 nuclear accumulation is associated with *TP53* gene mutations, significant discordance exists (Cordoncardo et al., 1994; Esrig et al., 1993). In a study involving complete exon sequencing of p53 and comparing the results with nuclear accumulation of the protein (Mitra et al., 2006),it was suggested that both nuclear accumulation and gene mutations play a role in progression, and that determining the status of both the gene and the protein provides additional synergistic information regarding prognosis. Further, it appears that not only a mutation per se, but also the site of mutation may be important in understanding the behaviour of the tumour (Mitra et al., 2006).

The $p21^{WAF1/CIP1}$ gene encodes the p21 protein, a cyclin-dependent kinase inhibitor (CDKI) which is transcriptionally regulated by the p53 protein (Eldeiry et al., 1993). p21 has also been shown to be regulated in a p53-independent manner (Eldeiry et al., 1993). Loss of p21 expression has been proposed to be a mechanism by which p53 alterations influence tumour progression. Results from studies have shown that loss of p21 expression can be a significant and independent predictor of urothelial carcinoma progression (Mitra et al., 2006) whereas the maintenance of

p21 expression appears to abrogate the deleterious effects of p53 alterations (Stein et al., 1998). Patients with p21-negative, p53-altered tumours had a greater recurrence and lower survival rate than those with p21-positive tumours, irrespective of the tumour grade or pathologic stage. This fact was especially notable in node-negative patients (Mitra et al., 2006).

The Mdm2 protein is involved in an auto-regulatory feedback loop with p53, thus controlling its activity (Wu et al., 1993). Increased p53 levels transactivate the *MDM2* promoter causing its upregulation. The translated protein then binds to p53 and transports it to the proteasome for ubiquitin-mediated degradation. The resultant lowered p53 levels then reduce the levels of Mdm2. The *MDM2* gene is, in turn, transcriptionally inhibited by p14 (Zhang et al., 1998), providing another fine level of control for p53 activity (**Figure 1.3**). The $p14^{ARF}$ gene may be inactivated by homozygous deletion (Berggren et al., 2003), or by varying degrees of methylation of the promoter region (Catto and Hamdy, 2005; Chang et al., 2003; Dulaimi et al., 2004; Kawamoto et al., 2006; Yates et al., 2006). Increased methylation levels of $p14^{ARF}$ have been shown to correlate with poor prognosis in UC (Kawamoto et al., 2006). Altered p53 protein expression in the presence of wild-type p53 may be caused in part by alteration in the p14/Mdm2 regulatory pathway (Mitra et al., 2006).

c) Retinoblastoma pathway

The retinoblastoma (*RB*) gene encodes a nuclear phosphoprotein (Rb), the phosphorylation of which plays a critical role in cell cycle regulation (Mitra et al., 2006). The active, dephosphorylated form of Rb binds and sequesters the transcription factor E2F (Chellappan et al., 1991). On phosphorylation by CDKs, Rb

releases E2F which, in turn, transcribes genes required for DNA synthesis (Mitra et al., 2006) (**Figure 1.4**). Inactivating mutations of the *RB* gene, which result in loss of protein expression, have been incriminated in low-grade and superficial bladder cancers, as well as in high-grade and invasive cancers (Miyamoto et al., 1995). It was originally believed that the detection of the Rb protein in the nucleus of the tumours cell (by immunohistochemistry or Western blot) indicated the presence of an intact functional gene, whereas loss of protein expression indicated mutations in the *RB* gene. However, Cote and co-workers has indicated that a significant proportion of tumours expressing Rb show the clinical consequences of loss of Rb function (Cote et al., 1998). Bladder cancer patients having tumours with high Rb protein had a clinical outcome similar to those with tumours showing loss of Rb expression. Studies have shown that tumours with the highest Rb protein expression levels demonstrated constitutively hyperphosphorylated Rb, which resulted from either loss of p16 expression (a product the *INK4A* gene and a CDKI) and/or cyclin D1 overexpression (Chatterjee et al., 2004). Mechanistic analysis of this association was performed *in vitro* by transfecting T24 cells, which express high levels of Rb, with p16 cDNA. The p16 transfection resulted in a marked decrease in Rb phosphorylation, decreased cell proliferation, and decreased levels of Rb expression as determined by immunohistochemistry. These studies provided the biological basis for constitutive alteration of Rb function in the presence of an intact gene through Rb phosphorylation (**Figure 1.4**) (Mitra et al., 2006).

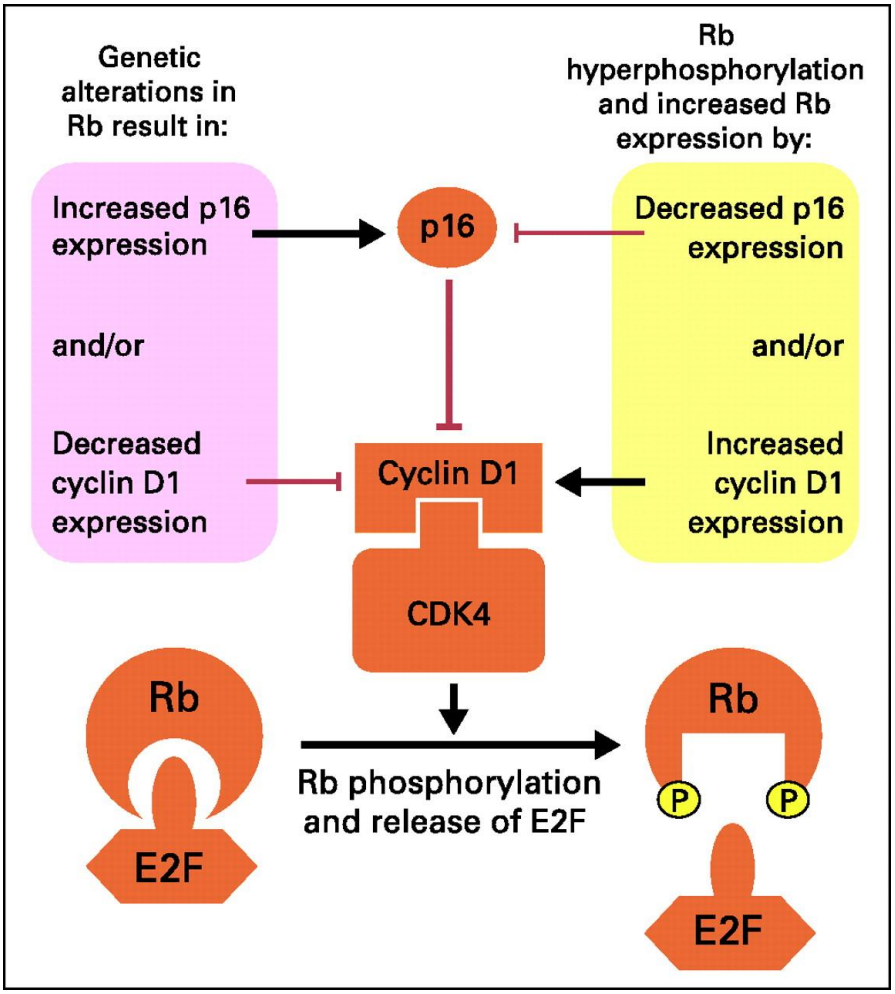


Figure 1.4: Proposed interrelationship between p16, cyclin D1/CDK4, Rb and E2F in bladder cancer. Genetically altered Rb may cause increased p16 and/or decreased cyclin D1 expression (pink panel). Rb hyperphosphorylation and increased Rb expression may be caused by decreased p16 and/or increased cyclin D1 expression (yellow panel). Either mechanism may lead to poor prognosis in bladder cancer. Rb, retinoblastoma protein; CDK4, cyclin-dependent kinase 4. (Adopted from Mitra *et al*., 2006)

There are several other factors that have been associated with the urothelial cell carcinogenesis process, for instance chronic urinary tract infections (Getliffe and Newton, 2006), cyclophosphamide use (Fernandes et al., 1996; Talar-Williams et al., 1996) and exposure to radiation therapy (Anderson et al., 2009; Pavlidakey and MacLennan, 2009), as well as inadequate consumption of fruits, vegetables and certain vitamins may be associated with higher risk of bladder cancer. Even coffee

consumption and artificial sweeteners are suggested to have an impact on tumourigenesis. However, hereditarily caused bladder cancer is fairly rare compared with other tumour sites (Colombel et al., 2008).

1.2.3 Clinical features

Haematuria (the passage of blood in the urine) is the fundamental symptom of bladder cancer. Therefore, microscopic haematuria requires investigation, especially in those aged over 40. Around 15% of patients will present with lower urinary tract symptoms of frequency, dysuria (pain on urinating) and urgency. This may be associated with or without haematuria (Phillips, 2002; Sengupta et al., 2004). A small number of patients present with uraemia (presence of excessive amounts of urea in the blood) due to bilateral ureteric obstruction or the symptoms of metastatic disease (Phillips, 2002).

1.2.4 Diagnosis

Cystoscopy is the primary tool in the investigation of bladder tumours. Most urologists employ a flexible cystoscope to examine the bladder following administration of local anaesthetic. If a bladder tumour is visualised, the patient is brought back for a transurethral resection of the bladder tumour under a general anaesthetic (Sengupta et al., 2004). Transitional cell carcinoma (TCC) or NMIBC is a pan-urothelial disease and may be multifocal. It is therefore mandatory to visualize the upper urinary tract, usually by intravenous urography (IVU) or occasionally retrograde pyelography, as there may be a synchronous tumour in the ureter or renal pelvis (Phillips, 2002). The other investigations for haematuria include a urine culture (for bacteria) and cytology (to look for malignant cells) (Sengupta et al., 2004). However,

whilst useful, urine cytology has a limited role in diagnosis. In grade I tumours, cytology is positive in only approximately 25% of cases but this rises to 75 – 80% with poorly differentiated disease or CIS (Phillips, 2002).

1.2.5 Bladder cancer staging

In general, bladder cancer is a rather heterogeneous disease, making classification, staging and grading a challenging task. The TNM system is the universally accepted method of classifying bladder tumours (the initial 'T' stands for tumour, 'N' for nodes and 'M' for metastases). This is based on the depth of invasion through the bladder wall and the presence or absence of pelvic lymph node involvement and distant metastasis. A tumour that is limited to the mucosa and lays flat (known as *carcinoma in situ*) and a tumour that is papillary and limited to the mucosa is pTa whilst a tumour that penetrates the lamina propria but not the muscle layer is pT1; both pTa and pT1 represent NMIBC. Stage T2 and above invade the muscle layer and are highly malignant with a strong potential to metastasise, preferentially to regional lymph nodes, lungs, liver and bone (**Figure 1.5**). Muscle-invasive tumours are staged clinically with a combination of bimanual examination under anaesthetic and imaging with computed tomography, chest radiograph and isotope bone scans (Phillips, 2002; Sengupta et al., 2004).

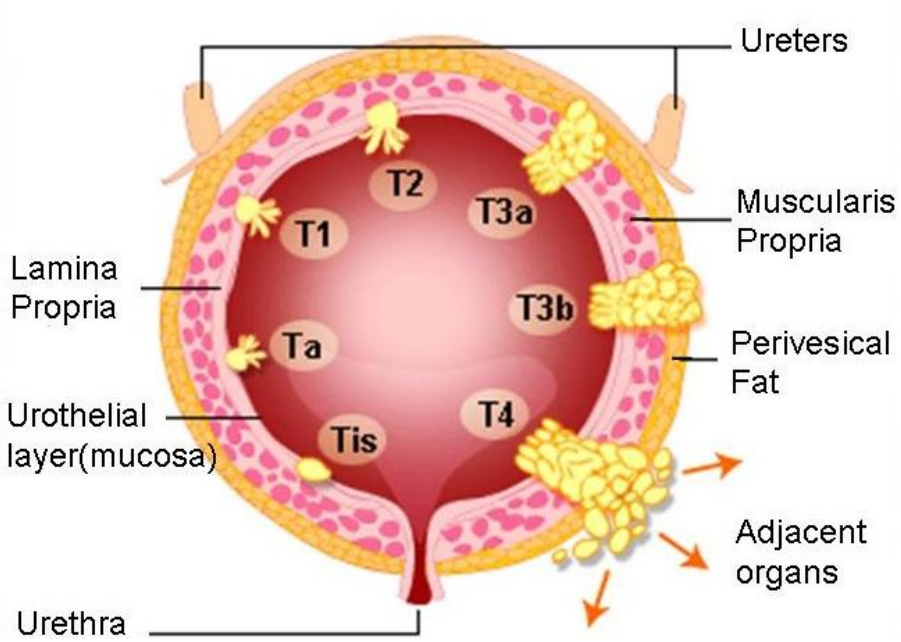


Figure 1.5: Grade and stage of bladder cancer based on TNM system (adapted from http://www.drugs-expert.com/health-conditions/cancer/bladder-cancer/)

On average, 70% of bladder urothelial cell carcinomas represent as superficial disease termed NMIBC, while the remaining 30% develop a muscle-invasive disease (muscle-invasive bladder cancer [MIBC]) bearing the risk of metastatic spread of the tumour (Kirkali et al., 2005).

1.2.6 Treatment of bladder cancer

Standard treatment of NMIBC is complete transurethral resection and subsequent cystoscopic surveillance. However, the major challenge in the management of NMIBC is not the removal of a lesion, but rather the prevention of tumour recurrence and progression. Thus, intravesical instillation of chemotherapeutic or immunotherapeutic agents for instance mitomycin C or live attenuated Bacille Calmette-Guerin (BCG) is applied to reduce the risk for recurrence and to delay or even prevent progression to a muscle-invasive disease (Phillips, 2002).

A tumour that has grown beyond the lamina propria and invades the muscularis propria is usually treated with radical cystectomy or radical radiotheraphy taking into account the performance status of the patient (Phillips, 2002). Based on the appropriate staging and planning investigation, MIBC can be treated with a course of radiotherapy (typically 64 Gy in 32 fractions). Radiotherapy has the advantage of bladder preservation, but may be associated with frequency and urgency along with future bladder contracture. Fifty per cent of patients will exhibit complete response to radiotherapy (Phillips, 2002).

Radical surgery in the case of MIBC therapy is usually accompanied by perioperative systemic chemotherapy to minimise metastatic dissemination and improve survival of patients. For patients with disseminated disease, aggressive systemic therapy with multiple chemotherapeutics is essential (Saxman et al., 1997).

1.2.7 Problems and barriers in bladder cancer research

Even though bladder cancer is ranked as the ninth common cancer worldwide, seventh most common cancer in the UK and fifth common cancer in the USA (CRUK, 2010; IARC, 2004; Lotan et al., 2009), on a per capita basis it is the most expensive cancer from the time of diagnosis to death when compared to the other leading malignancies the UK and USA (Lotan et al., 2009; Mostafid et al., 2009; Sangar et al., 2005; Sievert et al., 2009). Direct costs for bladder cancer treatment in the USA account for almost 3.7 billion US dollars (2001 values) (Botteman et al., 2003). In the UK, based on bladder cancer patients treated in 2001 – 2002 inclusive of 5 years follow up studies, the National Health Service (NHS) spends approximately £55.39 million to manage bladder cancer patients, of which NMIBC alone costs £35.25 million (Sangar et al., 2005).

Most patients (~70%) presenting with NMIBC have a long survival but, suffer from a high rate of tumour recurrence. Management of this population is costly because of the extended surveillance and repeated use of endoscopic and intravesical therapies. Patients with muscle-invasive and metastatic disease have a much more precarious survival outcome and also contribute greatly to the cost of bladder cancer care because of the expense of radical treatments (Lotan et al., 2009; Mostafid et al., 2009; Sangar et al., 2005; Sievert et al., 2009).

In addition, there is less attention given on the basic research of bladder cancer. Despite advances in the molecular characterization of urothelial tumours, there are relatively few detailed mechanistic studies investigating chromosomal regions and related function in urothelial carcinoma. A lack of relevant models has also hampered progress. Most of the available urothelial cancer-derived cell lines were created in the 1970s, and animal models have relied on dog and rodent carcinogen models with acknowledged caveats (Crallan et al., 2006). The uroplakin II transgenic mouse (Zhang et al., 1999) and normal human urothelium cultures (Southgate J, 2002) are powerful tools, but are greatly underused in testing novel therapeutic concepts. Even the NCI-60 panel of cancer cell lines, often used in experiments to represent the most common tumour types, does not include bladder cancer cell lines (Lotan et al., 2009).

In spite of the considerable financial resources spent on the management of bladder cancer there is another issue related to the efficacy of treatment that still needs to be addressed. Over the past two decades, despite the reported advances in the treatment of bladder cancer, approximately 50 – 70% of the NMIBC treated recur within 5 years of resection (CRUK, 2010; Lutzeyer et al., 1982). Furthermore, the five year survival rate for bladder cancer is approximately 60 per cent and has not changed for the past two decades (Deweerd et al., 1982; Mostafid et al., 2009). Over the same

period the survival rates of other urological cancer have improved considerably (Mostafid et al., 2009). Consequently, alternative strategies are clearly needed to have a significant impact on bladder malignancies. Cancer chemoprevention, the intervention with agents to prevent or retard cancer development, represents a relatively new approach with potential to reduce the occurrence/recurrence of bladder cancer.

1.3 Cancer Chemoprevention

Cancer chemoprevention, as first defined by Sporn in 1976, as the use of natural or synthetic agents to reverse, suppress, or prevent carcinogenic progression (Sporn, 1976). It is based on the concepts of multifocal field carcinogenesis and multistep carcinogenesis. In carcinogenesis, diffuse epithelial injury in tissues, such as the aerodigestive tract, results from generalized carcinogen exposure throughout the field and clonal proliferation of mutated cells (Tsao et al., 2004). Genetic changes exist throughout the field and increase the likelihood that one or more premalignant and malignant lesions may develop within that field. Multistep carcinogenesis describes a stepwise accumulation of alterations, both genotypic and phenotypic (O Shaughnessy et al., 2002). For instance, epithelial carcinogenesis is a multistep process in which an accumulation of genetic events within a single cell line leads to a progressively dysplastic cellular appearance, deregulated cell growth and finally carcinoma (O Shaughnessy et al., 2002).

Many types of cancer are caused, in part, by the exposure of cellular macromolecules to specific ROS produced during normal cellular metabolism (Weisburger, 1999). ROS produced by other means, including the metabolism of environmental toxins and carcinogens, by ionizing radiation and by phagocytic cells involved in the inflammatory response, also contribute to these diseases. There are five

major recognized types of ROS; i.e. singlet oxygen ($^1O_2$), superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and nitric oxide ($NO^{\bullet}$). Although some types are more injurious to cells than others, there is no question that, collectively, these ROS cause significant oxidative damage to cellular DNA, proteins and lipids leading to the development of chronic diseases including cancer (Ames and Gold, 1991).

1.3.1 Dietary and cancer prevention

Epidemiological studies indicate that persons who consume a diet rich in fruits and vegetables have a reduced risk of cancer at multiple sites (Block et al., 1992; IARC, 2003; McMichael, 2008; Steinmetz and Potter, 1991; Willett, 1995). Since fruits and vegetables are major sources of antioxidants (among other factors), it is hypothesized that these antioxidants are partly responsible for their cancer preventive effects. This hypothesis is supported by both epidemiological data, implicating micronutrient antioxidants in reduction of cancer risk and by experimental data *in vitro* and *in vivo* (Ames and Gold, 1991; Chu et al., 2002; Eberhardt et al., 2000; Sun et al., 2002). The antioxidant effects of fruits and vegetables are due, in a significant part, to their content of flavonoids and phenolic acids (Kahkonen et al., 2001; Stoner, 2004). Some of the extensively investigated polyphenolic compounds, with demonstrated cancer-preventative effects, such as, the catechins in green tea, theaflavins in black tea, curcumin in turmeric, resveratrol in red wine, quercetin in apples and the anthocyanins in berries, are antioxidants that exhibit significant radical scavenging activity (Kahkonen et al., 2001; Leonard et al., 2003; Rietveld and Wiseman, 2003; Wang and Jiao, 2000) .

Anthocyanins are a class of flavonoid compounds responsible for the bright attractive red, orange, purple, and blue colours of most fruits and vegetables. Interest in

anthocyanins as natural colorants and value-added ingredients has increased due to their colour characteristics and potential health benefits. Anthocyanins are the most abundant dietary flavonoids. Anthocyanin consumption has been estimated to be as high as ~200 mg/day/ person (Kühnau, 1976), although a more recent study (Wu et al., 2006) reported anthocyanin consumption at about 12.5 mg/day/person in the United States, compared to the average daily intake of other flavonoids (23 mg/person) (Hertog et al., 1993). More recently, Justesen and co-workers, estimated intake of flavonols, flavones, and flavanones (additional classes of flavonoids that differ in the oxidation state from the anthocyanins) in Denmark to be 25 mg/day (Justesen, 2000), which is similar to that reported by Hertog and co-workers.

Many fruits and vegetables are rich in anthocyanins, including berries, purple carrot, purple corn, red radish, red cabbage, and others. Their anthocyanin profiles may vary between types of fruits or vegetables, with differences in the type of aglycone, type and number of glycosylations, and presence of acylating groups (Jing et al., 2008).

Berries are among the most widely consumed fruits in the human diet. They are richly abundant in antioxidant flavonoids and phenolic acids, as well as antioxidant vitamins, carotenoids, tannins, lignans and stilbenes (Seeram, 2006). Commercially the most important berries include members of the genus *Vaccinium* (blueberry, lingonberry, cranberry, and bilberry), *Rubus* (blackberry, black raspberry, red raspberry, arctic raspberry/bramble, and cloudberry), *Fragaria* (strawberry) and *Sambucus* (elderberry and red elderberry) (Stoner, 2008).

Berries contain a wide range of bioactive phytochemicals, the most prominent of which are phenolic in nature; i.e. they have hydroxyl (OH) groups on aromatic rings. Phytochemicals are categorized as secondary metabolites that are of non-nutritive constituents produced in plants. They are known to defend plants against predators,

microbial infections and ultraviolet light, to regulate metabolic pathways and provide colour and flavour to the plant (Stoner, 2008). The bioactive phytochemicals in berries fall into several structural and chemical classes including phenolic acids (hydroxycinnamic and hydroxybenzoic acids), flavonoids (anthocyanins, flavanols, flavonols), condensed tannins (proanthocyanins), hydrolyzable tannins (ellagitannins and gallotannins), stilbenoids, lignans, triterpenes and sterols (Seeram, 2006). The structures of these berry bioactives are shown in **Figure 1.6**, and their chemical features, biological activities and content in different berry types have been described in detail by Seeram (Seeram, 2006). Among the most prevalent compounds in berries are the anthocyanins and ellagitannins that, collectively, are responsible for much of their antioxidant activity (Aaby et al., 2005; Cerda et al., 2005; Connor et al., 2002; Wada and Ou, 2002). In addition to the bioactives listed in **Figure 1.6**, berries contain many other constituents known to exhibit cancer-preventive effects including vitamins, such as A, C, E and folic acid, and minerals, such as calcium, selenium and zinc (Kresty et al., 2001). The inhibitory effects of most of these constituents are due, at least in part, to their antioxidant capacity (Stoner et al., 2008).

Berry bioactives may have many roles in cancer prevention. These include protection against oxidatively damaged DNA by the scavenging of ROS, chelation of free cellular metal ion, inhibition of the formation of carcinogen-induced DNA adducts, enhancement of DNA repair, inhibition of carcinogen-induced tumourigenesis in animals and modulation of signalling pathways involved with cellular proliferation, apoptosis, inflammation, angiogenesis and cell cycle arrest. The prevention of ROS-induced DNA damage is probably a first line of defence against the multistage process of carcinogenesis. Many studies with berry-based antioxidants have demonstrated their protective effects against oxidative DNA damage in human lymphocytes (Duthie, 2007;

Wilms et al., 2005). For example, pre-treatment of freshly isolated human lymphocytes from non-smokers with quercetin (1–100 µM) for 1 hour was shown to protect against oxidative DNA damage induced by H_2O_2 as measured by the Comet assay (Wilms et al., 2005).

Similar findings were reported by Duthie and co-workers who treated freshly isolated human lymphocytes with quercetin and myricetin. Inhibition of H_2O_2-induced DNA damage was achieved by quercetin at concentrations ranging from 10 to 50 µM and by myricetin at concentrations of ≥100 µM (Duthie and Collins, 1997). In one animal study, the administration of 2.5, 5 and 10% lyophilized black raspberry (LBR) diets, which are abundant in anthocyanins and other antioxidants, to rats treated with the colon carcinogen, azoxymethane (AOM), led to 73, 81 and 83% reductions, respectively, in the levels of urinary 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG) (Harris et al., 2001). These results suggest that the berries markedly reduced oxidatively damaged DNA in AOM-exposed rats.

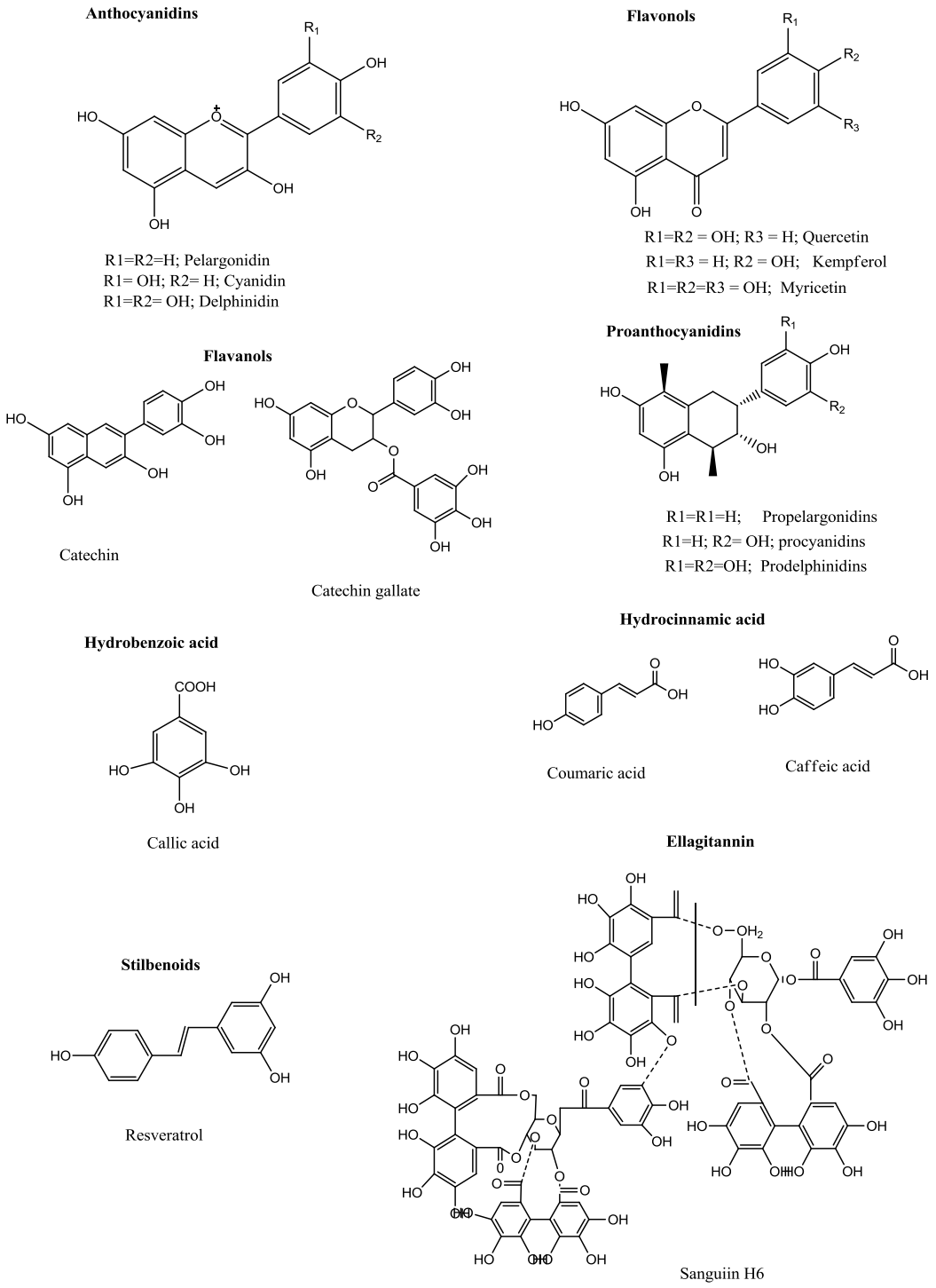


Figure 1.6: Structures of bioactives found in berries: anthocyanins, flavonol, flavanols, proanthocyanidins, hydroxycinnamic and hydroxybenzoic acids, stilbenoids and ellagitannins (adapted from Stoner et al., 2008)

1.3.2 Bladder cancer chemoprevention clinical trials

Bladder cancer chemoprevention trials have often focused on nutritional supplementation (**Table 1.1**) (Byar and Blackard, 1977; Lamm et al., 1994; Newling et al., 1995; Prout and Barton, 1992; Sabichi et al., 2008; Shibata et al., 1992; Studer et al., 1995). In a primary prevention study, Shibata and co-workers followed 11,580 retirement community residents who were cancer-free at enrolment. At eight years, an inverse relationship between vitamin C supplement use and bladder cancer risk was seen (Shibata et al., 1992). However, studies on retinoid and vitamin B6 trials have been conflicting (**Table 1.1**). There was no benefit in using 13-cis retinoic acid (13cRA) in patients with rapidly recurring bladder cancer, whilst other studies using etretinate, a synthetic retinoid, demonstrated decreased recurrence rates and a prolonged mean time interval to tumour recurrence in superficial papillary bladder tumours (Alfthan et al., 1983; Studer et al., 1995; Yoshida et al., 1986). Another study showed that *N*-(4-hydroxyphenyl) retinamide (4-HPR) can reverse abnormal cytology in patients (Decensi et al., 1992). In an early randomized study (Byar and Blackard, 1977), vitamin B6 was reported to decrease tumour recurrence rates in patients with Stage I bladder cancer; however, later trials showed no benefit (Newling et al., 1995). A study on fenretinide (a synthetic retinoid) against superficial bladder cancer failed to exhibit a beneficial effect against the recurrence of NMIBC (Decensi et al., 2000; Sabichi et al., 2008).

Combinations of high doses of vitamins (higher than Recommended Dietary Allowances) were reported to have a beneficial effect on preventing superficial and low-grade bladder tumour recurrence (Lamm et al., 1994). Sixty-five patients with prior bladder cancer were randomized to the recommended daily allowance of multiple vitamins or to the recommended daily allowance plus 40,000 IU of vitamin A, 100 mg of vitamin B6, 2,000 mg of vitamin C, and 400 IU of vitamin E (Lamm et al., 1994).

Although the first 10 months showed no difference in time to recurrence, the five-year estimates favoured the megavitamin arm which contributed towards low recurrence rates compared to the control group (40% versus 80%) (Tsao et al., 2004).

In summary, bladder cancer chemoprevention trials have demonstrated conflicting outcomes. Therefore, it is recommended that, those interventions producing positive trial result should be further corroborated prior to being accepted in the conventional management of bladder cancer (Tsao et al., 2004). On-going trials using targeted agents, for instance oltipraz (4-methyl-5-[2-pyrazinyl]-1,2-dithiole-3-thione), 4-HPR and celecoxib, are also underway (Tsao et al., 2004).

Table 1.1 Selected Bladder Cancer Chemoprevention Trials (Tsao et al., 2004**)**

Trial	Year	Patients (n)	Prevention	Population	Endpoint	Compounds	End Result
Shibata *et al*.	1992	11,580	Primary	Healthy elderly	Bladder cancer	Vitamin C (dietary)	Positive
National Bladder Cancer Collaborative Group	1992	20	Tertiary	Prior T_{a-1} superficial bladder cancer	Bladder cancer	13cRA (0.5 to 1 mg/kg)	Negative
Studer *et al*.	1995	90	Tertiary	Prior T_{a-1} superficial bladder cancer	Bladder cancer	Etretinate (25 mg)	Positive
Byar *et al*.	1977	121	Tertiary	Prior Stage I bladder cancer	Bladder cancer	Pyridoxine (25 mg)	Negative
EORTC Genito-Urinary Cooperative Group	1995	291	Tertiary	Prior T_{a-1} superficial bladder cancer	Bladder cancer	Pyridoxine (20 mg)	Negative
Lamm *et al.*	1994	65	Tertiary	TCC bladder cancer Receiving I-BCG	Bladder cancer	Vitamin A (40,000 IU) Vitamin B_6 (100 mg) Vitamin C (2000 mg) Vitamin E (400 U) Zinc (90 mg)	Positive
Decensi *et al*	2000	99	Tertiary	Prior T_{a-1} superficial bladder cancer	Bladder cancer	Fenretinide	Negative
Sabichi *et al*	2008	149	Tertiary	Prior T_{a-1} & TIS superficial bladder cancer	Bladder cancer	Fenretinide	Negative
Sabichi *et al*	On going	152	Tertiary	superficial bladder cancer	Bladder cancer	Celecoxib	?

Oltipraz (4-methyl-5-[2-pyrazinyl]-1,2-dithiole-3-thione), a compound that is able to prevent carcinogenesis by enhancing detoxification of [*N*-nitrosobutyl(4-hydroxybutyl)amine] (a bladder carcinogen) in the liver and urinary bladder (Iida *et al*., 2004), is currently at the Phase I clinical trial stage at the Robert H Lurie Comprehensive Cancer Centre, Northwestern University, Chicago Illnois and Duke Comprehensive Cancer Centre, Durham, North Carolina, United State (http://clinicaltrials.gov/ct2/show/NCT00006457). University of Texas MD Anderson has two National Cancer Institute (NCI)-sponsored Phase III trials using 4-HPR and Celecoxib (see http://www.cancer.gov/clinicaltrials/MDA-ID-99368) (Grossman, 2006; Tsao et al., 2004). The 4-HPR trial is designed for early-stage superficial bladder cancer patients, and the celecoxib trial is geared for more advanced stage patients receiving bacillus Calmette-Guérin adjuvant therapy. 4-HPR is a chemopreventive agent suggested to restrain cancer cell growth via apoptosis. Celecoxib has shown promise in animal studies for the prevention of many cancers (e.g., bladder, colorectal, esophageal, skin, breast, and prostate). It has also been proven to reduce colorectal polyps in patients with familial adenomatous polyposis (FAP), a condition that leads to the development of colorectal cancer. This trial will study the effectiveness of celecoxib in preventing the recurrence of superficial bladder cancer, which is characterized by high levels of the COX-2 enzyme (Gee et al., 2002; Grossman, 2006; La Rochelle et al., 2008).

1.3.2.1 Cancer biomarkers

Arresting one or several of the steps of carcinogenesis may impede or delay the development of cancer. This has been described particularly well in studies involving precancerous and cancerous lesions of the head and neck, which focus on oral

premalignant lesions (leukoplakia and erythroplakia) and their associated increased risk of progression to cancer. If the disappearance of lesions related to the specific cancer can be correlated with a reduction in cancer incidence and then markers of premalignancy may serve as intermediate endpoints for chemoprevention trials (Tsao et al., 2004). In addition to histologic assessment, intermediate markers of response are needed to assess the validity of these therapies in a timely and cost-efficient manner (Tsao et al., 2004).

Development of intermediate markers for chemoprevention trials is crucial. Improvements (that is, deceases) in cancer incidence among populations receiving a chemopreventive intervention may require years to evaluate. Monitoring intermediate markers that correlate with a reduction in cancer incidence would allow a more expeditious evaluation of potentially active chemopreventive agents (Tsao et al., 2004). It has been shown that assessment of altered biomarkers (p53, pRB, p21, p27 and cyclen E) in cystectomy samples improves the prediction of bladder cancer recurrence and survival of bladder cancer patients (Shariat et al., 2008). Recently, a protein identified as cystatin B present in urine and related tissues has been shown to positively correlated with tumour grade, stage and shorter time to disease recurrence and progression could be a useful predictive biomarker of NIMBC (Feldman et al., 2009). There is also an effort to utilise microarray technologies to reveal gene expression signatures as a predictive measures for the bladder cancer progression and recurrence (Kim et al., 2007).

1.3.3 Future directions for bladder cancer chemoprevention

The future of cancer chemoprevention remains open to innovation, with a specific need for emphasizing cancer prevention in public health policy. The design and

conduct of investigational studies for the prevention and management of bladder cancer should involve the rational integration of several key factors including: (a) *agents* (pharmaceuticals, biologics and natural products); (b) *biomarkers* (intermediate endpoints that reflect biologic activity and cancer risk reduction); (c) *cohorts* (well defined individuals with high risk for recurrence and progression) and (d) *designs* (efficient randomized controlled trials linked to guided drug development) and (e) *endpoints* (clinically meaningful outcomes) such as reduction of cancer recurrence and progression (Leppert et al., 2006; Lieberman, 2001).

Pre-clinical and limited clinical data demonstrate that bladder cancer is responsive to primary and secondary prevention efforts. Primary prevention is referring to preventing the risk factor, for instance quit smoking to avoid smoking related diseases, whilst secondary prevention is an action to prevent exacerbation of known disease by recomanding specific treatment/managment. Furthermore, epidemiologic studies imply that natural products, such as vitamins and herbal compounds, may provide preventive benefit. Bladder cancer is an attractive target for prevention efforts because renal excretion of agents enables prolonged exposure to high concentrations of drugs in the urine. Additionally, the urothelium is easily monitored using highly successful and relatively noninvasive methods. The frequent recurrence of NMIBC and low rate of progression to MIBC disease permits the safe and efficient assessment of strategies to prevent secondary bladder cancers with a modest number of patients and short follow-up (Leppert et al., 2006). Although improved understanding of the pathways of bladder carcinogenesis presents new prevention targets, the lack of accurate models of patient risk assessment (that integrate data on both genetic susceptibility and precise exposure metrics), novel interventions, and surrogate endpoint biomarkers limit the ability to implement prevention studies. The development

of validated surrogate endpoints will facilitate the future evaluation of novel chemopreventive agents and strategies (Leppert et al., 2006).

Therefore, this study to assess naturally occurring phytochemicals to prevent oxidatively damaged DNA formation in selected bladder cancer cell lines may be able to contribute valuable scientific evidence pertinent to the therapeutic properties of the anthocyanins in the prevention of bladder cancer in the future.

1.4 Aims and objectives of this project

The aim of this study is to investigate the ability of standardised anthocyanins extracts to reduce global and specific types of DNA damage in the human bladder cancer cell lines (RT112, RT4 and HT1376) in particular, to determine if a correlation could be established between the mirtoselect pre-treated bladder cancer cell lines and a reduced global oxidatively damaged DNA burden and/or a specific oxidative purine damaged, at either and endogenous level or when exposed to exogenous source of hydrogen peroxide.

To start this project, it is important to determine the optimal concentration of the mirtoselect that is going to be utilised for the whole duration of this study. Establishment of mirtoselect optimal concentration for RT112, RT4 and HT1376 treatment was carried out CyQUANT cell proliferation assay kit. It is paramount to ensure that the mirtoselect concentration used in this study should not kill majority of the bladder cancer cells treated but adequate to provide protection against oxidising agent in the subsequent experiment (**Chapter 2**).

The main technical component of this study focussed on the establishing of a modified version of the available standard alkaline Comet assay to specifically assess oxidative DNA damage and subsequently this technology was applied to assess the effect of anthocyanins against the selected bladder cancer cell lines.

The ability to measure the amount of oxidatively damage DNA formed in each bladder cancer cell line was achieved through the establishment of "modified" alkaline Comet assay protocols with the inclusion of the DNA glycosylase enzymes; formamidopyrimidine DNA glycosylase (Fpg) and human 8-oxoguanine DNA glycosylase (hOGG1). The utilisation of this modified version of alkaline Comet assay increased the specificity of the Comet assay via its ability to recognise specific damaged

purines and 8-oxoGua damage and then cleaving the oxidatively damage DNA to creating new strand breaks that can be measured with the existing Comet assay protocol and software (**Chapter 3**).

Subsequently, the standard and modified alkaline Comet assay protocol was used to investigate the anthocyanins effects on the endogenous and induced DNA damage (**Chapter 4**). The mechanism of action of the protection effect exerted by the mirtoselect was established by comparing the effect of mirtselect on H_2O_2-induced versus X-ray induced DNA damage (**Chapter 5**) and the mechanism observed was further established and verified via the LIP measurement. This study was carried out utilising fluorescent probe, Phen Green SK. In general this method is based on quenching of the fluorescence of the transition metal indicator PG SK by intracellular iron and its subsequent dequenching on addition of a strong membrane-permeable iron chelator. The increase in fluorescence produced by the chelator is considered proportional to the size of the intracellular redox active iron available in the cells (**Chapter 6**).

At the end of this study, parameters and data generated has been successfully applied to develop modified alkaline Comet assay protocol and subsequently utilised to determine the protective effect in mirtoselect pre-treated bladder cancer cell line. The mechanism of mirtoselect protection was revealed and established in the selected bladder cancer cell line. Observation recorded throughout this study was further discussed and future plan of action was suggested for the benefit of future works (**Chapter 7**).

Chapter 2.

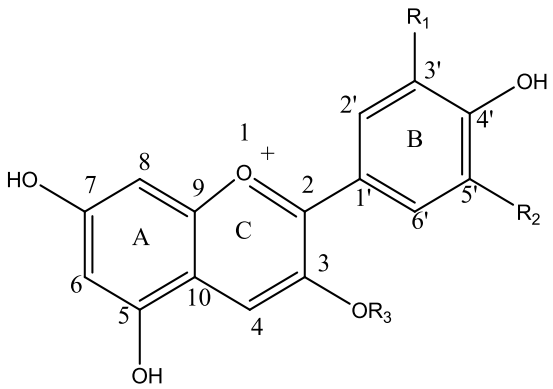
The effect of mirtoselect on the proliferation of bladder cancer cell lines *in vitro*.

2.1 Introduction

2.1.1 Plant phenolic compounds (anthocyanins)

Flavonoids are one of the major phenolic compounds found in plants (Harborne, 1986; Havsteen, 2002). One of the important classes of compounds in the flavonoid group is the anthocyanins. Anthocyanins, also known as the glycosides of anthocyanidins, give the red or blue pigment to flowers, fruits and berries. Recently, more than 400 different types of anthocyanins have been identified (Kong et al., 2003). The basic core structure of anthocyanins is based on the flavylium ion. The number of hydroxyl groups, the types of sugars attached, the position of attachment and the types of organic compounds determines the different types of anthocyanin compound found in nature. Six major anthocyanidin compounds are distributed ubiquitously in plant species; cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin, with cyanidin, delphinidin and pelargonidin glycosides being the most abundant anthocyanins found in nature (**Figure 2.1**) (Kong et al., 2003).

Anthocyanins are recognised as very potent antioxidant compounds (Kahkonen and Heinonen, 2003a; Tsai et al., 2002). Available experimental data, from anthocyanin studies both *in vivo* and *in vitro*, support the antioxidant properties claimed with respect to the benefits of anthocyanins against human diseases that are closely related to oxidative stress, for instance, coronary heart disease and cancer (Duthie and Crozier, 2000; Spormann et al., 2008; Wang and Stoner, 2008).



Cyanidin $R_1 = OH, R_2 = H$

Delphinidin $R_1 = OH, R_2 = OH$

Petunidin $R_1 = OCH_3, R_2 = OH$

Peonidin $R_1 = OCH_3, R_2 = H$

Malvidin $R_1 = OCH_3, R_2 = OCH_3$

R_3 = Glucose or galactose or arabinose

Figure 2.1: Structure formulas of common anthocyanins.

Furthermore, anthocyanins have been shown to be absorbed into the plasma in their intact glycate form (Cao et al., 2001; Noda et al., 2000) and are incorporated into the membrane and cytosol of *in vitro* cell culture systems (Youdim *et al*., 2000). Previously, anthocyanins which exist as glycosides compound has been long considered as nonabsorbable in human without prior hydrolysis by microflora in the guts. Therefore this finding has enable the absorption and bioavailability of the anthocyanins to be studied in human (Cao et al., 2001) Intact anthocyanins as well as glucoronide metabolites are also detected in the urine of animals used in *in vivo* studies administered with cyanidin-3-glucoside and mirtoselect (Cooke et al., 2006). A study on the antioxidant activity of cyanidin-3-rutinoside and delphidin-3-rutinoside has shown that delphinidin-3-rutinoside had a higher antioxidant activity against Fe(II)-induced liposome oxidation than cyanidin-3-rutinoside. This result indicates an important role of the OH group in the B ring (R_2C5') of delphinidin-3-rutinoside in its antioxidant action (Gabrielska and Oszmianski, 2005). Direct biochemical analysis of the antioxidant activity of anthocyanins, for instance via the trolox equivalent antioxidant activity (TEAC) assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay, have shown that anthocyanidins possess stronger antioxidants activities than anthocyanins (Kahkonen and Heinonen, 2003a; RiceEvans et al., 1997). The electron deficiency of

the flavylium cation makes the anthocyanidins highly reactive (Mazza and Miniati, 1993).

Anthocyanins are also reported to have anti-proliferative activity against selected cancer cell lines *in vitro*. Approximately 50% of HT29 cell growth was inhibited after 48 hours exposure to choke berry anthocyanin rich extracts (AREs) (Zhao et al., 2004). Açai (*Euterpe oleracea*), a native palm plant in northern South America, has recently emerged as a promising source of natural antioxidants. Anthocyanins extracted from this plant have been shown to suppress the proliferation of C-6 rat brain glioma cells, but has no effect on the growth of MDA-468 human breast cancer cells (Hogan et al., 2010). Lazze and co-workers have suggested that different effects exerted by delphinidin, as compared with cyanidin, against uterine carcinoma and colon adenocarcinoma cells and against normal human fibroblasts suggest that the presence of the three hydroxyl groups on the B ring in the molecular structure of delphinidin may be important for its greater biological activity (Lazze et al., 2004).

There are numerous commercial anthocyanin products on the market sold as food supplements. One of these is mirtoselect which is produced by Indena S.p.A in Italy. To date, and to the best of my knowledge, there has been no extensive/comprehensive study carried out on the effects or mechanism of anthocyanins against bladder cancer cell lines *in vitro* as an indicator of the appropriateness of anthocyanins as a cancer chemopreventive agent for this cancer type. These anthocyanins 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.

2.1.1.1 Mirtoselect

Mirtoselect is a standardised bilberry fruits (*Vaccinium myrtillus* L) extract containing 36% anthocyanins commercially produced by Indena S.p.A, Italy. This product contains at least 15 major anthocyanin compounds and has been extensively characterized by HPLC profiling (see http://www.mirtoselect.info/public/overview.asp). Predominant anthocyanin constituents are delphinidin-3-galactoside, delphinidin-3-glucoside, and delphinidin-3-arabinoside, and cyanidin-3-galactoside and cyanidin-3-glucoside. Other anthocyanins in mirtoselect are cyanidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-glucoside, petunidin-3-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside, malvidin-3-galactoside, malvidin-3-glucoside and malvidin-3-arabinoside. Mirtoselect also contains other polyphenols (phenolic acids, flavonols, proanthocyanidins; ~18%), carbohydrates and aliphatic organic alcohols (~29%), fats (~0.04%), nitrogen compounds (~1%), ash (~0.7%), with the remaining 15% undefined (Indena data sheet).

This standardised anthocyanin extract has been rigorously tested for its biological activities such as anti-inflammatory, vasoactive, hypolipemic, hypoglycaemic, cell-regenerating, antimicrobial and cancer chemopreventive (see http://www.indena.com/pdf/mirtoselect.pdf). Some of the stated pharmacological properties exhibited by anthocyanins are strongly related to the potent antioxidant properties of its constituents (Bao et al., 2008a; Bao et al., 2008b; Bell and Gochenaur, 2006; Yao et al., 2010).

The current study of the chemoprevention properties of mirtoselect towards bladder cancer follows on from the interesting findings of other researchers within this group concerning mirtoselect's beneficial properties. In a study by Cooke and co-workers, 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 (45% and 30%) respectively and, 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) (Cooke et al., 2006). Recently a small human intervention study involving colorectal cancer patients displayed similar pharmacodynamic effects for mirtoselect as was observed in the Apc^{Min} mice studied by Cooke and co-workers (Thomasset et al., 2009). Twenty-five patients with either primary colorectal cancer or colorectal liver metastases received 1.4, 2.8 or 5.6 g of mirtoselect (containing 0.5-2.0 g anthocyanins) daily for 7 days prior to colon/liver resection. Anthocyanin levels were measured by high performance liquid chromatography. Consumption of up to 5.6 g of mirtoselect daily was well tolerated. In the colorectal tumours of all patients who received mirtoselect, the proliferation index reflected by Ki-67 staining was significantly decreased by 7% compared with the pre-intervention value measured in biopsy samples. There was also a decrease in tumour tissue proliferation in patients taking 1.4 grams mirtoselect. These findings demonstrated pharmacodynamic effects commensurate with colorectal cancer chemoprevention (Thomasset et al., 2009).

2.1.2 RT4, RT112 and HT1376 Cell Lines

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 and co-workers. 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 (Crallan et al., 2006; 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.

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).

2.1.3 Determination of cell number.

There are several methods available that can be used to determine bladder cancer cell number after treatment with mirtoselect. The easiest, but most time consuming, is by direct counting of cell numbers using a haematocytometer. The technique is simple but is not suitable for high through put screening (Papadimitriou and Lelkes, 1993). Measurement of cellular metabolic activity, which is considered proportional to cell number, is also popular and widely used by many researchers. For

instance, the MTT assay is a standard colorometric assay (an assay which measures changes in colour) for measuring the activity of enzymes that reduce yellow MTT to formazan, giving a purple colour, and can be used to measure cytotoxicity, proliferation or activation of cells (Mosmann, 1983). Cell numbers can also be determined based on quantification of cytosolic acid phosphatase activity. This assay is based on the hydrolysis of the p-nitrophenyl phosphate by intracellular acid phosphatases in viable cells to produce p-nitrophenol. The absorbance of p-nitrophenol at 405 nm is directly proportional to the cell number in the range of 10^3 to 10^5 cells (Yang et al., 1996). However there are a few shortcomings of these assays that will be further discussed in the **Discussion section** (**Section 2.4**).

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).

2.2 Materials and Methods

2.2.1 Chemicals and Media

Eagle's Minimum essential medium (EMEM), Dubelco minimum Eagle's medium, McCoy's 5A growth medium, non-essential amino acid (100 x), phosphate buffer saline (PBS), glutamine and trypsin were all purchased from Sigma (Poole, UK), unless otherwise stated. Foetal calf serum (FCS) and CyQUANT® NF Cell Proliferation Assay Kits were purchased from Invitrogen (Paisley, UK). Mirtoselect was obtained from Indena S.p.A. (Milan, Italy). 75 cm^2 culture flasks were purchased from Techno Plastic Product (TPP) (Trasadingen, Switzerland).

2.2.2 Cells and culture conditions

Human bladder carcinoma RT112 cells (ECACC no: 85061106) were grown in EMEM supplemented with 1% non-essential amino acid and 10% FCS. Human Caucasian bladder transitional-cell carcinoma RT4 cells (ATCC no: HTB-2) were grown in McCoy's 5A supplemented with 2mM glutamine and 10% FCS. Human bladder carcinoma HT1376 cells (ATCC no: CRL-1472) were grown in DMEM, supplemented with 10% FCS. All cell lines were cultured and maintained in 75 cm^2 cell culture flasks in humidified 5% CO_2 at 37 ºC.

2.2.3 Establishment of standard curve for cell number assessment

Prior to the cell number assessment, standard curves of each cell line were prepared according to the protocols provided by the CyQUANT® NF Cell Proliferation

Assay Kit's manufacturer, Invitrogen. Briefly, the bladder cancer cells were seeded at densities of 0 to 50,000 cells in triplicate and incubated in humidified 5% CO_2 at 37 ºC for four hours to facilitate adherence. Subsequently, the cells were incubated with CyQUANT NF reagent for 30 minutes at 37 °C according to the standard adherent cell analysis protocol described in the product information sheet provided by the manufacturer. Fluorescence intensities of triplicate samples were measured using a FLUOstar Optima Microplate Reader (BMG Labtech, Ayelsbury, UK) with excitation at 485 nm and fluorescence detection at 530 nm.

2.2.4 Assessment of cell number

Antiproliferative and/or cytotoxicity properties of mirtoselect were determined using the CyQUANT® NF Cell Proliferation Assay kit. This kit is based on a patented green-fluorescent CyQUANT dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. 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 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 proliferation measurement. The assay was carried out in 96 well plates, with at least 4 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 quadraplicate 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), 0, 24, 48 and 72.

2.2.4.1 Cell Staining and Measurement of Fluorescence

Approximately 5,000 cells/well of RT112, 10,000 cells/well of RT 4 and HT1376 were seeded in 96 well plates in quadruplicate. After 24 hours incubation to allow cell adherence, the cells were washed twice with PBS solution and treated with 0, 2, 20, 50, 75 and 100 µg/ml mirtoselect, dissolved in respective complete growth medium, for 0, 24, 48 and 72 hours. The mirtoselect dosing was carried out daily. The cells were incubated in humidified 5% CO_2 at 37 ºC.

At the end of each incubation time, the mirtoselect solution was removed and replaced with the DNA binding dye mixture from the CyQUANT® NF Cell Proliferation Assay kits. 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 (Hank's Buffered Salt Solution) buffer. The binding solution was added directly to the cells and control wells, after removal of the culture media, using a multichannel pipette adding 50 µl 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 485 nm and emission of 530 nm. The gain value for each individual plate was set at 80% of the well with maximum fluorescence.

2.2.5 Data analysis

The number of bladder cancer cells remaining after treatment with different mirtoselect concentrations was determined by measuring the fluorescence intensity following each treatment. The fluorescence intensity measured was converted into actual cell numbers using the linear standard curve plotted for each bladder cancer cell line. The total numbers of cells present at the end of the experiment were deduced from an average of three experiments given as the mean cell number ± standard deviation (SD). Statistical analysis of each treatment was carried out by using the mean values of the proliferation data from the three independent experiments of every cell line and compared with the baseline data of the control cells by one way ANOVA (Statgraphics Centurion version XV; Herndon, USA). Significance was accepted at a 5 % significance level ($p<0.05$).

2.3 Results

a) RT112

RT112 cells can be categorized as fast growing cancer cells. The population doubling time for these cells is about 24 hours, which is faster than that of RT4 and HT1376 cells (Waters *et al*., 1986). For this reason, half the numbers of RT112 cells (5000) were seeded compared to the other cell lines. Therefore, after the 24 hours incubation, to facilitate the adherence of the cells to the surface of the plate, the number of RT112 cells may have already reached approximately 10,000 cells, which is equivalent to the initial number of the RT4 and HT1376 cells seeded.

The actual number of RT112 cells remaining after treatment with different concentrations of mirtoselect was determined via the prepared standard curve (**Figure 2.2**).

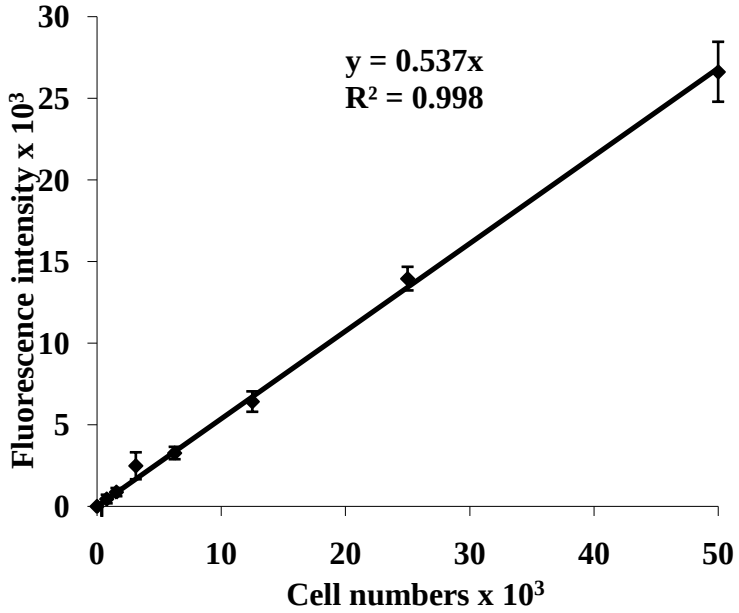


Figure 2.2: Quantification of RT112 cells using the CyQUANT® NF Cell Proliferation kit. Fluorescence intensities of triplicate samples were measured using a FLUOstar Optima Microplate Reader with excitation at 485 nm and fluorescence emission at 520 nm. Plotted data points represent averages of 3 independent experiments with triplicate incubations with background subtraction (mean ± SD).

From **Figure 2.3**, after 24 hours treatment with increasing mirtoselect concentrations, there was an increase in cell number to approximately 20,000 cells, for both control cells and those incubated with 2, 20 and 50 µg/ml mirtoselect.

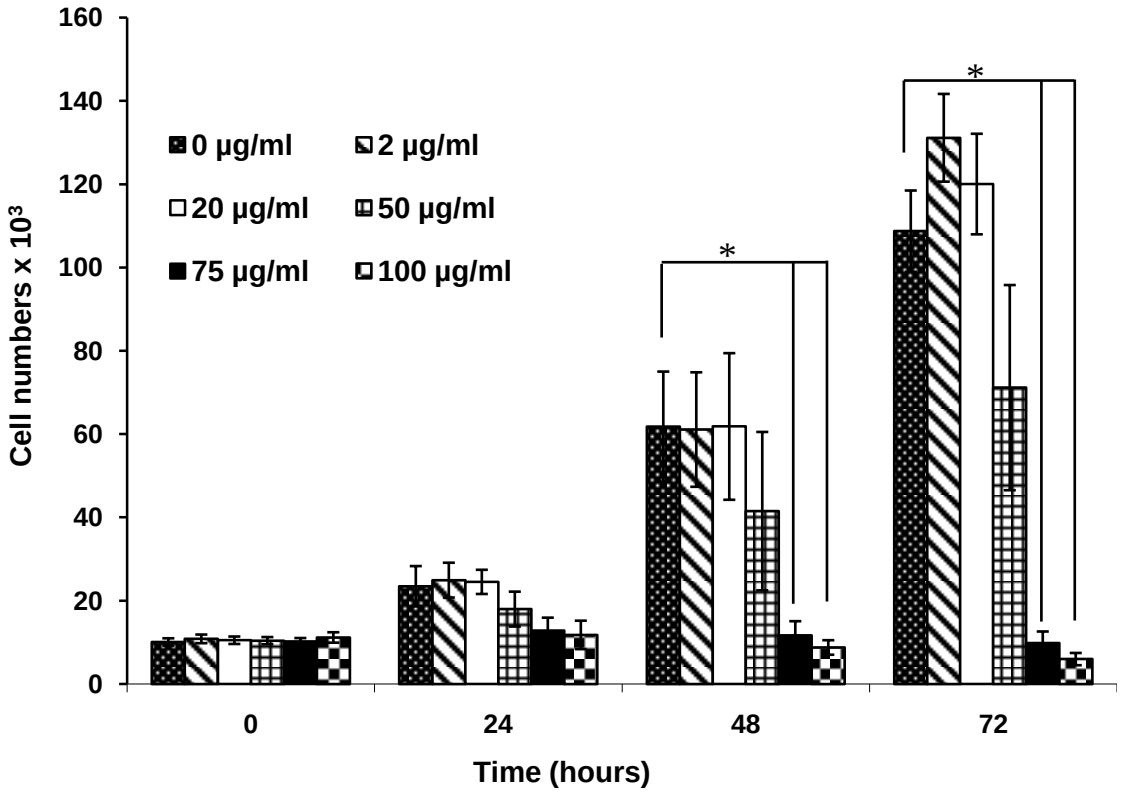


Figure 2.3: Effects on proliferation of RT112 cells after treatment with different mirtoselect concentrations (0, 2, 20, 50, 75 and 100 µg/ml) for 0, 24, 48 and 72 hours. Results are the mean ± SD of three independent experiments (four replicates per experiment). A significant difference between untreated control and treated cells was observed for the 75 and 100 µg/ml mirtoselect treatments at 48 and 72 hours (* = $p<0.0001$).

This indicated that the RT112 cells are able to proliferate in the growth media that was supplemented with mirtoselect between 0 and 50 µg/ml. However, there seems to be some inhibition of the proliferation of RT112 cells treated with 75 and 100 µg/ml mirtoselect, as after 24 hours there were approximately 15,000 cells per well.

The RT112 cells treated with 0, 2 and 20 µg/ml mirtoselect continued to show gradual proliferation approaching approximately 60,000 cells after 48 hours of

treatment, compared to 10,000 cells at the beginning of the treatment. There was now clear evidence of less cell proliferation observed in the 50 µg/ml mirtoselect treated RT112 cells with the cell number measuring approximately 40,000. At the same time, the cells treated with 75 and 100 µg/ml mirtoselect showed significant cessation of proliferation with the cell numbers determined to be around 10,000 and 8,000 cells, respectively.

After 72 hours treatment, for the 0, 2 and 20 µg/ml mirtoselect concentrations the number of RT112 cells exceeded 100,000 cells. The RT112 cells treated with 50 µg/ml only recorded approximately 70,000 cells. However the cells that were treated with 75 and 100 µg/ml mirtoselect showed significant reductions with the number being approximately 10,000 and 7,000 cells, respectively.

From **Figure 2.3**, the effect of mirtoselect against RT112 cells was, overall, dose dependant, as at low concentrations (0, 2 and 20 µg/ml) the cells still managed to proliferate, whilst 50 µg/ml mirtoselect treatment partially inhibited the proliferation of RT112 bladder cancer cells. However, there was significant inhibition of RT112 bladder cancer cell proliferation after 72 hours treatment with 75 and 100 µg/ml mirtoselect.

At the end of 72 hours incubation, the measured proliferation of the control cells has been exceeded slightly by both the 2 and 20 µg/ml mirtoselect treated cells. One possible reason for this observation in the controls cells was that these cells were already ‘over grown’ and maybe some of the cells had died as there was limited space for the cells to proliferate, and mirtoselect or any limiting factor was not available to slow down cell growth; however, one may of also expected this to apply to the 2 and 20 µg/ml mirtoselect treated cells. The other (and perhaps more likely) possibility that may explain the situation observed is that the mirtoselect at low concentration (2 and 20

µg/ml) has a small pro-proliferative effect. However, the difference between the control and the 2 and 20 µg/ml mirtoselect treated cells was not significant.

b) RT4

Overall there was a similar response observed when RT4 cells were treated with different concentrations of mirtoselect ranging from 0 to 100 µg/ml. The actual number of RT4 cells remaining after each mirtoselect treatment was determined via the prepared standard curve (**Figure 2.4**).

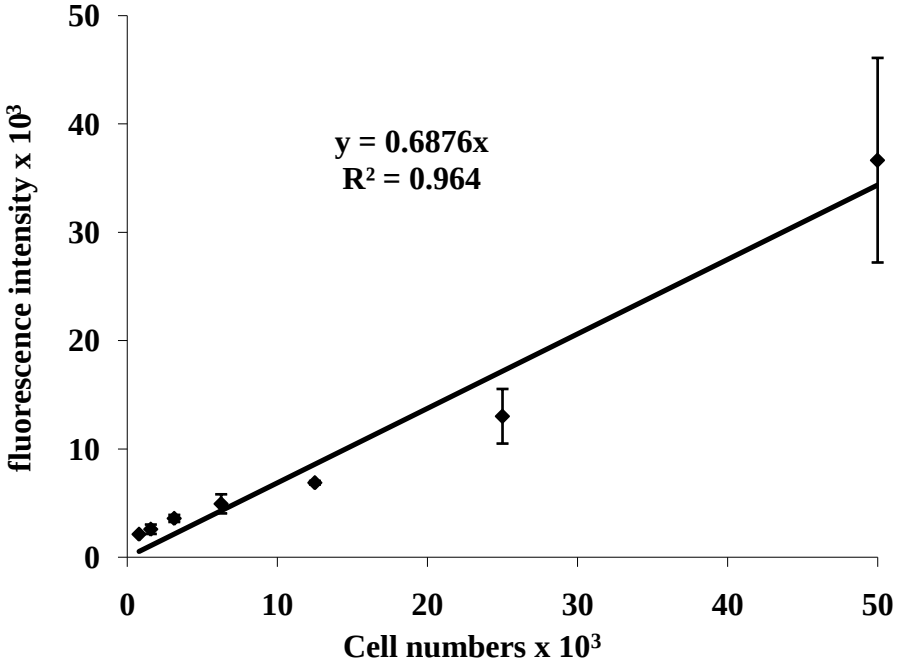


Figure 2.4: Quantification of RT4 cells using the CyQUANT® NF Cell Proliferation kit. Fluorescence intensities of triplicate samples were measured using a FLUOstar Optima Microplate Reader with excitation at 485 nm and fluorescence emission at 520 nm. Plotted data points represent averages of 3 independent experiments with triplicate incubations with background subtraction (mean ± SD).

After 24 hours of treatment with different concentrations of mirtoselect, a dose dependent anti-proliferative trend for the mirtoselect effect is clearly apparent. Proliferation of the control cells was greater than the cells that were treated with mirtoselect; there was a clear dose dependant decrease in cell proliferation for the

mirtoselect treated cells. There were ~18,000 control cells measured at 24 hours whilst the number of cells decreased by approximately half, to below 10,000 at the highest mirtoselect concentration used (100 µg/ml) (**Figure 2.5**).

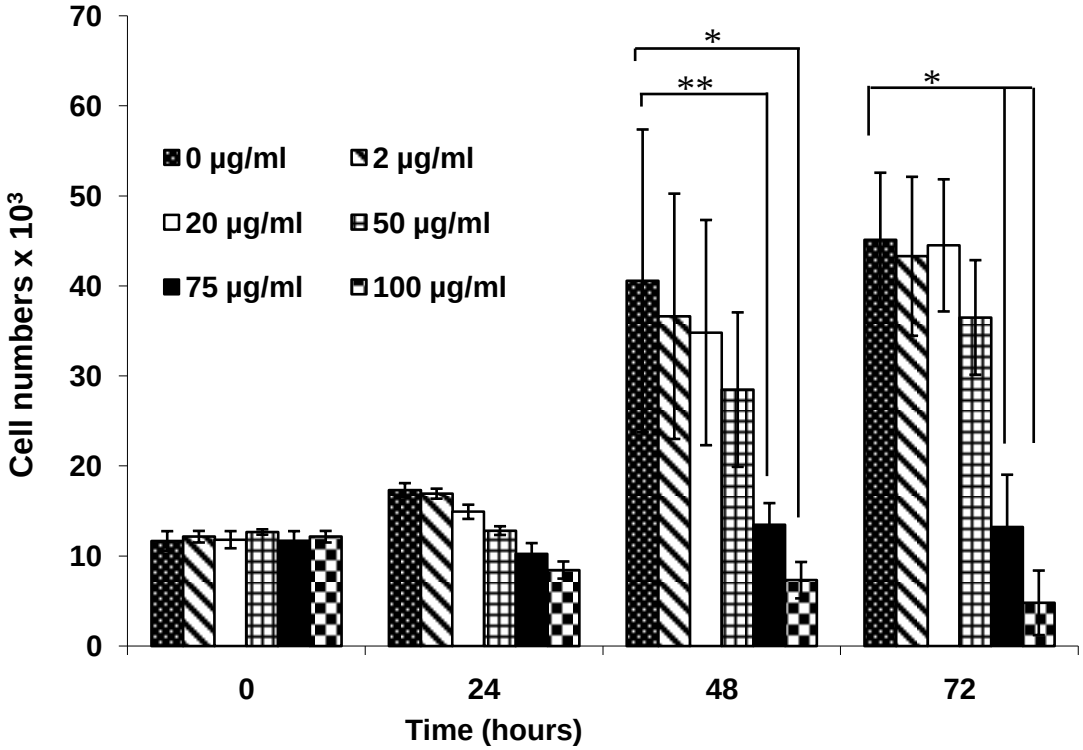


Figure 2.5: Effects on the proliferation of RT4 cells treated with different mirtoselect concentrations (0, 2, 20, 50, 75 and 100 µg/ml) for 0, 24, 48 and 72 hours. Results are the mean ± SD of three independent experiments (four replicates per experiment) Significant difference at 75 and 100 µg/ml mirtoselect at 48 and 72 hours time point (* = $p<0.0001$, ** = $p < 0.05$).

A dose dependant pattern was still observed after 48 hours of exposure to mirtoselect. The RT4 cells that were not treated with mirtoselect continued to proliferate to approximately 40,000 cells whilst the 2, 20, 50, µg/ml mirtoselect treated cell numbers were ~37,000, 34,000 and 29,000, respectively. There were significant inhibitions of growth observed in the 75 and 100 µg/ml mirtoselect treated cells, where there were approximately 15,000 and 7,000 cells measured, respectively.

After incubation with mirtoselect for 72 hours, proliferation of the cells treated with concentrations lower than 50 µg/ml did not show a significant difference when compared with the control cells. The quantities of the cells growing were measured between 35,000 and 43,000 cells. Similar to the observations made at 48 hours, there was a significant inhibition of cell proliferation detected in the 75 and 100 µg/ml mirtoselect incubations, where the numbers of RT4 cells were approximately 13,000 and 6,000, respectively.

c) HT1376

The HT1376 bladder cancer cell line was treated with the same mirtoselect concentrations as used in the treatment of RT112 and RT4 bladder cancer cells.The number of HT1376 cells remaining after different concentrations of mirtoselect treatment was determined using the prepared standard curve (**Figure 2.6**). There was no significant difference observed for the HT1376 cells after 24 hours treatment with any concentration of mirtoselect. Even cells treated with the highest mirtoselect concentration (100 µg/ml) managed to grow to approximately similar numbers as the untreated control HT1376 cells (~ 20,000 cells) (**Figure 2.7**).

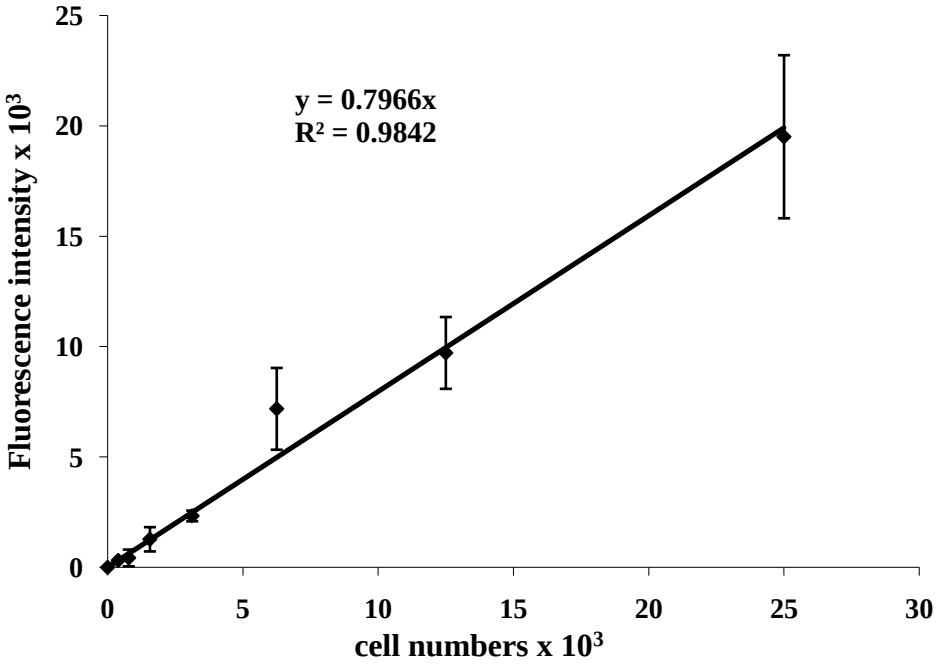


Figure 2.6: Quantification of HT1376 cells using the CyQUANT® NF Cell Proliferation kit. Fluorescece intensities of triplicate samples were measured using a FLUOstar Optima Microplate Reader with excitation at 485 nm and fluorescence emission at 520 nm. Plotted data points represent averages of 3 independent experiments with triplicate incubations with background subtraction (mean ± SD).

After 48 hours of mirtoselect treatment, there was evidence that the extent of HT1376 cells proliferation was being compromised in the 50, 75 and 100 µg/ml mirtoselect treated cells, but there was little apparent difference between the untreated control cells and those incubated with the lower concentrations of mirtoselect. Whilst mirtoselect did not significantly prevent the proliferation of the HT1376 cells, as the cell numbers measured were between 40,000 to 50,000, there is a clear dose dependency, whereby, as the mirtoselect concentration increased, proliferation is notably inhibited (**Figure 2.9**).

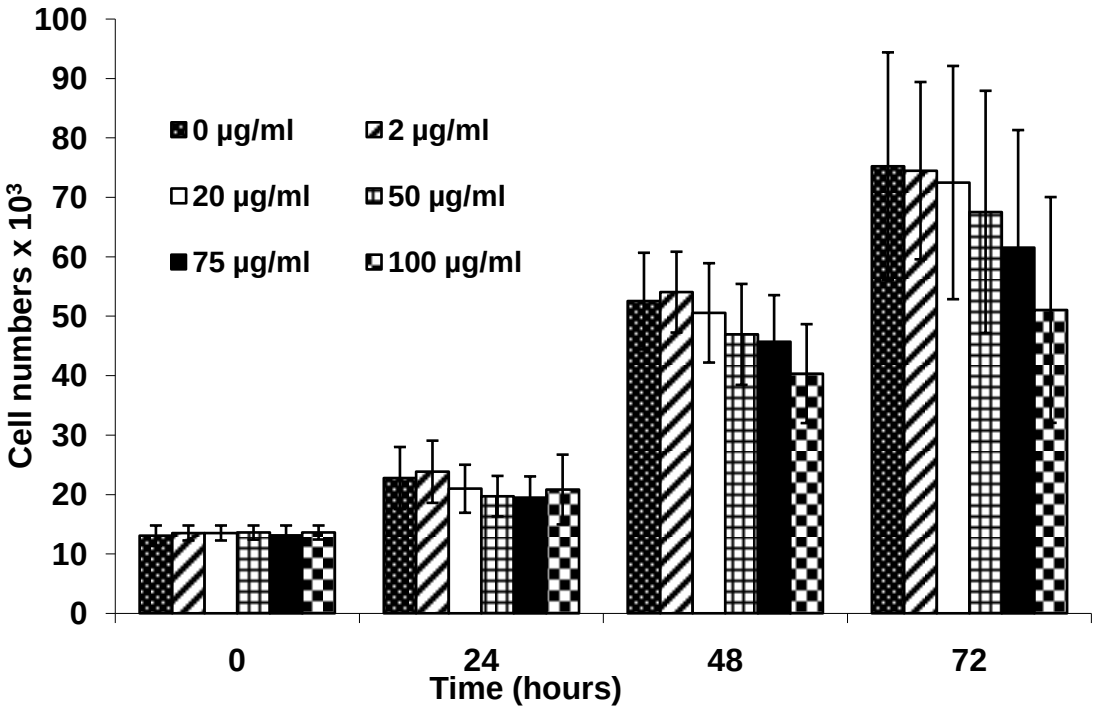


Figure 2.7: Effects on the proliferation of HT1376 cells after treatment with different mirtoselect concentrations (0, 2, 20, 50, 75 and 100 µg/ml) for 0, 24, 48 and 72 hours Results are the mean ± SD of three independent experiments (four replicates per experiment).

After 72 hours of treatment with different concentrations of mirtoselect, the graph plotted in **Figure 2.7** depicts mirtoselect interfering with the proliferation of HT1376 cells in a dose dependant manner. The highest concentration (100 µg/ml) resulted in a reduced number of HT1376 cells (~50,000) as compared to the other mirtoselect concentrations and control cells, which managed to proliferate to between 60,000 and 75,000 cells. Even though the HT1376 cells may share a similar response and pattern of growth with the other two bladder cancer cell line used in this study, there was no significant difference in proliferation inhibition capacity between the control and the highest mirtoselect concentration used in this study after 72 hours of mirtoselect treatment.

2.4 Discussion

This study was carried out to determine the effect of mirtoselect against the selected bladder cancer cell lines *in vitro*. The respective growth media used for each cell line were supplemented with 0, 2, 20, 50, 75 and 100 µg/ml mirtoselect daily dosing. 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. The daily dosing was carried out to mimic daily ingestion of mirtoselect, which is what patients need to do with chemopreventive agent.

From the graphs plotted for the three bladder cell lines used in this study (**Figures 2.3, 2.5 & 2.7**), mirtoselect supplemented at 75 and 100 µg/ml in the growth media inhibited approximately 90-70 % of the RT112 and RT4 bladder cancer cell line's growth capacity, respectively. On the other hand, at the same concentrations, only 20 % to 30 % inhibition of the HT1376 cell line was noted. Taking into account the presented data, it was justified that 75 and 100 µg/ml mirtoselect concentrations were not to be used in the subsequent Comet assay experiment (**Chapter 4**), to determine antioxidant potential of mirtoselect, due to the significant antiproliferative activity of mirtoselect observed. In a parallel PhD study, the mirtoselect sensitive cell lines, RT112 and RT4, showed a significant increase in apoptosis compared to control, whereas the HT1376 cell line, that showed little response to mirtoselect in proliferation and cell survival assays, had little or no induction of cell death as determined by markers of apoptosis (Higgins, 2010). RT112 and RT4 cell lines treated with 100 µg/ml mirtoselect exhibited small

dose dependent increases in caspase activity after 48 hours of mirtoselect treatment and significant increases in caspase activity were observed after 72 hour of treatment (1.7 fold and 2.7 fold, compared to respective control cells). 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. There was also a trend of slower cell division in RT4 and HT1376 after 72 hours of mirtoselect treatment (Higgins, 2010). Therefore, apoptosis and slowing of cell division could presumably contributed to the antiproliferative activities observed in the bladder cancer cell lines used in this study.

Mirtoselect dosing strategies gave 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 (G1pTa) (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.

Therefore, 50 µg/ml mirtoselect was chosen as the most suitable concentration to be used in the subsequent studies; 50 µg/ml mirtoselect treatment lead to approximately 40%, 25% and 15% growth inhibition in RT112, RT4 and HT1376 cells, respectively. In the study by Cooke and co-workers, mice fed a diet containing mirtoselect had levels of excreted anthocyanins in their urine (12.3 µg/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 50 µg/ml mirtoselect (based on the anthocyanin concentration of mirtoselect being 25%).

Anthocyanins from different varieties of fruits and berries have been extensively investigated in numerous antioxidant and anti-proliferative studies of cancer cells either *in vitro* or *in vivo* (Cai et al., 2010; Cooke et al., 2006; Jing et al., 2008; Zhao et al., 2004). In many cases, the anthocyanins have been shown to possess antioxidant and anti-proliferative properties that could potentially inhibit the growth of cancer cells and tumour development. However there was no direct correlation described between both properties studied (Gorinstein et al., 2009; Liu et al., 2002b; Meyers et al., 2003).

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 ahaemocytometer 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). This assay also failed to demonstrate a linear relationship between the MTT and cell number at high cell densities, more than 2×10^4 cells (Plumb et al., 1989). 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. 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 green 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 proliferation 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.

Mirtoselect, 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 (Lazze et al., 2004; Reddivari et al., 2007), 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. The main focus of this project is on the ability of mirtoselect to act as antioxidant and so reduce the occurrence of oxidatively damaged DNA in bladder cancer cell lines *in vitro*. In demonstrating the ability of mirtoselect to disrupt the proliferation of bladder cancer cell lines, this study has successfully determined the appropriate concentration of

mirtoselect to be utilised in the subsequent antioxidant study. The concentration determined has proven activity but not too great an antiproliferative/cytotoxic effect that we don't have enough cells remaining after treatment to do the Comet assay analysis (**Chapter 4**).

To the best of my knowledge, to date, there are no articles published that have specifically described the effect of mirtoselect, or any single entity of mirtoselect, against the selected bladder cancer cell lines used in the project (RT112, RT4 and HT1376). However, there was a case-control study using specific carotenoids (α-carotene, β-carotene, lutein, and lycopene) and flavonoids (quercetin, kaempferol, myricetin, and luteolin) to investigate the relationship of these phytochemicals with bladder cancer (Garcia et al., 1999). The study included 497 cases first diagnosed with bladder cancer, 547 neighbourhood controls, and 566 hospitals controls, matched by gender, age, area of residence, and hospital. None of the specific carotenoids and none of the specific flavonoids have been found to be significantly associated with bladder cancer risk in this analysis.This study does not support the hypothesis that intake of specific carotenoids and flavonoids is protective against bladder cancer risk (Garcia et al., 1999). There was also a poster presentation from Ohio State University describing a study of black raspberry extract against a panel of bladder cancer cell lines (RT4, HT1376, J82 and T24) presented at Ohio Agricultural Research and Development Center (OARDC) Annual Research Conference in 2007 (see http://hdl.handle.net/1811/24717). Therefore, the current study is probably the first attempt to determine the activity of mirtoselect (a standardised commercially available extract) towards bladder cancer cell lines *in vitro*.

2.5 Summary statement

The current findings have demonstrated mirtoselect to possess powerful antiproliferative effects against the bladder cancer cells studied, demonstrating at an initial proof-of-principle level that it may serve as a possible candidate chemopreventive agent. Based on the current findings, the concentration of mirtoselect that will be used in the subsequent antioxidant properties studies will be 50 µg/ml. The mirtoselect treatment will be carried out as daily dosing for specific periods of time.

Chapter 3.

Development of a Modified Version of the Comet Assay to Detect Oxidatively Damaged DNA in Bladder Cancer Cells *In Vitro*.

3.1 Introduction

3.1.1 Alkaline Comet assay

The Comet assay, also known as single cell gel electrophoresis, is a microscopy-based method for detecting DNA damage at the level of individual cells. It is a relatively inexpensive, robust, simple and sensitive method for measuring DNA damage and it has been used in studying genotoxicity in situations of chemical exposure, clinical studies, environmental bio-monitoring and human monitoring (Rojas et al., 1999).

The first Comet assay protocol was developed by Ostling and Johanson in 1984 where mammalian cells, irradiated with gamma rays, were suspended in agarose gels, cast on microscope slides. The embedded cells were then lysed in detergent solution prior to electrophoresis under neutral conditions (pH~9.5). The electric current pulls the negatively charged DNA from the nucleus in the direction of the anode, resulting in characteristic bodies that look like a comet with a head and a tail; more fluorescence is observed in the tail relative to the head in damaged cells. Visualisation of the extent to which the comet was formed, and therefore a measure of the extent to which damage is present, was achieved by staining of the DNA using ethidium bromide (Ostling and Johanson, 1984).

Due to the limitation of the neutral conditions used, which primarily allows for the detection of DNA double strands breaks, both Singh and co-workers and Olive and co-workers independently further improved the protocol by introducing an alkaline version of the Comet assay. The protocol introduced by Singh and co-workers was carried out using alkaline electrophoresis to analyze the DNA damage induced in X-ray irradiated and hydrogen peroxide (H_2O_2)-treated cells. The cells were lysed at pH 10 with 2.5 M sodium chloride (NaCl), Triton X-100 and sarcosyl for 1 hour, following

this with a treatment with alkali (0.3 M NaOH) and electrophoresis at the resulting high pH (>13). Their method allows for the detection of strand breaks (both single and double) and alkali labile sites (ALS) (Singh *et al*., 1988). The strand breaks in DNA could be a result of different types of reactions for instances scission of DNA backbone by chemical or radicals attack, scission following the binding of intercalating agents, alkali-labile DNA adducts, endonuclease or topoisomerase action and DNA hydrolase release from lysosome (Rhaese and Freese, 1968). On the other hand base modifications by alkylation may weakens the *N*-glycosilic bond which leads to the depurination/depyrimidination and the appearance of alkali labile abasic sites (Rhaese and Freese, 1968). Alkali labile sites may be converted into strand breaks if incubated in comet assay electrophoresis buffer at pH 13 (Collins, 2004; Collins et al., 2008; Singh et al., 1988) Alternatively, Olive and co-workers adapted the protocols introduced by Ostling and Johanson by conducting lysis under weak alkaline conditions (0.03 M NaOH) but the electrophoresis was conducted under either neutral or mildly alkaline (pH 12.3) conditions (Olive *et al*., 1990). Even though both methods are quite similar, the method of Singh and co-workers had a one- to two-fold greater sensitivity compared to that of Olive and co-workers (Tice et al., 2000).

The most widely used version of the Comet assay is the alkaline Comet assay, due to its greater sensitivity and it also being the simplest and most robust protocol. Briefly, for the alkaline version of the assay, the cells of interest are suspended in low melting point (LMP) agarose and then placed on a microscope slide, pre-coated with normal melting point agarose, and the LMP allowed to set under a coverslip. Then, with the coverslip removed, the embedded cells are lysed (pH 10) for between 1 hour and overnight, producing nucleoid bodies consisting of supercoiled loops of DNA linked to a lysis resistant/residual nuclear matrix. The nucleoid bodies generated are then allowed

to unwind by submerging the slides in alkaline buffer (pH > 13) prior to electrophoresis. During electrophoresis the unwound DNA, as it is negatively charged, migrates towards the anode of the electrophoresis tank. The slides are then neutralised with buffer and finally stained with fluorescence DNA-binding dye, for instance ethidium bromide, propidium iodide, YOYO or DAKO (Hartmann et al., 2003). The dye either intercalates the double-stranded DNA or may bind electrostatically under specific conditions of pH and ionic strength. Observations of comet formation are then carried out either on fresh wet or dry/hydrated slides using fluorescence microscopy (Benitez-Bribiesca *et al*., 2001; Rojas *et al*., 1999). The microscope may be attached to a digital camera and the captured images analysed by image analysis software. Currently, there are several computer software systems that can be used to analyse the resulting comets. These systems are able to provide DNA migration parameters such as the primary Comet measures of "tail length" (TL) and "percentage tail DNA" (% TD) as well as "tail moment" (TM) and "Olive tail moment" (OTM), which take into account several criteria of the migrated DNA. The DNA migration parameter of tail DNA is considered to be most acceptable as it covers a wider range of damage and it is linearly related to break frequency up to a certain degree of DNA damage (Collins et al., 2008). TL is only suitable for measuring low levels of DNA damage because as the level of damage increases, beyond a certain point the intensity of the staining in the tail increases rather than the actual TL; TL therefore becomes limiting. TM is a value describing DNA damage through a combination of TL and % TD; however, at low doses, TM tends to deviate in its linearity (Collins et al., 2008). OTM combines length of the comet and the % TD by calculating the product of the percentage DNA in the tail and the difference between the head and the tail centres of gravity (Lovell, 2009). More importantly, except for % TD, the other DNA migration parameters mentioned are

reported arbitrary units. Consequently, using these latter parameters, it is difficult to 'visualize' the comet's formation or to evaluate the degree of damage being discussed (Collins et al., 2008). **Figure 3.1** shows a simplified diagram of the standard alkaline Comet assay protocol.

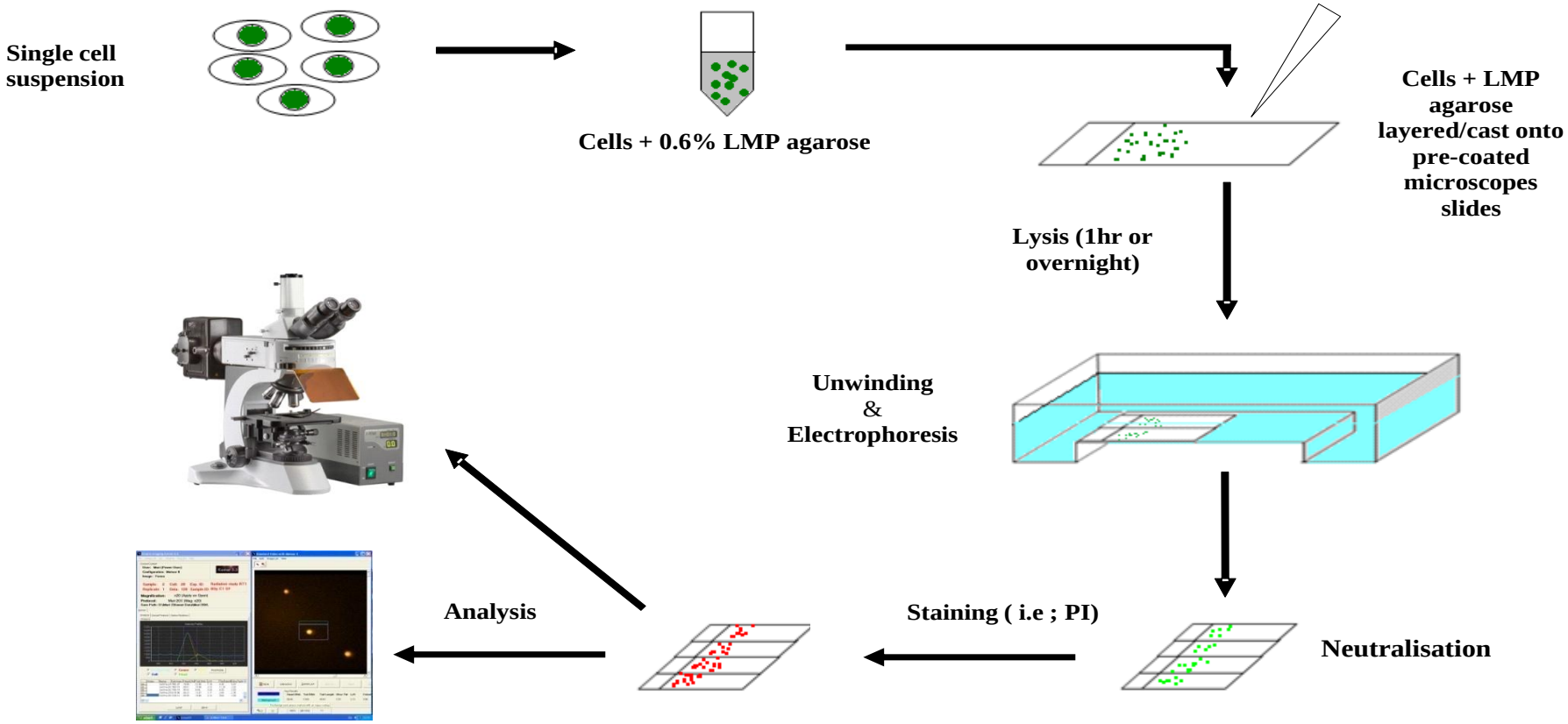


Figure 3.1: Schematic diagram of the standard alkaline Comet assay protocol. A single cell suspension (e.g. from blood, cells in culture or tissue) is suspended in low melting point agarose and aliquoted/cast onto a pre-coated slide. The cells are then lysed (removing membranes and proteins) and the remaining nucleoid bodies subjected to unwinding and electrophoresis. The alkaline pH of the electrophoresis buffer is neutralised. Subsequently, the slides maybe oven dried and rehydrated for staining with propidium iodide. Slides are analysed using a fluorescence microscope with the aid of image analysis software.

An alternative to image analysis is visual scoring. Visual scoring of the extent of comet formation can be achieved by grouping the visually determined level of DNA damage into a series of "classes" ranging from class 0 (an undamaged comet) to class 4 (a highly damaged comet). As, typically, 100 cells are scored per slide (50 cells per gel), by multiplying the number of observed comets (100) by the comet classification (0 – 4) and summing the values obtained in each sample, possible values from ranging 0 to 400 are calculated (Garcia *et al*., 2004).

Despite the advantages mentioned, the standard alkaline protocol provides limited information on the type/nature of the damage being measured and consequently it is not always possible to determine the source of damaging agents. However, the specificity of the Comet assay protocol can be enhanced by introducing lesion-specific endonucleases after the lysis process. The endonucleases recognise specific damaged DNA bases, and in removing the damaged moiety, produce, further single strand breaks (Collins et al., 1996; Smith et al., 2006; Tice et al., 2000).

The most common endonucleases used in the modified Comet assay are the bacterial enzymes, Formamidopyrimidine DNA-Glycosylase (Fpg), human 8-oxoguanine DNA glycosylase (hOGG1) and endonuclease III (ENDO III) for the assessment of oxidatively damaged DNA base lesions. Fpg recognises specific oxidised purines such as 8-oxoGua, 2,6-4,6 diamino-5-formamidopyrimidine (FaPyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua), abasic apurininc and apyrimidinic (AP) sites and ring-opened N-7 guanine adducts. hOGG1 has more limited substrate range with a higher specificity for 8-oxoGua and methyl-fapy-guanine (Collins et al., 1996; Smith et al., 2006; Speit et al., 2004).

Fpg and hOGG1 both possess N-glycosylase and AP-lyase activities. These enzymes remove oxidatively damaged purines by cleaving the glycosidic bond between

the modified base and sugar moiety, so removing the modified base and creating an AP site. The AP-lyase activity of Fpg acts on the produced AP site by hydrolysing the 3' oxygen bond (β-elimination) and then subsequently the 5' oxygen bond (δ-elimination) creating a SSB consisting of a single nucleoside gap. The corresponding activity of hOGG1, on the other hand, is just associated with a β-elimination which cleaves the 3' oxygen bond of the DNA back bone so generating a SSB with a 3' α,β-unsaturated aldehyde (Schiff base) (Asagoshi et al., 2000b; Dizdaroglu et al., 2000; Hamm et al., 2007). Endonuclease III is specific for oxidised pyrimidines for instance, thymine glycol and uracil glycol. Endo III's mechanism of action on the pyrimidine lesions is also via N-glycosylase and AP-lyase activities similar to Fpg's activity (Lindahl, 1993). **Figure 3.2** and **3.3** are schematic diagrams illustrating the mechanisms of action of Fpg and hOGG1.

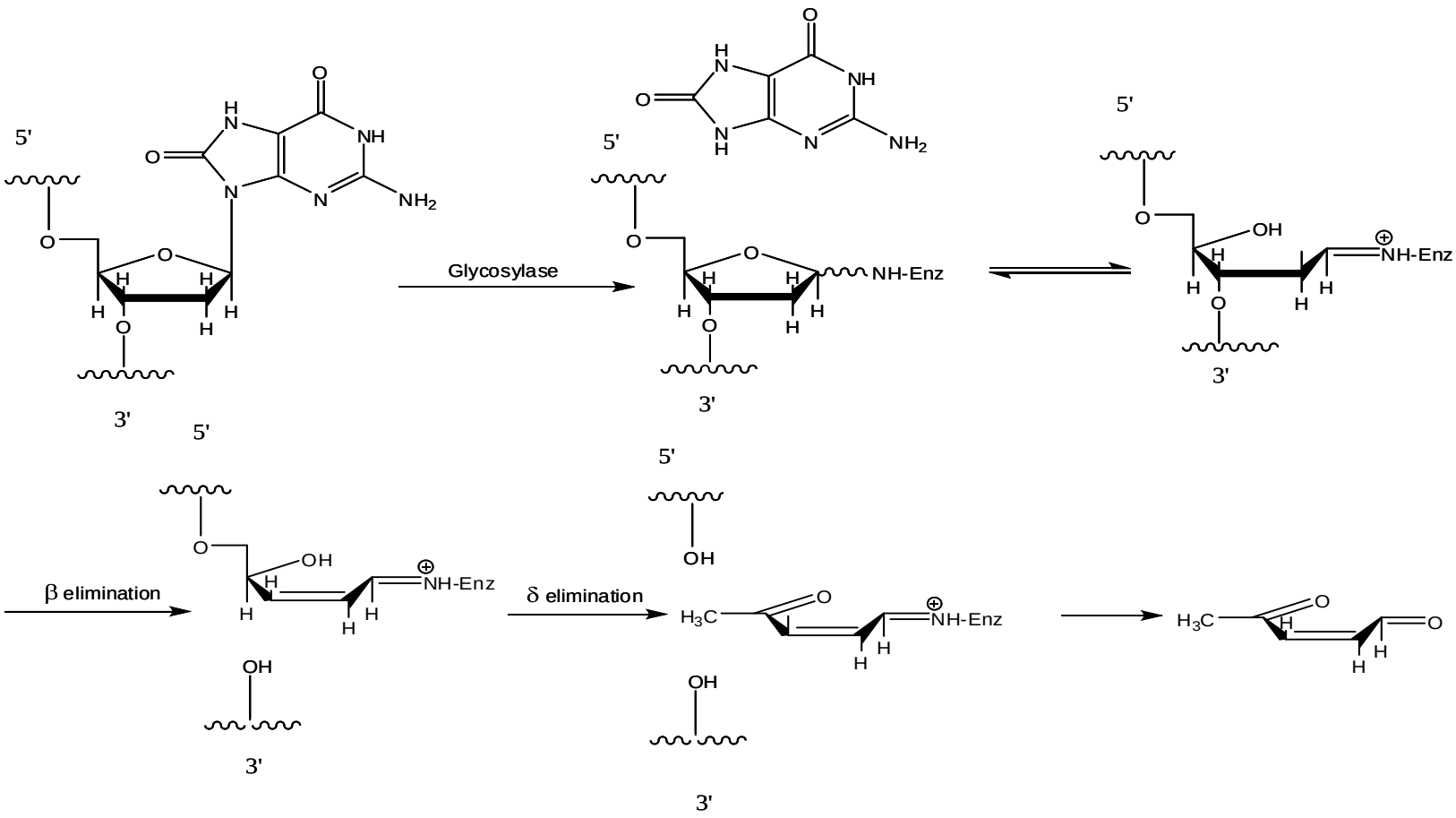


Figure 3.2: Mechanism of Fpg mediated 8-oxoGua excision via glycosylase and both β and δ -lyase activities. Free 8-oxoGua and a modified deoxypentose fragment are liberated with formation of 3' and 5' phosphorylated DNA end groups (adapted from Hamm *et al*., 2007).

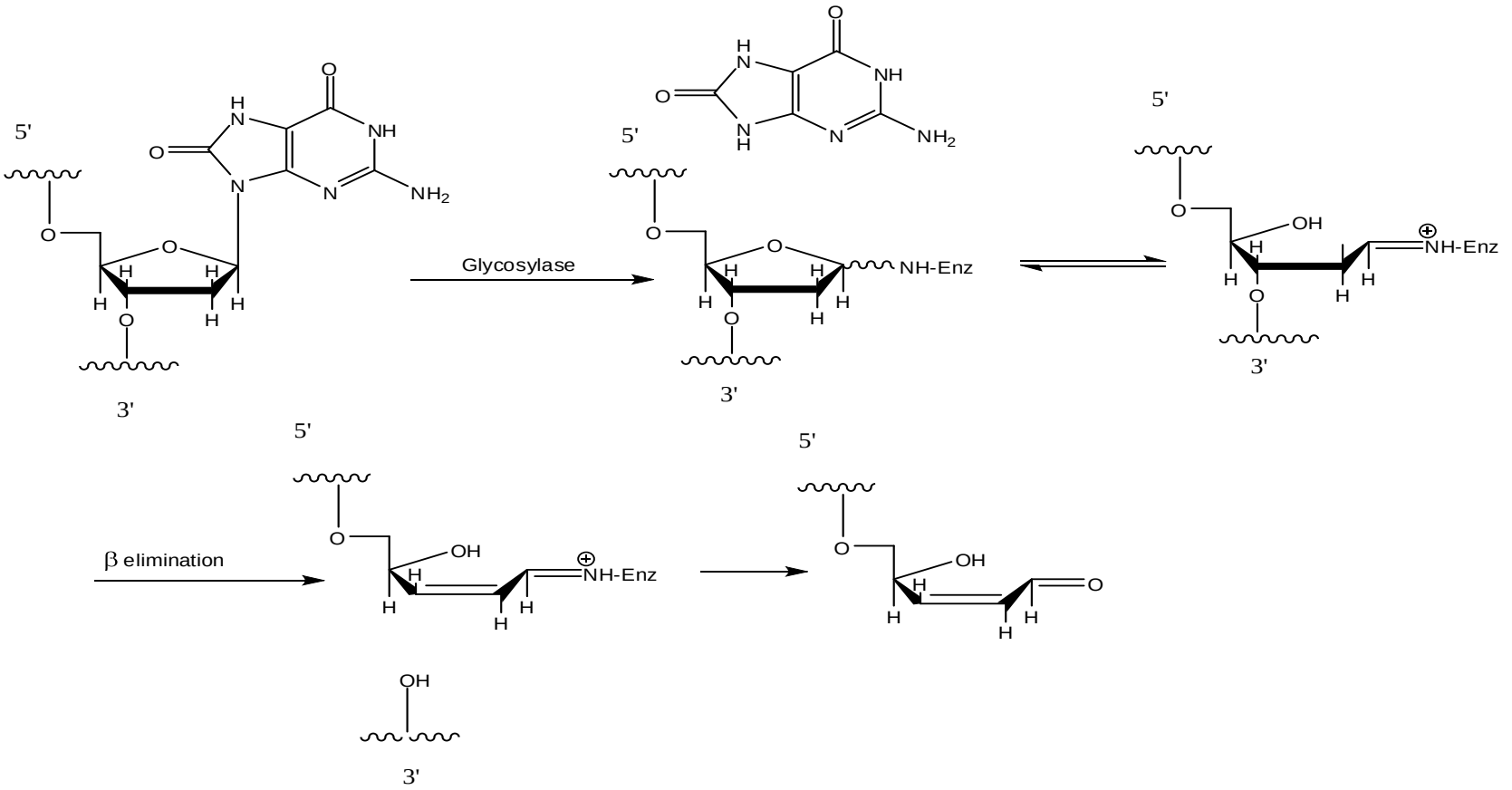


Figure 3.3: Mechanism for base excision and β-lyase activity of hOGG1. hOGG1 interacts with 1‘C, resulting in removal of the 8-oxoGua base and the β elimination cleaves the backbone 3’ to the formed AP site, forming a Schiff base (adapted from Hamm et al., 2007)

In experiments which involve the use of endonucleases, it is important to be able to distinguish between any immediate DNA SSBs and the further strand breaks that arise from cleavage of the endonucleases sensitive sites in the DNA. Interestingly, Collins and co-workers failed to detect any additional DNA SSBs immediately after irradiation of lymphocytes by the ENDO III Comet assay, but when the lymphocytes were allowed to repair for 30 minutes, the ENDO III sensitive sites became more apparent (Collins et al., 1995). One procedure to allow for the clear differentiation between the immediate DNA SSBs and the new strand breaks that arise from the endonucleases sensitive sites, is for the immediate DNA SSBs to be allowed to be repaired prior to the addition of the DNA endonucleases. Examples of this are: human pulmonary carcinoma cells (NCI-H292) exposed to cigarette smoke exhibited oxidative DNA lesion measured by Fpg Comet assay after 16 to 20 hours incubation, allowing all the DNA SSB to repair (Thorne et al., 2009); also in a study by Collins and co-workers, HeLa cells treated with hydrogen peroxide (H_2O_2) were allowed to repair the immediate DNA SSBs for one hour to prior the estimation of ENDO III sensitive sites (Collins et al., 1995).

Overall, the addition of specific endonucleases, which recognise specific oxidised purines or pyrimidines, improves the specificity and sensitivity of the alkaline Comet assay protocol in detecting oxidatively damaged DNA. Better estimation of the endonucleases sensitive site is achievable if the immediate DNA SSBs are allowed to repair before the treatment with the specific endonucleases of interest. However, a precaution that should be taken into account, is that the endonucleases such as Fpg and ENDO III may not only recognise oxidatively damaged DNA but also other type of damage, for instance alkylation damage (Speit *et al*., 2004); but induced levels of these lesions will be low/negligible following exposure to a strong oxidant.

Accordingly, as it is a strong oxidant, H_2O_2 was chosen as the oxidising agent to be used in this study. It is well established that H_2O_2 is one of the by-products of normal cellular metabolism activities (formed by dismutation of superoxide) and high levels of H_2O_2 have been documented as being present in many type of cancerous cells (Halliwell et al., 2000; Schumacker, 2006; Szatrowski and Nathan, 1991). H_2O_2 is itself poorly reactive and readily diffuses through the cell membrane (Bienert et al., 2006). It is believed that H_2O_2 does not damage cells by itself, but rather by reacting with intracellular transition metals to form much more damaging species such as the hydroxyl radical (Halliwell, 1989; Imlay et al., 1988).

3.1.2 Outline of the present study

For many years, the laboratory in which the present project was undertaken has been using the standard alkaline Comet assay protocol to determine the level of DNA damage induce by either chemicals or physical insults against cells of interest (Almeida et al., 2008; Almeida et al., 2006; Button et al., 2010; Duarte et al., 2007; Duarte and Jones, 2007; Moneef et al., 2003; Sayan et al., 2009), the available protocol has been proven to be very reliable. One of the key early objectives to achieve this is the optimisation of the amount of DNA glycosylase enzyme to be utilised in the modified alkaline Comet assay protocol. This objective is of the utmost importance, as, if too little enzyme is used, this may be insufficient to adequately/optimally detect the majority of the oxidatively damaged DNA (leading to an underestimation), whereas if too much enzyme is being used, it may begin to cut the DNA strand adventitiously/indiscriminately (leading to an overestimation).

Determining the optimal amount of the DNA glycosylases enzyme requires the creation of cells that contain sufficient amounts of specific oxidatively damaged DNA

bases in their DNA; these cells are designated ‘substrate cells’. The optimal parameters that were required in the development of the substrate cells are the determination of a H_2O_2 exposure concentration, determination of the duration of H_2O_2 exposure and determination of the time required to achieve DNA SSB repair. Briefly, these substrate cells were created by treating the cells of interest with H_2O_2 in the dark on ice. These treated cells were then allowed to proceed with the immediate DNA SSB repair prior to the cells being harvested and used.

Terminating the repair process at a point where all the immediate DNA SSB are virtually completely repaired will generate cells enriched with respect to oxidatively damaged DNA bases. Oxidatively damaged DNA bases require a longer time to be fully repaired by the endogenous DNA repair systems. Therefore, the nuclear material of the substrate cells, following exposure and repair, will contain high amounts of oxidatively damaged DNA bases compared to normal untreated cells. Then incubating the prepared substrate cell-nucleoid bodies with different dilutions of DNA glycosylases enzyme will enable the optimal dilution of the DNA glycosylase enzyme to be determined.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Growth media were the same as described in **Chapter 2, Section 2.2.1**. Low melting point (LMP) agarose, normal melting point (NMP) agarose, sodium chloride (NaCl), disodium ethylenediaminetetraacetic acid (Na_2EDTA), sodium hydroxide (NaOH), tris-HCl, propidium iodide (PI), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), potassium chloride (KCl), potassium hydroxide (KOH), triton X-100, hydrogen peroxide (H_2O_2) (30% w/w), formamidopyrimidine-DNA glycosylase (Fpg) and propidium iodide (PI) were all purchased from Sigma (Poole, UK), unless stated otherwise. Human 8-hydroxyguanine DNA-glycosylase (hOGG1) was purchased from New England Biolabs (Hitchin, UK). Frosted end glass microscope slides, 22 x 22 glass cover slips and eppendorf tubes were obtained from VWR International (Lutterworth, UK). Six well plates were purchased from NUNC (Roskilde, Denmark).

3.2.2 Cells culture protocol

3.2.2.1 Cells and culture conditions

The bladder cancer cell lines were cultured as described in **Chapter 2**, **Section 2.2.2**.

3.2.2.2 Trypan blue exclusion assay

This dye exclusion test is used to determine the number of viable cells present in a cell suspension. Generally, cells with an intact membrane are able to exclude the dye while cells without an intact membrane take up the colouring agent. The dye used for exclusion stain is usually trypan blue, but erythrosin and naphthalene black have also

been used (Freshney, 1994). In addition to dye exclusion, a dye uptake stain can be used to measure viability as well. In this case, the dye is normally taken up by viable cells but not by the non-viable cells (Freshney, 1994).

20 µl of the harvested cell suspension and 20 µl trypan blue dye were mixed together in a 0.5 ml eppendorf tube. An aliquot (20 µl) of the cell mixture was then applied to a haemocytometer and viewed using an inverted NIKON TMS microscope (20 X). Total cells counts, comprising both live and dead cells were counted in the 4 quadrants of the haemocytometer (**equation 3.1**). Percentage cell viability was determined by dividing the number of live cells by the total cell count and multiplying this by one hundred (**equation 3.2**). Cell number per ml of original cell suspension was estimated by the following formula:

$$\text{Number of cells/ml} = (\text{number cells counted/number grids counted}) \times 2 \times 10^4 \quad (\textbf{eq. 3.1})$$

Where 10^4 is the conversion of cells/0.1 mm^3 (volume of grid) and 2 is the dilution factor.

Percentage of cell viability was estimated by the formula:

$$\text{Percent viability} = \text{‘white’ cells} / (\text{‘blue’ cells} + \text{‘white’ cells}) \times 100\% \quad (\textbf{eq. 3.2})$$

As well as determining the viability of the cells, appropriate cells number (~ 20,000 cells/slide) were determined, and these were then embedded in LMP (see **Section 3.2.4**).

3.2.3 Comet Assay Procedures

3.2.3.1 Microscope slide pre-coating

The ultimate goal of slide preparation is to obtain uniform gels sufficiently stable to survive through to data collection, as well as to ensure easily visualized comets with minimal background noise (Tice et al., 2000). Initially, fully frosted slides were used most commonly because they offered increased gel bonding and thus stability. However, within the last few years, either conventional microscope slides (Klaude et al., 1996) or slides specifically modified to increase gel stability have been increasingly used. In this study, normal clear end-frosted glass microscope slides were used. These were pre-coated with normal melting point agarose by dipping the clear glass ends of the slides into the molten agarose solution (1% NMP agarose in distilled water), until the molten agarose was slightly below the boundary of the frosted end, then one side of the slide was wiped clean and they were placed into an oven overnight for drying at 40 ºC. It is best to work in a low-humidity environment to ensure agarose adhesion (Olive and Banath, 2006).

3.2.3.2 Embedding the cell samples in agarose gels on the microscope slides

The concentrations of cells in agarose, as well as the concentration of agarose, are important parameters for ensuring a successful analysis. The optimal number of cells (at least for image analysis) is typically not more than a few per visual field. Higher cell densities can result in a significant proportion of overlapping comets, especially at high levels of DNA migration. Higher agarose concentrations can affect the extent of DNA migration, as well as the accessibility of the DNA to other manipulations (Tice et al., 2000). To avoid any deleterious environmental influence, for instance adventitious exposure to ultraviolet light, the slide preparations were carried

out under low, dim light. Approximately 20,000 cells per gel were prepared from every sample of the bladder cancer cell lines. Eppendorf tubes containing an appropriate number of cells were centrifuged at 2000 rpm for 5 minutes at 4 ºC. After the supernatant was removed, 170 µl of 0.6% LMP agarose in PBS (melted and held at 37 ºC) was added into the eppendorf tube. The cells and LMP agarose suspension was quickly mixed using a micropipette to give a good dispersion. An aliquot (80 µl) of the mixture was then applied onto the chilled pre-coated slides that were placed on a level ice-chilled stainless steel plate. Subsequently and immediately, 22 x 22 mm glass coverslips were placed on top to level the agarose. The samples were prepared in duplicate (two gels per slide, but with each duplicate on a different slide). Once the LMP agarose had set, the glass coverslip was gently removed.

3.2.3.3 Lysis

Pairs of microscope slides, with the bladder cancer cells embedded in LMP agarose gels, were placed back-to-back into a polypropylene coplin jar filled with cold lysis buffer (2.5 M NaCl, 100 mM Na2EDTA,10 mM Tris, pH 10 and 1% Triton X-100 added fresh, 4 ºC) and incubated overnight at 4 ºC. Banath and co-workers have demonstrated that longer lysis duration at higher pH lead to an increase in efficiency for detecting strand breaks, probably by allowing more time for DNA unwinding and diffusion before electrophoresis (Banath et al., 2001); however, earlier studies in this lab (and others elsewhere) revealed no difference in assay response between 1 hour and overnight lysis using the above mentioned buffer.

3.2.3.4 DNA unwinding and electrophoresis

Following lysis, the microscopes slides were removed from the lysis buffer and washed with ice-cold deionised water for 15 minutes. The slides were then placed into an electrophoresis tank, which was level and surrounded by packed ice, and the slides were arranged in the same orientation with the duplicates from each sample placed in the adjacent lanes (so they are not together). The slides were then submerged under ice-cold alkaline electrophoresis buffer (300 mM NaOH, 1.0 mM Na2EDTA, pH >13 at 4 ºC) and were further incubated for 20 minutes prior to electrophoresis in the dark, to allow for DNA unwinding and to permit ALS expression as SSB. After the DNA unwinding stage, electrophoresis was carried out in the same buffer at 30 V (0.66V/cm), 300 mA for 20 minutes in the dark.

3.2.3.5 Neutralisation

After electrophoresis, the slides were neutralised with 1 ml of neutralisation buffer (0.4 M Tris-HCl, pH 7.5) applied to each agarose gel for 20 minutes prior to washing with chilled deionised water for another 10 minutes. The washed slides were then dried in the oven (40 ºC) for 3 hours. These dried slides can be stored in the dark indefinitely.

3.2.3.6 Staining

For staining, the slides were re-hydrated in deionised water for 30 minutes at room temperature prior to staining with propidium iodide. Approximately one ml of propidium iodide solution (2.5 µg/ml) was applied on top of the gel area and incubated for 20 minutes. The propidium iodide solution was drained off from the slides and these stained slides were then washed with deionised water for another 30 minutes. This procedure was also conducted under dim light, as bright light could interfere with the

fluorescence dye intensity. The stained slides were then dried in the oven (40 ºC) for 3 hours so that the cells were in a similar plane; the slides were then ready for comet scoring.

3.2.3.7 Scoring

The extent of comet formation was visualised using a fluorescent microscope. A total of 100 cells per sample (50 per duplicate gel) were analysed to give a representative result for the population of cells. Comet image capture and analysis utilised Komet software (version 5.5, Andor^{TM} Technology, Belfast, UK) and an epifluorescence microscope (Olympus BH2) fitted with an excitation filter of 515-535 nm, a barrier filter of 590 nm and a 100 W mercury lamp, and operated at a magnification of X 200. Percentage tail DNA (%TD) was selected as the parameter that best reflected DNA damage. %TD covers the widest range of damage, and it is linearly related to break frequency over most of this range (Collins *et al.,* 2008). %TD was measured from three independent experiments, each containing duplicate measures and presented as the mean %TD ± SEM (standard error of mean).

Mean values of the %TD from each DNA damage repair experiment was compared with the baseline data of the control cells by Student's *t*-test (Statgraphics centurion version VX; Herndon, USA). Significance was accepted at a 5 % significance level ($p<0.05$).

3.3 Development of the modified alkaline Comet assay

3.3.1 Preparation of 'substrate cells'

Cells growing in *in vitro* systems without any exogenous interference are exposed to low steady state DNA damage by endogenous ROS (Musarrat and Wani, 1994; Wiseman and Halliwell, 1996), but this damage will be quickly repaired by the cellular repair mechanisms. Therefore, the use of these cells to determine the optimal concentration of the DNA glycosylase enzymes would not be successful as it will be very difficult to assess the low amount of damage available in these cells. Therefore, cells containing higher amounts of induced oxidatively damage DNA bases needed to be developed as 'substrate cells'. In order to develop this substrate, the cells of interest were exposed to H_2O_2, inducing DNA damage chiefly in the form of SSBs and oxidatively damaged DNA bases. The cells will then be allowed to repair their SSBs but the repair process will be terminated prior the majority of the oxidatively damaged DNA bases being repaired.

To achieve an appropriate level of oxidatively damaged DNA bases, both the concentration of the H_2O_2 to be used and the repair time for the induced SSBs to be virtually completely repaired need to be determined.

3.3.1.1 H_2O_2 dose response

The bladder cancer cell lines were plated at a density 5.0 x 10^5 cells per well in six well plates. After 24 hours incubation to facilitate cell adherence, the cells were washed twice with PBS to remove any trace of the growth medium and were then treated with 0, 10, 20, 30, 40, 50 and 80 µM H_2O_2 in serum free media in duplicate. The treatments were carried out on ice, in the dark for 15 and 30 minutes. The treated cells

were then washed twice with PBS and harvested using trypsin/EDTA. Subsequently, the cells were prepared for Comet assay analysis as described above (see **Section 3.2.3)**

3.3.1.2 SSB-damage repair

The bladder cancer cell lines were plated at a density 5.0 x 10^5 cells per well in six well plates. After 24 hours incubation to facilitate cell adherence, the cells were washed twice with PBS. The cells were then treated with 30 and 50 µM H_2O_2 in serum free media in duplicate, on ice and in the dark for 30 minutes. Following the treatment, the treated cells were washed twice with PBS. Following this, pre-warmed complete growth media (37 ºC) was added and the cells incubated at 37 ºC in 5% CO_2. The time course used in the damage repair analysis was 0, 5, 15, 30, 45 and 60 minutes. At the end of each time point, the cells were washed with PBS and then harvested using trypsin/EDTA. Subsequently, the cells were prepared for Comet assay analysis as described above (see **Section 3.2.3**)

3.3.2 Preparation of the 'substrate cells'

The bladder cancer cell lines were plated at a density of 1.0 x 10^6 cells in 75 cm^2 culture flasks. After 24 hours incubation to facilitate cell adherence, the cells were washed twice with PBS. Cells were then treated with 50 µM H_2O_2 in serum free media on ice and in the dark for 30 minutes. The H_2O_2 treated cells were then washed twice with PBS. Pre-warmed complete growth media (37 ºC) was added into the flasks and incubated at 37 ºC in 5% CO_2 for 60 minutes to allow for the repair of immediate SSBs. After 60 minutes incubation, the repair process was terminated by removing the complete growth media and the cells were washed twice with PBS and harvested using trypsin/EDTA. The cells were kept on ice prior to Comet slide preparation. **Figure 3.4**

(**A & B**) schematically illustrates the preparation of the substrate cells and subsequently how the optimisation of the Fpg and hOGG1 levels to be used was determined.

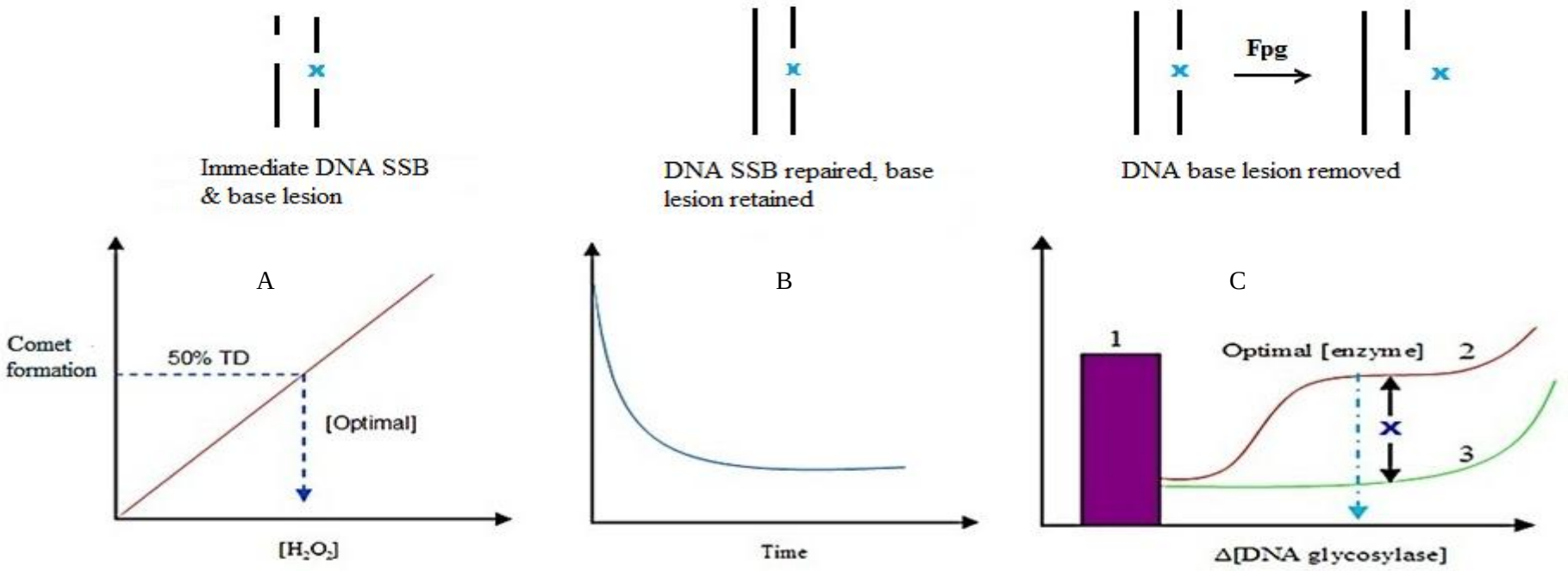


Figure 3.4: Schematic diagram illustrating the procedure undertaken to prepare the 'substrate cells' and to determine the optimal amounts of Fpg and hOGG1to be used in the modified alkaline Comet assay. A - The optimal concentration of H_2O_2 was determined which induced up to 50% tail DNA (immediate DNA SSBs). B – The cells treated with the optimal H_2O_2 concentration were allowed to undertake SSB repair for periods of time, leaving the DNA base lesion in the DNA. C – Lysed substrate cells (nucleoid bodies) prepared following A & B were treated with different dilutions of DNA glycosylase enzyme (Fpg or hOGG1). With increasing amounts of enzyme the substrate cells (line 2) show a 'plateau' effect indicating an optimal concentration of the enzyme had been reached (before adventitious cutting at the very high concentration of enzyme). The untreated control cells (line 3) were employed to confirm the adventitious cutting effect observed at the higher concentration of enzyme. Treated control cells (solid bar 1) were employed to confirm the H_2O_2 induction of immediate DNA SSBs and to allow for the judgement of the relative levels of the oxidatively damaged base lesions induced *vs.* SSB.

3.3.3 Determining the optimal Fpg and hOGG1 concentration

In order to determine the optimal amounts of the DNA glycosylase enzyme to be used, the 'substrate cells', prepared as described above, were used as follows. After lysis, microscope slides bearing nucleoid bodies of the substrate cells embedded in LMP gels were exposed to different dilutions of Fpg and hOGG1 enzyme. Firstly, the slides were removed from the lysis buffer and placed onto a transparent polystyrene chamber (24.5 x 24.5 cm) for washing with ice cold water for 5 minutes. Next, the slides were transferred into a fresh plastic chamber (24.5 x 24.5 cm) filled with enzyme reaction buffer [ERB] (40 mM HEPES, 0.1 M KCl, 0.6 mM EDTA, 0.2 mg/ml bovine serum albumin, adjusted to pH 8.0 with KOH at 4 ºC). The slides were initially washed for 5 minutes and this was repeated a further two times. About 50 µl of serial Fpg enzyme concentrations (0, 0.49, 1.47, 4.89, 14.66, 29.33, 48.88 and 244.25 U/ml) and hOGG1serial enzyme concentrations (0, 0.05, 0.16, 0.53, 1.60, 3.20, 5.33 and 32 U/ml) prepared using the ERB was then applied onto the respective gels of designated slides and covered with 22 x 22 mm glass coverslips. Slides were then placed onto a sealed black humidified polystyrene chamber and incubated at 37 ºC for 30 minutes for Fpg or 45 minutes for hOGG1.

In parallel with the preparation of substrate cells, two types of control cells were also prepared. The first, **treated control cells**, were treated with 50 µM H_2O_2 (40 µM H_2O_2 was used for HT1376 cells). At the end of the H_2O_2 treatment the cells were washed with PBS, harvested using trypsin/EDTA and kept on ice prior being used in the slide preparation. These treated control cells were used to determine the immediate SSBs level after the cells were exposed to H_2O_2. The second, **untreated control cells** were untreated (non-H_2O_2 exposed) cells (*i.e.* normal growing bladder cancer cell *in vitro*), which were used to assess both endogenous oxidatively damaged base lesion

levels and adventitious enzyme activity. Both substrate cells and the untreated control cells were treated with the respective Fpg and/or hOGG1 dilutions. Following the DNA glycosylase enzymes treatment, the three types of cells were subject to alkaline Comet assay analysis. At the end of these experiments, graphs of percentage tail DNA versus different dilutions of Fpg and hOGG1 were plotted to determine the optimal dilution of the DNA glycosylase enzymes to be subsequently used. **Figure 3.4 C** represents the hypothetical graph that should be acquired from this study.

3.4 Data analysis

Mean values of the %TD from each dose response and DNA damage repair experiment were compared with the baseline data of the control cells by non-parametric test, Mann-Whitney U, Kruskal-Wallis H test and Box-and-Whisker Plot were used wherever appropriate. Significance was accepted at a 5 % significance level ($p<0.05$).

3.5 Results

3.5.1 Optimisation of H_2O_2 concentration to induce an appropriate level of SSB.

The cell viability, as determined by the trypan blue exclusion assay (see **Section 3.2.2.2**), before and after 50 and 40 µM H_2O_2 treatment of each bladder cancer cell line was >95%. The high percentage of viable cells indicated that the concentration of H_2O_2 used in this study did not immediately kill the cells, thus enabling them to mediate the required SSB repair.

The first stage in developing the required 'substrate cells' was to determine the optimal H_2O_2 concentration to be used to induce damage and the optimal exposure time. Two different exposure times (15 and 30 minutes) and six different H_2O_2 concentrations (10, 20, 30, 40, 50 and 80 µM) were used to optimise both parameters. The RT112, RT4 and HT1376 bladder cancer cells were treated with H_2O_2 on ice in the dark and then the treated cells were analysed using the standard alkaline Comet assay protocol.

Figures 3.5 A, B and C compares the averages of three independent experiments carried out following 15 or 30 minutes exposure to H_2O_2 for the RT112, RT4 and HT1376 cells, respectively. Each graph shows the level of DNA damage measured as % TD and all graphs indicate clear dose-dependant responses. Significant increases above the background level of % TD for both treatment times were achieved after the first dose of H_2O_2 (10 µM) for the RT112 and RT4 cells whilst a significant increase above the background level of % TD for both treatment times was achieved after the second highest dose of H_2O_2 (20 µM) for the HT1376 cells. There was no significant difference between each dose when comparing between the different durations of H_2O_2 exposure. For 15 minutes exposure, the highest mean % TD measured was approximately 50%, when HT1376 cells were treated with 80 µM H_2O_2, whilst exposing these cells to this concentration of H_2O_2 for 30 minutes, again produced the

highest measure of damage; 66% tail DNA. From the data presented in **Figures 3.5 A** and **B**, it was decided that the H_2O_2 concentrations for the subsequent DNA SSB-repair study (see **Section 3.3.1.2**) using the RT112 and RT4 cells should be 30 and 50 µM, with an exposure time of 30 minutes. Similarly, from the data presented in **Figure 3.5 C**, it was decided that the H_2O_2 concentrations chosen for the subsequent DNA SSB-repair study (see **Section 3.3.1.2**) using the HT1376 cells should be 20 and 40 µM, with an exposure time of 30 minutes

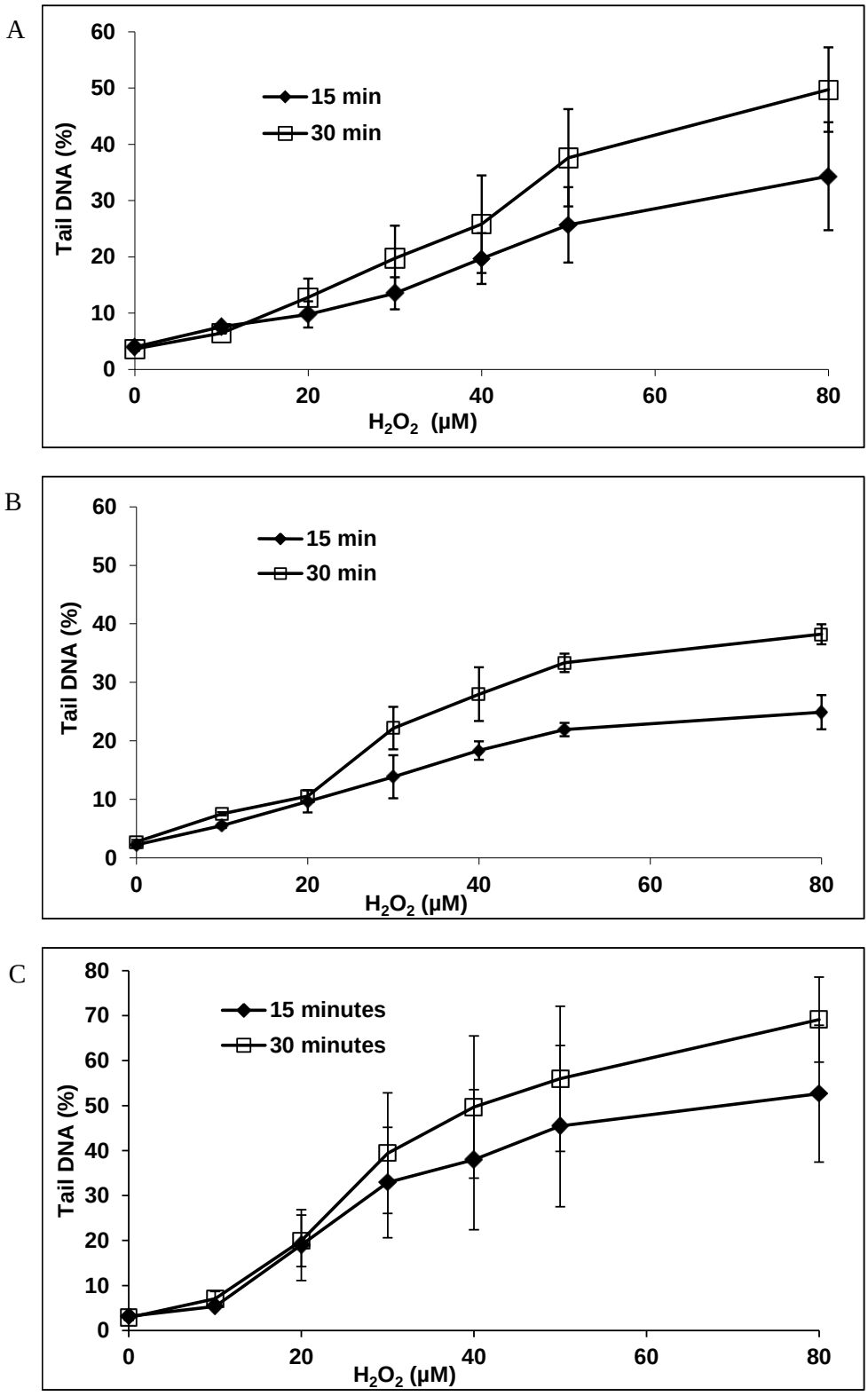


Figure 3.5: Results of standard alkaline Comet assay analysis used to determine the effect of H_2O_2 on immediate SSBs on selected bladder cancer cell lines. Average % TD of three independent experiments for the (A) RT112, (B) RT4 and (C) HT1376 bladder cancer cells following treatment with a series of H_2O_2 concentrations (0, 10, 20, 30, 40, 50 and 80 µM) for either 15 or 30 minutes. The results are presented as the mean %TD ± SEM.

3.5.2 Optimisation of incubation time for DNA SSB repair

It was necessary to determine the SSB repair time in order to allow for the SSBs to be virtually completely repaired by the cells, so enhancing the relative level of the oxidatively damage DNA bases induced in the cells after treatment with H_2O_2. The repair of immediate SSBs is faster than the repair of oxidatively damaged DNA bases (Collins et al., 1995).

Figures 3.6 (a) A, B and **C** show the actual repair kinetics for the cells treated with two doses of H_2O_2 (RT112 & RT4: 30 and 50 µM; HT1376: 20 and 40 µM - determined from the previous dose response study; see **Section 3.5.1**). **Figures 3.6 (b) A**, **B** and **C** depicted the relative percentage TD of the repair kinetics for the same treatment. The SSB repair process was terminated at 60 minutes at which point the level of strand breaks measured approached the level measured in the untreated control cells (**Figure 3.6 (a)**). In **Figures 3.6 (a) and (b) A, B** and **C**, the repair profiles were generally characterised by an initial rapid decrease in SSBs levels during the first five minutes, followed by a more modest repair activity for the remaining period of time until 60 minutes, where approximately 10% TD was measured. There was no appreciable difference between the repair capacity of the 30 and 50 µM H_2O_2 treated cells, as determined when the means of each experimental time point were compared (**Figure 3.6 (a) and (b)**). 50 µM H_2O_2 plus 60 minutes repair was chosen to be used for the preparation of the RT112 and RT4 'substrate cells' whilst 40 µM H_2O_2 plus 60 minutes repair was chosen for the preparation of the HT1376 'substrate cells' (see **Section 3.3.2**); the higher H_2O_2 concentrations were chosen as these gave higher levels of immediate damage without evidently compromising repair capacity.

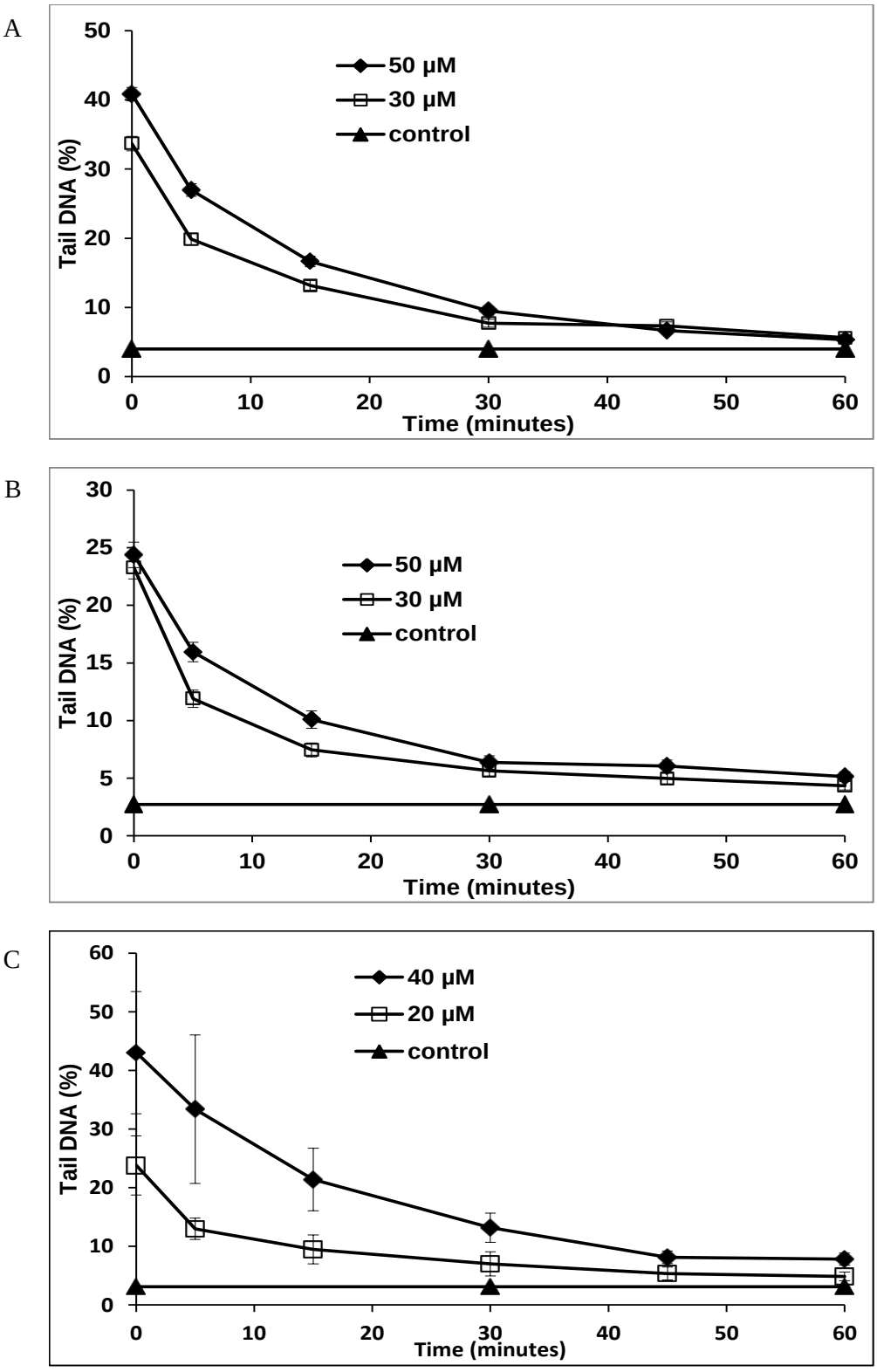


Figure 3.6 (a): Results of standard alkaline Comet assay analysis used to determine the kinetic of SSBs damaged-repair after H_2O_2 treatment on selected bladder cancer cell lines.Average of the three independent DNA damage-repair experiments for (A) RT112, (B) RT4 and (C) HT1376 cells treated with noted concentration of H_2O_2 (see text) for thirty minutes and allow to repair for up to 60 minutes. The results are presented as the mean ± SEM.

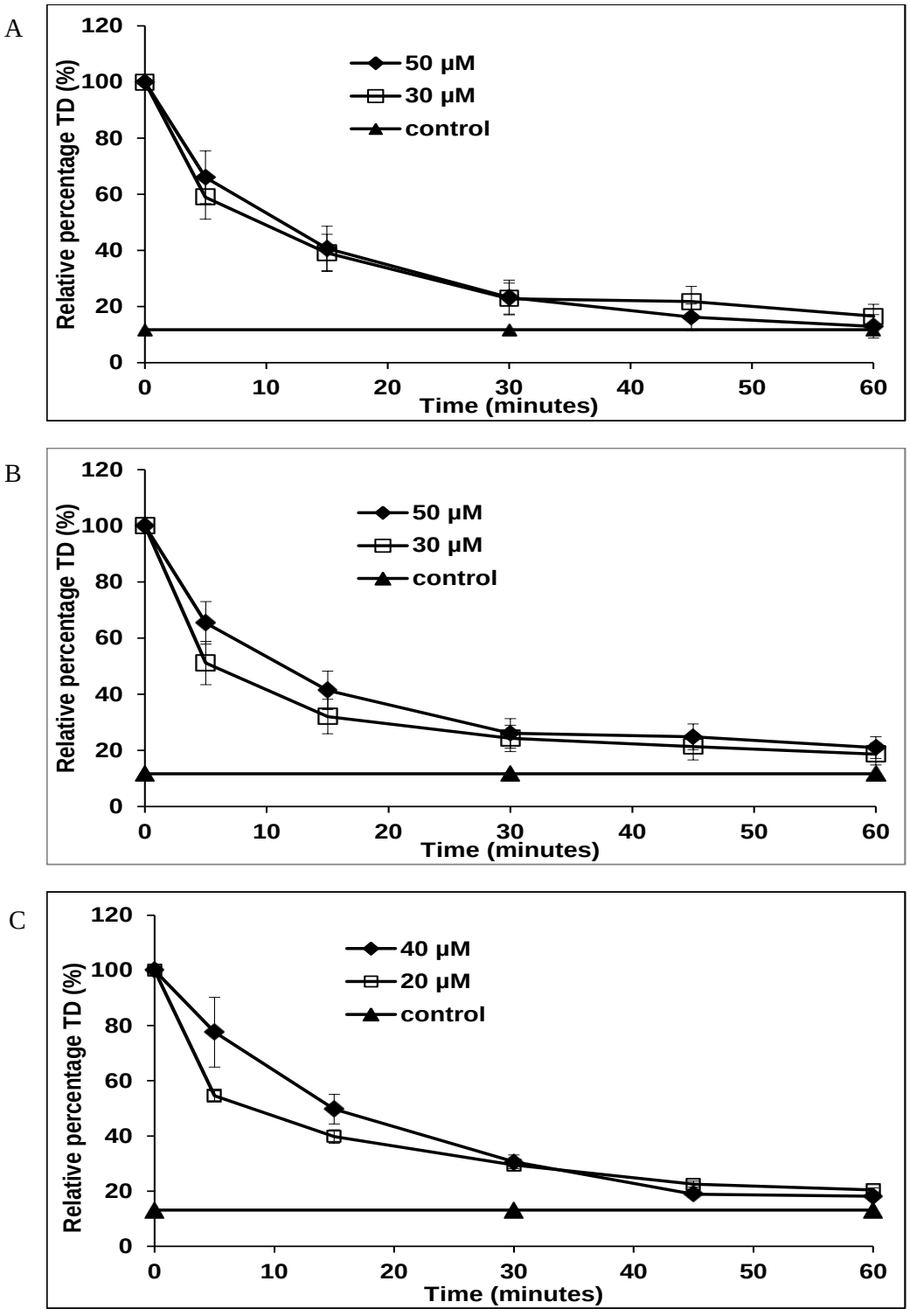


Figure 3.6 (b): Results of standard alkaline Comet assay analysis used to determine the kinetic of SSBs damaged-repair after H_2O_2 treatment on selected bladder cancer cell lines. Average of the three independent DNA damage-repair experiments for (A) RT112, (B) RT4 and (C) HT1376 cells treated with noted concentration of H_2O_2 (see text) for thirty minutes and allow to repair for up to 60 minutes. The results are presented as the mean ± SEM of the relative percentage TD of the SSB damaged-repair kinetics.

3.5.2 Determination of Fpg and hOGG1 dilution for the optimal assessment of DNA oxidatively damaged DNA base lesions.

3.5.2.1 Formamidopyrimidine glycosylase (Fpg).

Using the optimal parameters deduced from **Sections 3.5.1** and **3.5.2**, RT112, RT4 and HT1376 substrate cells were prepared ready for the subsequent experiments to determine the optimal concentration of the Fpg required for the assessment of DNA oxidatively damaged DNA base lesions.

During an initial attempt to determine the optimal Fpg enzyme concentrations, serial Fpg concentrations were prepared and co-incubated with lysed RT112 substrate cells (nucleoid bodies), embedded in the low melting point agarose on the microscope slides, at 37 °C for 30 minutes. From **Figure 3.7**, the ability of 50 µM H_2O_2 to induce SSBs in the RT112 cells was demonstrated in the treated control cells where approximately 35% TD was measured. The Fpg concentrations used during the initial developmental stage were 0.49, 1.47, 4.89, 14.66 and 48.88 U/ml. Even though the protocol seemed to be successful (i.e. Fpg treatment gave a greater response compared to the non-Fpg treatment, as shown in **Figure 3.7**), the optimal Fpg dilution could not be determined as there was only limited evidence of a 'plateauing' effect (indicative of an optimal Fpg concentration being achieved). Therefore, in the subsequent experiments, additional Fpg concentrations of 29.33 and 244.25 U/ml were added to the series of Fpg concentrations studied, bringing the total number of the concentrations used to eight (0, 0.49, 1.47, 4.89, 14.66, 29.33, 48.88 and 244.25 U/ml).

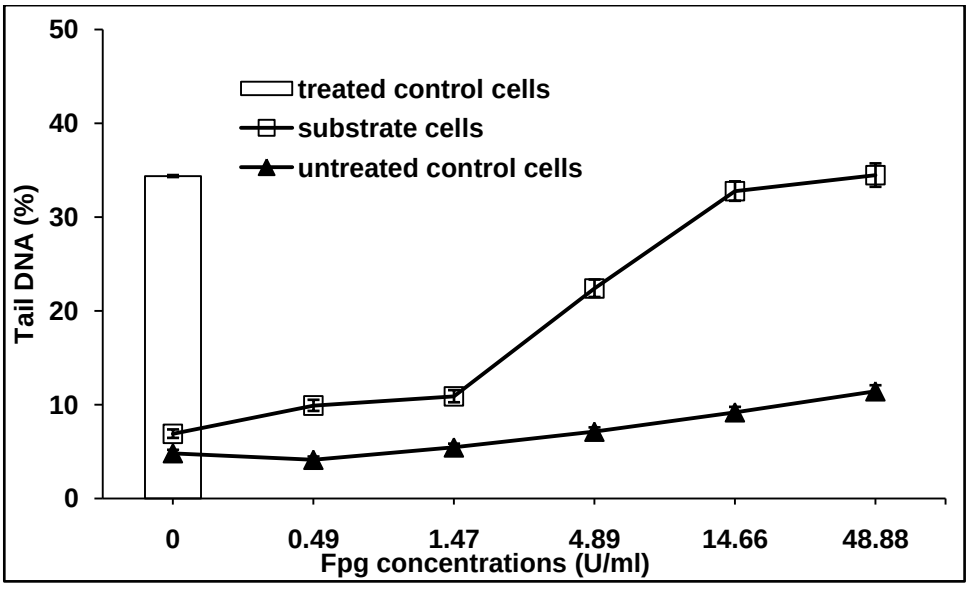


Figure 3.7: Determination of optimal Fpg concentration. Effect of serial Fpg enzyme concentrations (0, 0.49, 1.47, 4.89, 14.66 and 48.88 U/ml) added to RT112 nucleoids body for the initial establishment of the Fpg modified version of alkaline Comet assay. The results are presented as the mean ± SD of two independent experiments.

From **Figure 3.8 A**, it can be seen that treatment of RT112 cells with 50 µM H_2O_2 for 30 minutes induced approximately 30% TD (immediate SSB) in the treated control cells. For the untreated control cells, exposure to lower Fpg enzyme concentration (0.49 U/ml) failed to reveal any Fpg sensitive sites but there was a slight increase of the Fpg sensitive sites noted in the untreated control cells as the Fpg enzyme concentrations was increased from 4.89 to 48.88 U/ml. Clear evidence of indiscriminate adventitious cutting was detected at the highest Fpg enzyme concentration (244.25 U/ml), with this level of enzyme inducing ~ 25% TD in the untreated control cells.

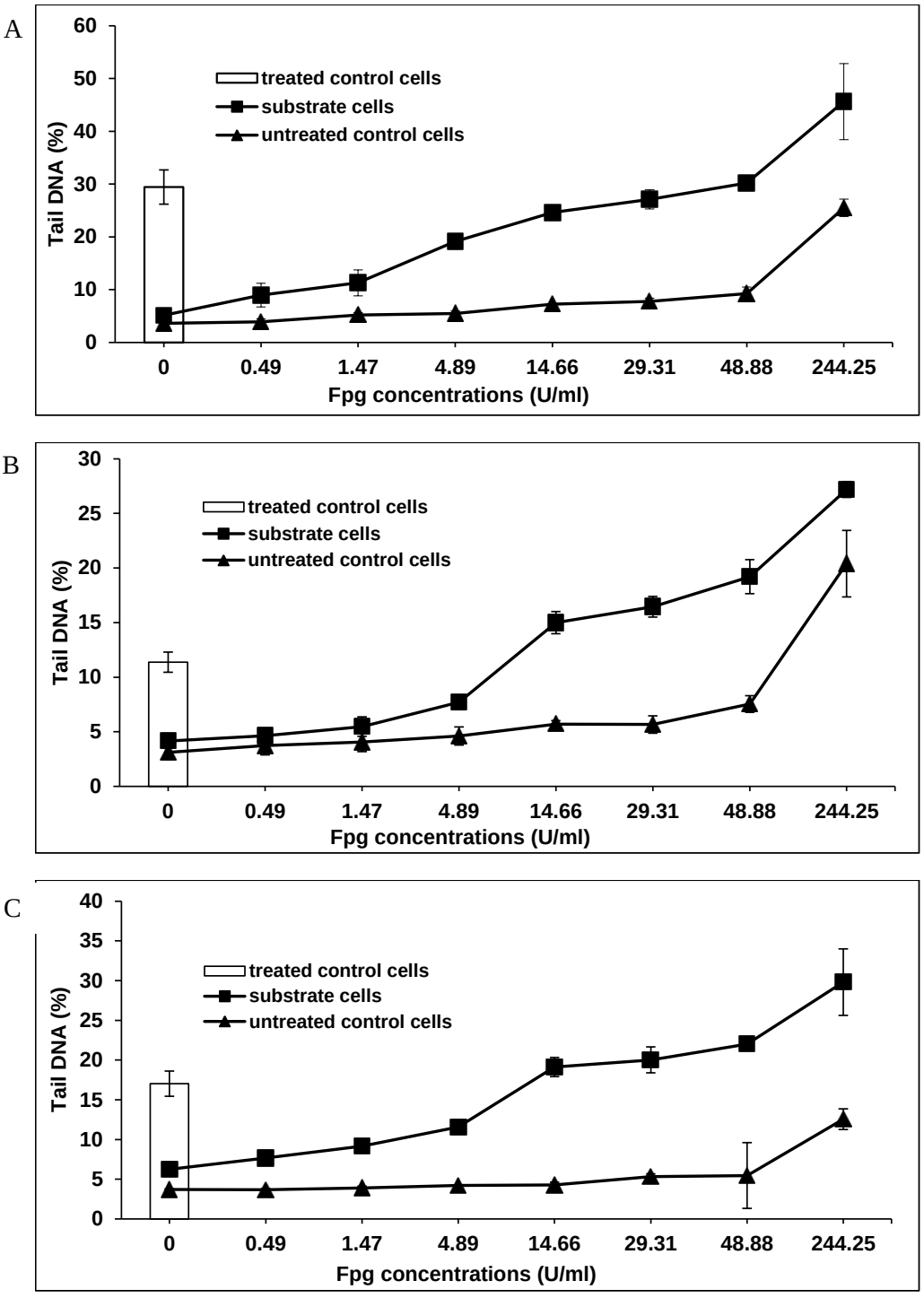


Figure 3.8: Determination of optimal Fpg concentrations. Effects of serial Fpg enzyme activity (0, 0.49, 1.47, 4.89, 29.31, 48.88 and 244.25 U/ml) added into the modified alkaline Comet assay protocols of (A) RT112, (B) RT 4 and (C) HT1376 bladder cancer cells. Results are means ± SEM of three independent treatments

For the prepared lysed substrate cells, gradual increases in the amount Fpg applied yielded higher measures of percentage tail DNA compared to the untreated control cells. For instance, there was approximately a 5% increase in TD measured after incubation with 1.47 U/ml Fpg whilst after incubation with 4.89 U/ml diluted Fpg, there was approximately a 14% increase in TD measured. Subsequently, as the amount of Fpg applied was increased (at 14.66 and 29.31 U/ml Fpg), evidence of a plateauing effect (indicative of the enzyme having a maximal effect) was apparent. The amounts of additional strand breaks measured at these two points are approximately 17% and 19% TD, respectively. Statistical analysis of RT112 data showed no significant increase in % TD measured between 0 and 0.49; 0.49 and 1.47; 14.66 and 29.31; 29.31 and 48.88 U/ml of the Fpg enzyme concentrations. There was a significant increased between 1.47and 4.89; 4.89 and 14.66; 48.88 and 244.25 U/ml of the Fpg enzyme concentrations. Similar to the untreated control cells, evidence of adventitious activity were observed for the substrate cells at the highest level of enzyme concentration used (244.25 U/ml). From **Figure 3.8 A**, the optimal Fpg to be used in the studies of mirtoselect's effect on the endogenous and exogenous DNA base lesions (see **Chapter 4**) were determined to be 14.66 and 29.33 U/ml.

From **Figure 3.8 B**, it can be seen that treatment of RT4 cells with 50 µM H_2O_2 for 30 minutes induced approximately 11% TD (immediate SSB) in the treated control cells. For the untreated control cells, treatment with low amounts of Fpg (0.49 U/ml) failed to reveal any the Fpg sensitive sites but there was a slight increase of the Fpg sensitive sites noted in the untreated control cells as the concentration of Fpg was increased from 1.47 to 244.25 U/ml Fpg. Clear evidence of indiscriminate adventitious cutting was detected at the highest concentration of the Fpg (244.25 U/ml), with this level of enzyme inducing ~ 20% TD in the untreated control cells. For the prepared

lysed substrate cells, gradual increases in the amount Fpg applied yielded higher measures of % TD compared to the untreated control cells. For instance, there was approximately 1.5% increase in TD measured after incubation with 1.47 U/ml Fpg whilst after incubation with 4.89 U/ml Fpg, there was approximately a 3% increase in TD measured. Subsequently, as the amount of Fpg applied was increased (at 14.66 and 29.33 U/ml Fpg), evidence of a plateauing effect (indicative of the enzyme having a maximal effect) was apparent. The amounts of additional strand breaks measured at these two points are approximately 10% and 12% TD, respectively. There were no significant increase between the % TD measured between 0 and 0.49; 0.49 and 1.47; 14.66 and 29.33; 29.33 and 48.88 U/ml Fpg concentrations. Significant increase in the RT4 data measured between 4.89 and 14.66; 48.88 and 244.25 U/ml of the Fpg. Even though it was noted that there was no significant difference in % TD measured between 29.33 and 48.88 U/ml of the Fpg, there is a clear trend evident for an increase in the measures between 29.33 and 48.88 U/ml of the Fpg, indicating the indiscriminate adventitious cutting is starting to take place. Therefore, the 48.88 U/ml Fpg was considered to be too high. Similar to the untreated control cells, evidence of adventitious activity was observed at the highest level of enzyme used (244.25 U/ml). From **Figure 3.8 B**, the optimal Fpg concentrations to be used in the studies of mirtoselect's effect on the endogenous and exogenous DNA base lesions (see **Chapter 4**) were determined to be 14.66 and 29.33 U/ml Fpg.

From **Figure 3.8 C**, it can be seen that treatment of HT1376 cells with 40 µM H_2O_2 for 30 minutes induced approximately 17% TD (immediate SSB) in the treated control cells. For the untreated control cells, treatment with low amounts of Fpg (0.49 U/ml) failed to reveal any of the Fpg sensitive sites but there was a slight increase in the Fpg sensitive sites noted in the untreated control cells as the concentration of Fpg was

increased up to the 48.88 U/ml of the Fpg. Clear evidence of indiscriminate adventitious cutting was detected at the highest concentration of Fpg (244.25 U/ml), with this level of enzyme inducing ~ 13% TD in the untreated control cells. For the prepared lysed substrate cells, gradual increases in the amount of Fpg applied yielded higher measures of percentage tail DNA compared to the untreated control cells. For instance, there was approximately a 4% increase in TD measured after incubation with 1.47 U/ml Fpg, whilst after incubation with a 4.89 U/ml Fpg, there was approximately a 7% increase in TD. Subsequently, as the amount of Fpg applied was increased (at 14.66 and 29.33 U/ml Fpg), evidence of the plateauing effect (indicative of the enzyme having a maximal effect) was again apparent. The amounts of additional strand breaks measured at these two points are approximately 15% and 16% TD, respectively. There were no significant increase in the % TD measured between 0.49 and 1.47; 14.66 and 29.33; 29.33 and 48.88 U/ml Fpg used in this study. Significant increase was measured between 4.89 and 14.66; 48.88 and 244.25 U/ml of the Fpg concentration. Even though it is noted that there was no significant difference of % TD between the 29.33 and 48.88 U/ml Fpg, there is a trend towards an increase in the measures between 29.33 to 48.88 U/ml Fpg, indicating that indiscriminate adventitious cutting is starting to take place. Therefore, the 48.88 U/ml Fpg was not considered an optimal Fpg enzyme activity, as it was too high. Similar to the untreated control cells, evidence of adventitious activity was observed at the highest level of enzyme used (244.25 U/ml). From **Figure 3.8 C**, the optimal Fpg dilutions to be used in the studies of mirtoselect's effect on the endogenous and exogenous DNA base lesions (see **Chapter 4**) were determined to be 14.66 and 29.33 U/ml.

3.5.2.2 human 8-oxoguanine DNA glycosylase (hOGG1).

As for the determination of the optimal dilution of Fpg (see **Section 3.5.2.1**), serial hOGG1concentrations (0, 0.05, 0.16, 0.53, 1.60, 3.20, 5.33 and 32.0 U/ml) were used to determine the dilution required for the optimal assessment of DNA oxidatively damaged DNA base lesions.

From **Figure 3.9 A**, it can be seen that treatment of the RT112 cells with 50 µM H_2O_2 for 30 minutes induced ~ 32% TD (immediate SSB) in the treated control cells. In the untreated control cells, treatment with hOGG1 failed to reveal any endogenous hOGG1 sensitive sites; similar levels of % TD were measured over almost the entire range of enzyme studied; ~4% TD. There was some evidence of indiscriminate adventitious cutting at the highest level of hOGG1 used (32.0 U/ml), where the amount of strand breaks measured was 7% TD in the untreated control cells; though this was more evident in the prepared substrate cells.

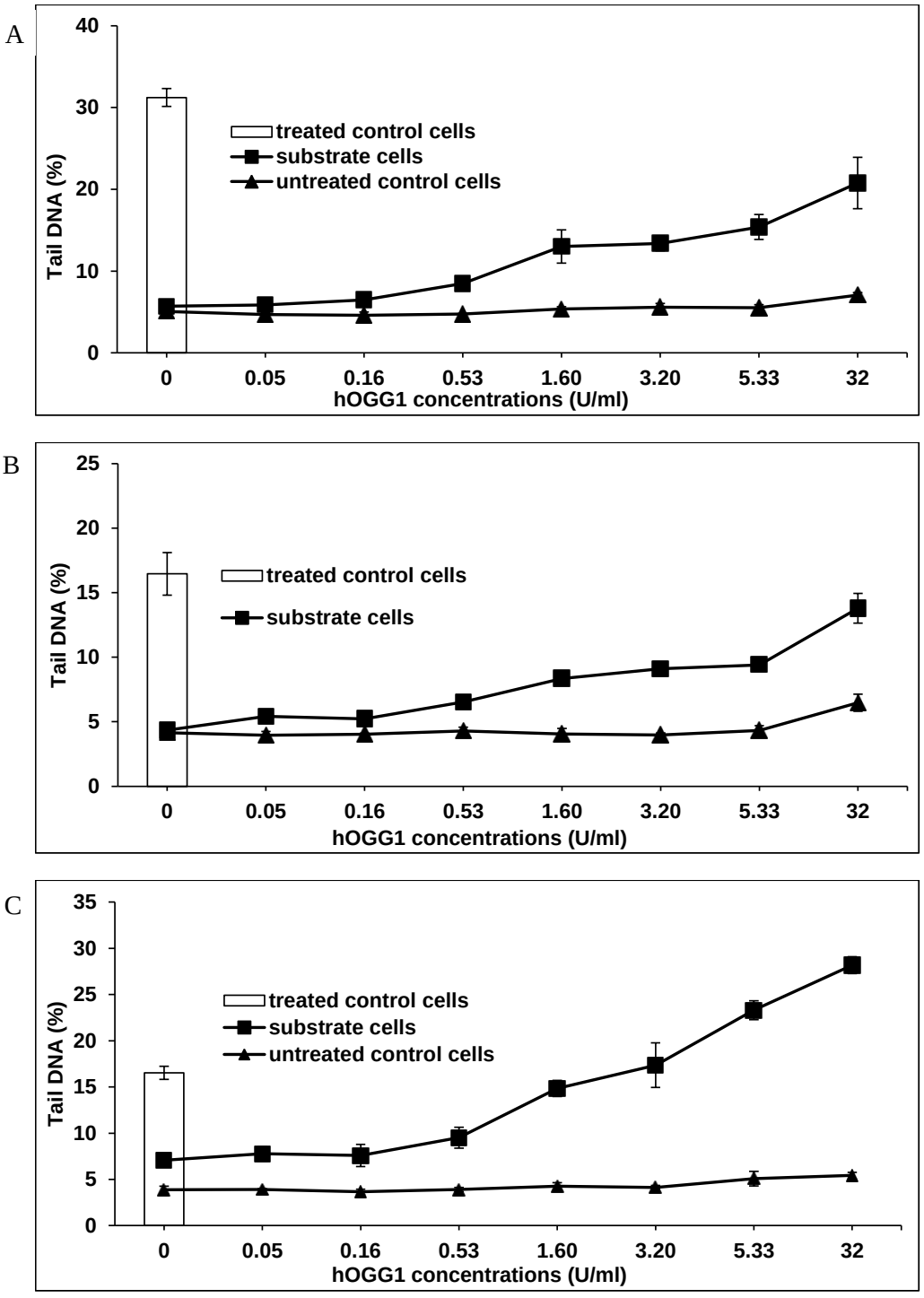


Figure 3.9: Determination of optimal hOGG1 concentration. Effects of serial hOGG1 concentrations (0,0.05, 0.16, 0.53, 1.60, 3.20, 5.33 and 32 U/ml) added into the modified alkaline Comet assay protocols of (A) RT112, (B) RT4 and (C) HT1376 bladder cancer cells. Results the mean ± SEM of three independent experiments.

For the prepared substrate cells, gradual increases in the amount of hOGG1 used yielded higher measures of % TD compared with the untreated control cells. There was an approximate 2% and 4% increase in TD measured at 0.16 and 0.53 U/ml hOGG1 respectively. Subsequently, as the amount of hOGG1 was increased (at 1.60 and 3.20 U/ml hOGG1) a plateauing effect (indicative of a maximal effect) was apparent. The amounts of additional strand breaks measured at these two points are approximately 8% TD each. There were no statistically significant difference in the % TD measured between 0 and 0.05; 0.05 and 0.16; 1.60 and 3.20 U/ml of hOGG1. Significant increase was measured between 0.53 and 1.60; 3.20 and 5.33; 5.33 and 32.0 U/ml of hOGG1. Similar to the untreated control cells, evidence of adventitious DNA strand cutting was observed with the highest amount of enzyme used (32.0 U/ml). From **Figure 3.9 A**, the optimal hOGG1 dilutions to be used in the studies of effect of mirtoselect on the endogenous and exogenous DNA base lesions (see **Chapter 4**) were determined to be 1.60 and 3.2 U/ml.

From **Figure 3.9 B**, it can be seen that treatment of the RT4 cells with 50 µM H_2O_2 for 30 minutes induced approximately 16% TD (immediate SSB) in the control cells. In the untreated control cells, treatment with hOGG1 failed to reveal any endogenous hOGG1 sensitive sites, with similar levels of % TD measured over almost the entire range of enzyme investigated (~ 4% TD), apart from the highest concentration (32.0 U/ml hOGG1), where there was some evidence of indiscriminate adventitious cutting, with a TD of 6.5%. As with the RT112 cells, this effect was again more evident in the substrate cells. For the prepared substrate cells, gradual increases in the amount of hOGG1 used yielded higher measures of percentage tail DNA compared with the untreated control cells. There was an approximate 1% and 2% increase in TD measured at 0.16 and 0.53 U/ml, respectively. Subsequently, as the amount of hOGG1 was

increased (at 1.60 and 3.20 U/ml hOGG1) a plateauing effect (indicative of a maximal effect) was again apparent. The amounts of additional strand breaks measured at these two points are approximately 4 and 5% TD, respectively. There were no significant increase in the % TD measured between 0 and 0.05; 0.05 and 0.16; 0.16 and 0.53; 1.60 and 3.20; 3.20 and 5.33 U/ml of hOGG1 concentration. Significant increase in % TD was measured between 0.53 and 1.6; 5.33 and 3.20 U/ml hOGG1. Similar to the untreated control cells, evidence of adventitious DNA strand cutting was observed after the highest amount of enzyme was used (32.0 U/ml). From **Figure 3.9 B**, the optimal hOGG1 concentrations to be used in the studies of mirtoselect's effect on the endogenous and exogenous DNA base lesions (see **Chapter 4**) were identified as 1.60 and 3.20 U/ml. The decision not to use the 5.33 U/ml hOGG1 as one of the optimal concentrations will be discussed later (see **Section 3.7**)

From **Figure 3.9 C**, it can be seen that treatment of the HT1376 cells with 40 µM H_2O_2 for 30 minutes induced approximately 17% TD (immediate SSB) in the treated control cells. In the untreated control cells, incubation with hOGG1 failed to reveal any endogenous hOGG1 sensitive sites; similar levels of % TD were measured over almost the entire range of enzyme being studied at ~ 4% TD. There was a very slight indication of indiscriminate adventitious cutting at the highest levels of hOGG1 used (5.33 and 32.0 U/ml), where the amount of strand breaks reached ~5.5 % TD. For the prepared substrate cells, gradual increases in the amount of hOGG1 yielded higher measures of percentage tail DNA compared with the untreated control cells. There was an approximate 4% and 6% increase in TD measured at 0.16 and 3.20 U/ml, respectively. Subsequently, as the amount of hOGG1 was increased (at 1.60 and 3.2 U/ml) a plateauing effect (indicative of a maximal effect) was slightly apparent when compared to the RT112 and RT4 cells. The amounts of strand breaks measured at these

two points are approximately 10 and 13% TD, respectively. There were no significant increase in the % TD measured between 0 and 0.05; 0.05 and 0.16; 1.60 and 3.20 U/ml of hOGG1. Significant increase in % TD was measured between 0.53 and 1.60; 3.20 and 5.33; 5.33 and 32.0 U/ml of hOGG1 enzyme concentrations. Similar to the untreated control cells, possible evidence of adventitious DNA strand cuttings were observed with the highest amounts of enzyme used (5.33 and 32.0 U/ml). From **Figure 3.9 C**, the optimal hOGG1 dilutions to be used in the studies of mirtoselect's effect on the endogenous and exogenous DNA base lesion (see **Chapter 4**) were identified as 1.60 and 3.20 U/ml.

Overall, for **Figures 3.8 and 3.9** the greater relative response of Fpg versus hOGG1 at each of the two optimal enzyme concentrations chosen is in line with the broader substrate specificity of Fpg (Collins et al., 2008). At the determined enzyme concentration, the increase in % TD was always greater using Fpg than for hOGG1. The smaller response for hOGG1 again reflects the narrower/more specific substrate requirements of hOGG1 (Smith et al., 2006).

3.6 Comparison between Fpg and hOGG1 specific activities.

The optimal concentrations of Fpg and hOGG1 to be used to reveal oxidatively damaged DNA base lesions has been successfully determined for the three bladder cancer cell lines employed in this study. This was facilitated by preparation of the substrate cells containing oxidatively damaged DNA base lesions. The incubation of lysed substrate cells embedded in low melting point agarose with Fpg (14.66 and 29.33 U/ml) and hOGG1 (1.60 and 3.20 U/ml) revealed a maximal amount of Fpg or hOGG1 sensitive sites in the form of further, additional DNA strand breaks that could be readily differentiated from the immediate DNA SSB induced by H_2O_2. This was based on the

assumption that all the SSBs induced by H_2O_2 in the substrate cells have completely repaired. The untreated control cells, which were also treated with the same dilutions of enzyme, revealed only minimal amounts of Fpg or hOGG1 sensitive sites. Furthermore, comparison of the percentage tail DNA differences between the substrate cells and untreated control cells is in line with the known substrate specificities of both enzymes.

Comparing the value of SSB induced by H_2O_2 with the DNA strand breaks caused by the activity of the Fpg and hOGG1offered valuble information on the amount of strand breaks caused by two different mechanisms. The data in **Figure 3.10 A** indicates no significant difference between the increases in % TD noted for each individual cell line when the 14.66 U/ml and 29.33 U/ml Fpg were used. Taking into account the % TD SSB measured in the RT112, RT4 and HT1376 treated control cells (25%, 7% and 17%), there were significant variations observed in the extent of Fpg sensitive sites revealed between each individual cell line. RT112 cells yielded the most Fpg sensitive sites, followed by HT1376, with RT4 having the lowest level of Fpg sensitive sites; this is a reflection of the immediate SSB yield. Overall, the percentage of Fpg sensitive sites revealed was between 71% to 180% relative to the level of immediate SSBs measured in control cells treated with H_2O_2.

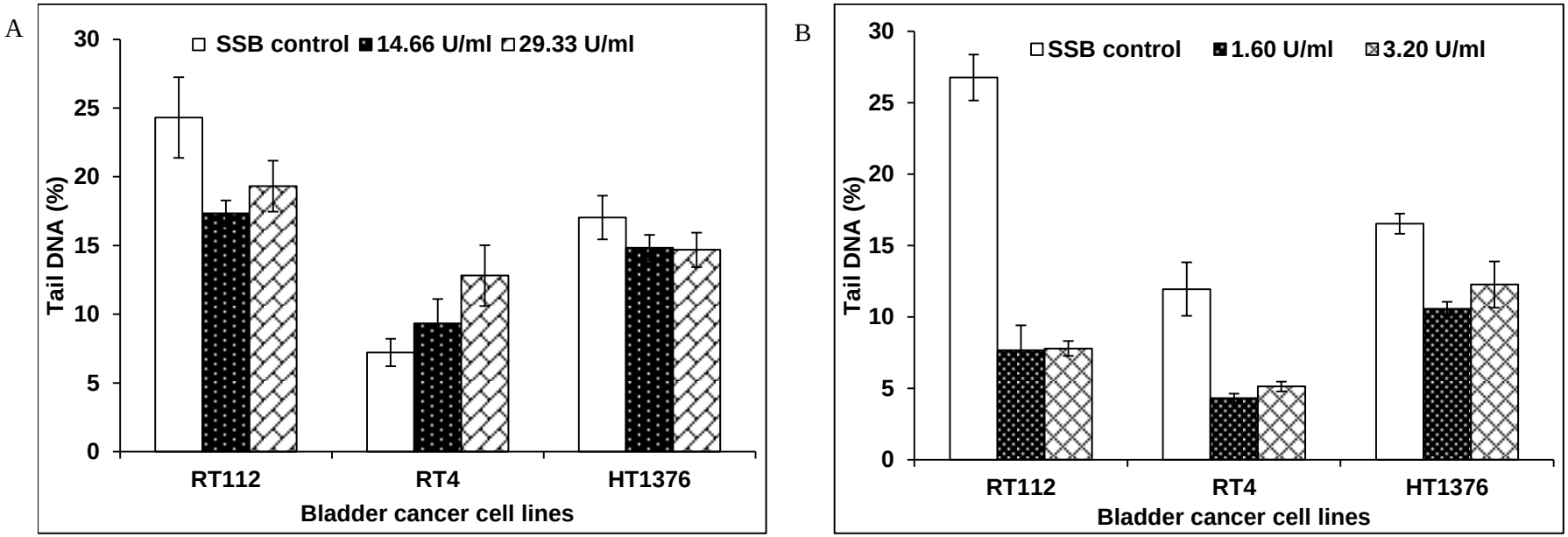


Figure 3.10: Percentage TD of the (A) Fpg and (B) hOGG1 sensitive site measured in three bladder cancer cell lines using modified alkaline Comet assay protocols. Difference in percentage tail DNA (representing Fpg and hOGG1 sensitive sites) were determined by substracting the value of TD measured in the untreated control cells from TD value of the "optimal" Fpg enzyme activity (14.66 and 29.33 U/ml) and hOGG1 (1.60 and 3.20 U/ml) of the substrate cells. In panel A, the control cells (induced by H_2O_2) produced 25%, 7% and 17% TD of SSB. DNA strand breaks cause by Fpg activities were about 20% in RT112, 12% in RT4 and 16% in HT1376.Whilst in panel B the control cells produced approximately 26%, 12% and 17% TD of SSB. DNA strand breaks caused by the hOGG1 activities were measured at 8% in RT112, 5% in RT4 and 11% in HT1376. Results are the mean of three independent experiments (± SEM).

Results have shown that the optimal dilutions for hOGG1 to maximally reveal oxidatively damaged DNA base lesions were 1.60 and 3.2 U/ml for all three bladder cancer cell lines studied. Again, the data in **Figure 3.10 B** reveals no significant difference between the increases in % TD noted for each individual cell line when the 1.60 and 3.2 U/ml hOGG1 used. However there were significant variations observed in the extent of hOGG1 sensitive sites revealed between each individual cell line. HT1376 cells yielded the most hOGG1 sensitive sites (despite only being originally treated with 40 µM H_2O_2, compared to 50 µM H_2O_2 for the other cells) followed by RT112, with RT4 having the lowest level of hOGG1 sensitive sites. This was probably caused by the oxidation of guanine by the free radicals in HT1376 leading to more formation of 8-oxoGua compared to the RT112 and RT4 cell lines. Overall, however, the percentage of hOGG1sensitive sites revealed was between 29% and 74% relative to the level of immediate SSBs measured in control cells treated with H_2O_2.

From **Figure 3.10 A** and **B**, the observation of differences between the levels of Fpg and hOGG1 sensitive sites indicates that approximately half of the Fpg sensitive sites revealed in RT112 and RT4 cells are 8-oxoGua, with the other half probably consisting of ring opened purines and other DNA base damage products that are recognised by Fpg. However, in HT1376 cells, nearly three quarters of the products revealed by Fpg are also hOGG1 sensitive sites (i.e. 8-oxoGua). From this observation it is deduced that there were dissimilar patterns of Fpg and hOGG1 activity towards the panel of bladder cancer cell lines used in this study.

Overall, the amount of Fpg and hOGG1 sensitive sites revealed by both enzymes used in this study were lower than the initial amounts of immediate SSBs induced in the treated control cells (except for RT4 Fpg sensitive sites). Dahm-Daphi and co-workers estimated that the ratio of base damage compared to SSB is ~1:1.95 (Dahm-Daphi et al.,

2000). Similarly, Collins and co-workers found that the amount of SSBs was higher than the yield of oxidised pyrimidines in HeLa cells treated with H_2O_2 (Collins et al., 1995). From **Section 3.5.1**, the initial dose response study indicated that the rank order of H_2O_2 sensitivity is as follows, HT1376 > RT112 > RT4 cells, with HT1376 therefore being most sensitive to H_2O_2; it was therefore thought that HT1376 may also be most prone to oxidatively damage DNA base lesion induction, followed by RT112 and RT4, respectively. There was a further inconsistency for the RT4 cells whereby the amount of SSBs measured was less than the strand breaks produced by Fpg activities (**Figure 3.10 A**). This inconsistency will be discussed in the following discussion section.

Overall, the differences observed in the relative levels of Fpg and hOGG1 sensitive sites are indicative of the specificities of both enzymes towards their substrates. Fpg recognises more oxidised purine moieties compared to the hOGG1 (Collins et al., 2008). Observations similar to this study have been reported by Smith et al., 2006.

3.7 Discussion

Exposure of the bladder cancer cells to hydrogen peroxide at 50 µM (for RT112 and RT4 cells) and 40 µM (for HT1376 cells) for 30 minutes induced an adequate amount of immediate DNA SSB and oxidatively damaged DNA bases (Fpg and hOGG1 sensitive sites). The initial dose responses did not demonstrate any significant difference between the two exposure times (15 and 30 minutes), especially at a H_2O_2 concentration of less than 30 µM; furthermore, there was no significant difference between the exposure times for the RT112 and HT1376 cells at any of the H_2O_2 concentration studied, however, for the RT4 cells there was a significant difference in the amount of immediate DNA SSBs induced following 15 and 30 minutes exposure to H_2O_2 concentrations of 40 µM and above.

An important criterion, with respect to the level of immediate SSBs induced, is that the H_2O_2 concentrations chosen [(50 µM (RT112 and RT4 cells) and 40 µM (HT1376 cells)] do not cause more than 50% TD, as there is the possibility of the Comet assay delivering a nonlinear response if the H_2O_2 induces too high a level of damage. At high levels of damage the amount of DNA in the "comet" head begins to decrease, ultimately to the point of depletion, and as this is approached the assay begins to deviate from linearity. Furthermore, longer exposure times and higher concentrations of H_2O_2 need to be avoided as they may cause excessive damage and subsequently lead to induction of apoptosis. Exposure of L6 rat muscle cells to 50-300 µM of H_2O_2 for between one and six hours caused cell death, whereby at 300 µM activation of the apoptotic pathway was detected two hours after the H_2O_2 exposure (Caporossi et al., 2003). Cells that have undergone apoptosis may lead to a false positive genotoxic response when analysed by the alkaline Comet assay (Choucroun et al., 2001).

The two concentrations of H_2O_2 (30 and 50 µM for RT112 and RT4; 20 and 40 µM for HT1376) used in the DNA repair experiments produced similar repair profiles. The SSBs induced in each cell line showed typical repair profiles (with both an immediate fast and subsequent slow component) and at 60 minutes the amount of % TD measured was similar to the control cells, indicating ~ 90% SSB repair. Similar observations have been reported by Collins and co-workers (Collins et al., 1995).

The optimisation of the H_2O_2-mediated induction of damage and establishing the time course of repair allowed for the subsequent creation of the 'substrate cells'. Production of the substrate cells was crucial, as these were needed to establish the amount of DNA glycosylase enzyme (Fpg & hOGG1) to be used for the optimal assessment of DNA oxidatively damaged DNA base lesions. The substrate cells were prepared in such a way that their DNA contained high amounts of oxidatively damaged DNA bases compared to normal untreated cells. To ensure that the lesions of interest are quantitatively detected, and that nonspecific breakage is not occurring, the optimal reaction conditions need to be established by varying the concentration of Fpg and hOGG1 enzymes and/or the time of incubation (Collins et al., 2008).

Inclusion of the DNA glycosylase enzymes Fpg and hOGG1 are useful modifications to the standard alkaline Comet assay because this increases both the assay's sensitivity and specificity. The Fpg enzyme is widely used for the detection of oxidatively damaged DNA bases, in particular 8-oxoGua and other oxidatively damaged purines (Collins et al., 1997; Collins et al., 1996). Fpg recognizes the common oxidised purine 8-oxoGua, 8-oxoAde, ring-opened purines (formamidopyrimidines) and abasic sites (Tchou et al., 1994), and its introduction into the Comet assay enabled the assay to be used to examine the effect of antioxidant supplementation in human volunteers (Moller and Loft, 2004). The human analogue of Fpg, hOGG1, has been demonstrated

to be more specific than Fpg, recognizing mostly 8-oxoGua and ring opened purines (methyl FapyGua) (Smith et al., 2006). Methyl FapyGua is produced upon the one-electron reduction of purines (Dizdaroglu et al., 2008).

The studies using various dilutions of Fpg indicated no significant difference in the amount of Fpg sensitive sites revealed when the lower enzyme concentrations, over the range of 0.49 to 4.89 U/ml, were used. Therefore, use of these lower amounts of enzyme would result in an underestimation of the Fpg sensitive sites actually present. Furthermore, it was also noted that for the bladder cancer cell lines treated with Fpg used in this study there was no significant difference between the measures obtained over the range of 14.66 U/ml up to 5.33 U/ml and that at the highest amounts of enzyme used (i.e. at a dilution of 244.25 U/ml) there was evidence of indiscriminate adventitious cutting of the DNA. Statistically, these results may indicate that the 48.88 U/ml dilution is an optimal dilution. However, there was an indication of some indiscriminate adventitious cutting of the DNA happening at this concentration. The beginning of an increase in the % TD response between 29.33 U/ml and 48.88 U/ml Fpg was evident in the non-treated control cells, this being most noticeable for the RT4 cells treated with Fpg (**Figure 3.8 B**). Therefore, the 48.88 U/ml Fpg could not be considered an optimal dilution. Consequently, to ensure that as much as possible of the Fpg sensitive sites will be revealed, but still avoiding the potential over estimation of the Fpg sensitive sites, only the 29.33 U/ml Fpg will be utilised in the subsequent experiments of this project.

Similar observations were noted when establishing the dilution of hOGG1 required to optimally detect oxidatively damaged base lesions. Non-significant increases in the responses were measured over the range 0.05 – 0.53 U/ml hOGG1 for all the cell lines tested and over 1.60 - 5.33 U/ml hOGG1 for the RT112 and RT4 cells

(but not for HT1376 cells); again as higher amounts of enzyme are used there was evidence of indiscriminate adventitious cutting of the DNA. For instance, in both the RT112 and RT4 cells (**Figure 3.9 A** and **B**), evidence of indiscriminate adventitious cutting of the DNA is apparent at lower than 3.20 U/ml. The beginning of an increase in the response between the range of 5.33 and 32.0 U/ml was evident in the non-treated control cells. Therefore, to ensure that as much of the hOGG1 sensitive sites will be revealed, but still avoiding any potential over estimation of the hOGG1 sensitive sites, only the 3.20 U/ml hOGG1 will be utilised in the subsequent experiments of this project.

Whilst studies to establish the required Fpg and hOGG1 dilution for the optimal assessment of DNA oxidatively damaged DNA base lesions were conducted, exhaustive variable time incubation experiments were not carried out, as there are numerous reports by other researchers concerning the optimal duration for the Fpg and hOGG1 incubation to reveal oxidatively damaged DNA base lesions (Smith et al., 2006; Williams and David, 1998). However, in addition to the information acquired from other researchers and studies, preliminary studies did indicate that incubation with hOGG1 required slightly longer (15 minutes more) to reveal the hOGG1 sensitive sites, when compared to Fpg incubation; 30 minutes incubation with hOGG1 did not reveal substantial amounts of hOGG1 sensitive sites, indeed, there was no difference noted between the non-treated control and prepared substrate cells after 30 minute hOGG1 treatment. The different incubation times required possibly reflect the different enzymes kinetics with respect to specific individual base lesions. Asagoshi and co-workers carried out a study on reaction kinetics of Fpg and hOGG1 using define oligonucleotides containing 8-oxoGua and a methylated analogue of Fapy (me-Fapy) at the same site. The reaction efficiency (k_{cat}/K_m) values of Fpg for 8-oxoGua and me-Fapy were comparable (0.14

and 0.13), this was also the case for hOGG1 (0.0015 and 0.0017). However, the k_{cat}/K_m values for hOGG1 both lesion were approximately 80 fold lower than Fpg. This implies that the hydrolysis of the imine bond and/or subsequent dissociation of hOGG1 proceeds more slowly (~ 10 fold) than that of Fpg (Asagoshi et al., 2000a).

It has been documented that higher amounts of SSB are produced compared to oxidatively damage DNA bases after exposure to H_2O_2 (Collins et al., 1995; Dahm-Daphi et al., 2000). This is in broad agreement with the data reported in **Figures 3.10 A** and **B,** which indicates greater levels of immediate SSBs being induced compared to both the levels of Fpg and hOGG1 sensitive sites induced, for the cell lines tested.

There have been situations reported where oxidative DNA lesions were not detected quantitatively through the use of DNA glycosylase enzymes. According to Collins and co-workers, shortly after the exposure of cells to ionising radiation, hardly any additional lesions were detected with Fpg-Comet assay, although it is known from alkaline elution, that the number of oxidative lesions induced was initially about the same as the number of single strand breaks. However, after 30 minutes repair incubation, the large majority of lesions present were Fpg sensitive (Collins et al., 2008). This phenomenon is most probably related to the tendency of X-ray-induced lesions to be clustered, with a mixture of strand breaks and oxidised bases in close proximity, and it's possible that only when the strand breaks have been repaired and the duplex structure restored, will the oxidised bases be recognized by the Fpg enzyme (David-Cordonnier et al., 2001; Olsen et al., 2003). To avoid any interference from the immediate SSBs induced by H_2O_2 treatment, the bladder cancer lines used in this study were allowed to undertake repair processing for 60 minutes following H_2O_2 treatment, which this study has indicated to be adequate for the near complete (~ 90%) repair of the SSBs. However, following the repair incubations there are still significant amounts

of oxidatively damaged DNA bases remaining, as determined by incubations with Fpg and hOGG1.

Despite the noted advantages of combining the Comet assay with endonuclease enzymes, there are some limitations that need to be considered. Fpg-Comet was used to determine the basal level of 8-oxoGua in human cells in a multicentre collaboration orchestrated by the European Standards Committee on Oxidative DNA Damage (ESCODD). The enzyme-based approach gave a median value for background oxidation of 0.34 per 10^6 Gua (in lymphocyte DNA), at least 10 times lower than the median values (4.24 per 10^6 Gua) obtained with chromatographic techniques (Gedik et al., 2005). The latter may suffer from oxidation of DNA during sample preparation, while the Comet assay, requiring much less sample manipulation, is relatively free of this artefact. However, as mentioned above, if some lesions are inaccessible to the enzyme, if they occur in clusters (i.e. several lesions in one DNA helical turn) or as tandem lesions (Bergeron et al., 2010), the assessed level of oxidatively damaged DNA base lesions may be underestimated. On the other hand, the level of damage detected with Fpg-Comet should not be taken as a reflection of 8-oxoGua levels, particularly if formamidopyrimidines are also present; accordingly, it is best to consider Fpg-Comet to measure of oxidised purines following exposure to a strong oxidant (unless one is sure that the oxidant specifically leads to 8-oxoGua). Furthermore, as Fpg has an associated AP lyase activity (nicking 3' to a baseless sugar), it may also cleave (and so detect) AP sites. However, on the assumption that AP sites are alkali-labile, these should be included in the breaks detected on the control slide, so that net enzyme-sensitive sites should include only the altered bases (Collins et al., 2008).

3.8 Summary statement

The following elements of this research project rely on the ability of Fpg and hOGG1, when combined with the Comet assay, to optimally reveal (and so allow assessment of) the relative levels of oxidatively damaged base lesions present in the DNA of the selected bladder cancer cell lines following their treatment with the putative chemopreventive agent mirtoselect. The failure to determine the optimal amount of Fpg and hOGG1 would compromise the future results acquired. For instance, using too lower an amount of the enzymes would lead to an underestimation of the amount of oxidatively damaged DNA base lesions present, whilst using too higher a concentration of the enzymes may lead an overestimation, due to indiscriminate cleavage of the DNA by the enzymes.

Using various serial enzyme concentrations of Fpg and hOGG1 against the prepared oxidatively damaged DNA base lesion-containing substrate cells, allowed for the successful establishment the optimal amount of Fpg and hOGG1 to maximally reveal oxidatively damaged base lesions, as being the amount of enzyme present in a (Fpg; 29.33 U/ml and hOGG1: 3.20 U/ml) of the respective enzyme stock, as supplied by the respective manufacturers; this being the dilution taken forward and used in the following studies.

Chapter 4.

The Effect of Mirtoselect on DNA Single Strand Break and Base Lesion Repair Capacity for both Endogenous and Exogenously-Induced Oxidatively Damaged DNA .

4.1 Introduction

4.1.1 DNA Damage and bladder cancer

All living organisms, both unicellular and multicellular, are constantly exposed to stress from environmental and endogenous DNA damaging agents, causing a series of different alterations in the DNA primary structure that range from simple to complex. It is estimated that a mammalian cell has to cope with around 55,000 single strand breaks, 13,000 depurinations and numerous other DNA lesions per day (Kohn, 2002). Endogenous damage can result from the production of ROS that arise as a side effect of mitochondrial energy metabolism. These ROS can cause a series of DNA base changes and are thought to act as causatives of cancer, ageing and neurological disorders (Floyd, 1999). External sources of damage can include UV irradiation (from exposure to sunlight), gamma rays and X-ray sources, food constituents that can directly damage the DNA or do so after metabolism (*e.g.* aromatic amines, found in a variety of foods, cause damage to the DNA and are highly mutagenic) (Berrington de Gonzalez and Darby, 2004; Lang et al., 1994; Rohrmann et al., 2009). If the damage is not repaired and the cell survives, this can give rise to permanent modifications in the DNA by the formation of mutations and other types of genomic damage (Kohn, 2002).

DNA damage either in the form of SSBs or base damage can occur in the DNA as the result of spontaneous hydrolysis or an attack by ROS arising from normal cellular metabolism or from exogenous sources (Kawanishi and Inoue, 1988; Lindahl, 1993). The consequences of unrepaired SSB in a proliferating cell can lead to blockage or collapse of DNA replication forks during the S phase of the cell cycle, which may lead to the formation of DSBs (Kouzminova and Kuzminov, 2006; Kuzminov, 2001). So far, SSBs have not been associated with carcinogenesis in urothelial cells.

Another type of DNA damage that has received a lot of attention is oxidatively damaged DNA. Normal cellular metabolism processes and inflammatory-based cellular defence mechanisms have been identified as contributors to the endogenous production of ROS that may contribute to the formation of oxidatively damaged DNA. The mitochondria electron transport chain has been established as one of the cellular producers of the ROS, such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$) (Chance et al., 1979; Liu et al., 2002b; Loschen et al., 1974). The mitochondria electron transport chain may leak electrons during the process of oxidative metabolism, with these electrons then reacting with surrounding oxygen molecules to form superoxide. Dismutation of these superoxide molecules, in turn, produces H_2O_2 (Loschen et al., 1974). ROS are also produced by human polymorphonuclear leukocytes (PMNs), for instance, by neutrophils that participate in defence mechanisms against the intrusion of microorganisms such as bacteria and fungi (Kobayashi et al., 2003; Nauseef, 2000). In addition to endogenous inflammatory mechanisms, exongenous organisms can also contribute to ROS formation through inflammatory mechanisms. A parasite known as *Schistosomiasis haematobium* is strongly associated with the occurrence of bladder cancer in Egypt, Sudan and Iraq (Mostafa et al., 1999). *Schistosomiasis haematobium* causes chronic inflammation by depositing its eggs in the bladder mucosa and submucosa and this can lead to the development of squamous cell carcinoma in humans (Mostafa et al., 1999; Rosin et al., 1994a).

Exogenous ROS can result from exposure to ionising radiation, carcinogens found in cigarette smoke and exhaust fumes, plus chemicals used in dye and latex industries. Ionising and ultraviolet radiation cause oxidative damage to DNA through either a ‘direct route’ or ‘indirect route’. The ‘direct route’ refers to the direct ionisation

of DNA by the radiation whilst the 'indirect route' involves the formation of free radicals such as hydroxyl radicals from the ionisation of DNA 'associated' water molecules, and these radicals can then interact with the DNA. For low-linear energy transfer (LET) ionising radiations, in cells, the indirect effect is thought to cause more damage and cell lethality than the former direct route (ca. 70:30) (Cadet et al., 2005). The metabolism of carcinogens prior to (& requisite for) their being excreted out from the body can also be a source of ROS (Murata et al., 2001; Zenser et al., 2002). Carcinogens, for instance 4-aminobiphenyl (4-ABP), are abundant in cigarette smoke and are closely linked to the initiation of bladder cancer. Murata and co-workers have suggested that the metabolism of 4-ABP produces ROS as a by-product and that these lead to oxidatively damaged DNA in epithelial bladder cells, in addition to DNA adduct formation, leading to the carcinogenesis in bladder cells (Kawanishi et al., 2002; Murata et al., 2001; Ohnishi et al., 2002).

The aetiology of bladder cancer is strongly associated with carcinogens, notably aromatic amines found in cigarette smoke, dye and latex industries and other related sources. Cigarette smokers have been shown to possess higher risk of developing bladder cancer compared to non-smokers (Castelao et al., 2001; Samanic et al., 2006). 2-nitroso-1-naphthol (NO-naphthol), a metabolite of 2-naphtylamine (2-NA), which is another bladder carcinogen found in cigarette smoke, has been shown to promote DNA damage preferentially at T>C>G residues in the presence of Cu (II) and NADH (Ohnishi et al., 2002). NO-naphthol has also been shown to induce the formation of 8-oxoGua in the presence of NADH and Cu (II) (Ohnishi et al., 2002) (**Figure 4.1**). This finding strengthens the involvement of oxidatively damaged DNA in the smoking related bladder cancer, in addition to DNA adduct formation (Ohnishi et al., 2002). The level of oxidatively damaged DNA 8-oxodeoxyguanosine (8-oxodGuo), was also

determined in DNA isolated from peripheral blood leukocytes of healthy adults (n=22) and patients (n=29) with superficial transitional cell carcinoma. Patients with transitional cell carcinoma of the bladder and control individuals were similar in age. The levels of 8-oxodG (8-oxodG/10^6 dG) in DNA from leukocytes of bladder cancer patients were significantly higher than those in controls (7.52 ± 0.58 versus 4.36 ± 0.19) (Akcay et al., 2003).

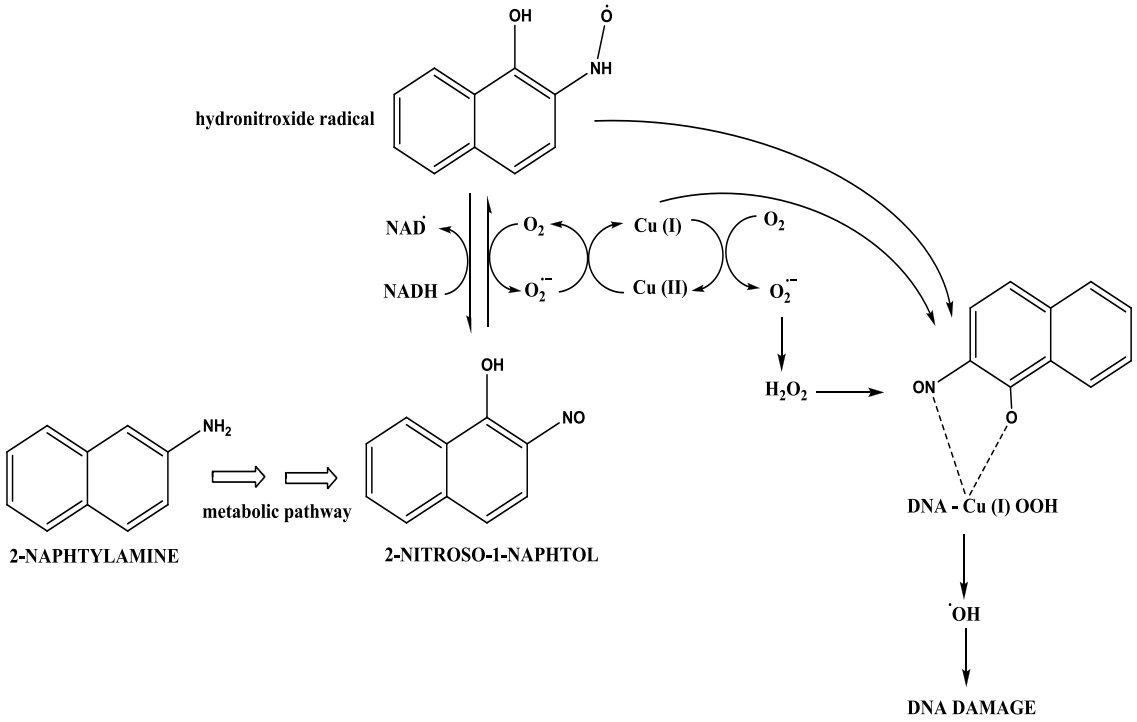


Figure 4.1: A proposed mechanism for oxidatively damaged DNA induced by 2-NA metabolites in the presence of Cu (II) and NADH. Adapted from Ohnishi et al., 2002.

Therefore, preventing ROS, especially •OH, from being formed and/or from attacking DNA could be a key strategy to limit the threat of oxidatively damaging mechanisms contributing to bladder carcinogenesis. Anthocyanins, which are known to be potent antioxidants, may be suitable candidates to limit/'neutralise' the free radical mechanisms responsible for DNA damage associated with bladder cancer.

4.1.2 Anthocyanins as antioxidants

Numerous studies carried out with anthocyanins have shown these compounds to be potent antioxidants (Kahkonen and Heinonen, 2003b; Liu et al., 2002a; Meyers et al., 2003; Sun et al., 2007; Zafra-Stone et al., 2007). An anthocyanin extract from roselle (*Hisbiscus sabdariffa*) exhibits high antioxidant capacity as determined using either the Ferric Reducing Ability of Plasma (FRAP), the Oxygen Radical Absorbance Capacity (ORAC) or the Total Antioxidant Status (TAS) tests (Tsai et al., 2002). Anthocyanins compounds isolated from bilberry, blackcurrant and cowberry which were identitified by High Performance Liquid Chromatography (HPLC) and HPLC and an electrospray interface with a mass spectrometer (HPLC/ESI-MS) , including malvidin-3-glycosides, , cyanidin-3-glycosides and delphidin-3-glycosides, have been shown to exhibit antioxidant properties when tested with studied in emulsified methyl linoleate, human low-density lipoprotein (LDL) *in vitro* and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay (Kahkonen and Heinonen, 2003b).

Part of the antioxidant properties of the anthocyanins are believed to be due to free radical scavenging. The anthocyanin compounds from three non-*Vitis vinifera* grape varieties (Marechal Foch, Norton, and Concord) and anthocyanins extracted from the berry fruits of the genera Rubus, Rhibes and Aronia, exhibit free radical scavenging activities when analysed by the DPPH radical assay (Benvenuti et al., 2004; Munoz-Espada et al., 2004).

The antioxidant properties of anthocyanins depend upon the arrangement of the functional groups on the compound structure. The positions of hydroxyl groups at position 3 of ring C and also at the 3´, 4´and 5´ positions of the B ring are closely related to the free radical scavenging properties of anthocyanins and other flavonoids (Burda and Oleszek, 2001; Heijnen et al., 2001). B ring hydroxyl groups more readily

donate electrons to hydroxyl, peroxyl and peroxynitrate radicals (Cao et al., 1997). Proficient radical scavenging activity relies on the ease of electron donation/hydrogen abstraction, from the above stated groups and also on the termination of the newly formed radicals (Seyoum et al., 2006; vanAcker et al., 1996). Anthocyanidin free radical scavenging activities seem to be better than that of anthocyanins (Wang and Stoner, 2008). Scavenging by hydroxyl groups is better than by glucoside moieties, whilst disaccharide is slightly stronger than monosaccharide, which is not present in anthocyanidins since these are aglycons (Ji-Wu, 2002).

Anthocyanins have also been demonstrated to induce phase II antioxidant and detoxifying enzymes *in vitro*. A study of anthocyanins on rat liver Clone 9 cells showed an elevation of the antioxidant capacity, including activated expression of glutathione-related enzymes (glutathione reductase, glutathione peroxidise and glutathione-S-transferase) as well as increased glutathione content. Moreover, the activity of NAD(P)H: quinine oxidoreductase (NQO1) was also promoted due to the treatment with anthocyanins (Shih et al., 2007). Shih and co-workers believe the molecular mechanism is associated with the activation of antioxidant response elements upstream of the genes involved in antioxidation and detoxification. Anthocyanins seem to play an important role in influencing ARE-regulated phase II enzyme expression, which is important in defending cells from oxidative stress (Shih et al., 2007).

The antioxidant protection exhibited by anthocyanins is also associated with the capability of these compounds to chelate free metal ions (Barbouti et al., 2001). The presence of copper and/or iron metal ions mediates the production of hydroxyl radicals ($^{\bullet}OH$) and hydroxide ion (^{-}OH) from hydrogen peroxide, the latter originating from either endogenous or exogenous sources. Sestili and co-workers have demonstrated the protective effect of selected plant derived phenolic compounds against DNA single

strand break induced by tert-butylhydroperoxide through metal ion chelation (Sestili et al., 2002). Silybin, which is a flavonoid compound, has also been shown to chelate Fe III when added into Jurkat cells (Gharagozloo et al., 2008).

There is also the possibility of anthocyanins upregulating DNA repair enzyme activity of the target cells. Collins and co-workers have demonstrated the ability of kiwi fruit extract to enhance antioxidant levels and stimulate DNA repair in lymphocytes (Collins et al., 2003). Utilising the hormone-responsive human prostate cancer cell line (LNCaP), Gao and co-workers have demonstrated the ability of a flavonoid compound, known as naringenin, to stimulate hOGG1 and DNA poly β *in vitro* (Gao et al., 2006). Recently, Duarte and co-workers have demonstrated the ability of the vitamin C derivative, ascorbic acid 2-phosphate (AA2P) to increase the expression of genes associated with DNA replication and repair (Duarte et al., 2009); AA2P treated fibroblasts also exhibited faster repair of oxidatively damaged DNA (Duarte et al., 2009).

4.1.3 Mirtoselect

Anthocyanins used in this study are a standardised extract of bilberry fruits obtained from Indena S.p.A, Italy. The extract is commercially known as mirtoselect. Full details are given in **Chapter 2, Section 2.1.1**

4.1.4 Outline of the present study

To determine the effect of mirtoselect on measures of endogenous oxidatively damaged DNA, the selected bladder cancer cell lines (RT112, RT4 and HT1376) will be treated with mirtoselect for seven days. At the end of day seven, the mirtoselect exposed

bladder cancer cells will be harvested and the level of endogenous damage assessed by the modified alkaline Comet assay protocols.

Similar to the endogenous approach above, the levels of exogenously-induced immediate SSBs and oxidative DNA damage will be assessed following treatment of the mirtoselect-treated cells with determined concentrations of H_2O_2. Subsequently, the treated cells were analysed by modified alkaline Comet assay protocols.

Besides determining the effect of mirtoselect on the amount of oxidatively damaged DNA induced by H_2O_2, this study also determined the effect of mirtoselect on the DNA SSBS and base lesion repair capacity of the bladder cancer cells.

4.2 Materials and Methods

The chemicals used in this section of the study were the same as those used in the previous section and are fully described in **Chapter 3**, **Section 3.2**.

4.2.1 Cells and culture conditions

A) For the determination of endogenous strand break damage and oxidatively damaged DNA base lesions

Approximately 5,000 and 30,000 RT112 cells, for control and mirtoselect treatment, respectively, 20,000 RT4 cells for both control and mirtoselect treatment and 25,000 HT1376 cells for both control and mirtoselect treatment were seeded in 25 cm^2 culture flasks. Five replicates of each control and treatment were prepared for each cell line. After 24 hours incubation to facilitate cell adherence, the growth media from each flask was removed. Cells designated for mirtoselect treatment, were treated with 50 µg/ml mirtoselect dissolved in the respective growth media. For control cells, complete growth media were used without any mirtoselect added. The mirtoselect dosing was carried out daily for a period of seven days. At the end of day seven, the growth media from each treatment and control culture flasks was removed. Cells were washed with PBS and harvested using trypsin/EDTA. The cells were kept on ice prior to Comet assay analysis for endogenous strand break damage and oxidatively damaged purine DNA base lesions as described below (**Section 4.3.2**).

B) For the determination of exogenously-induced strand break damage and oxidatively damaged DNA base lesion formation and repair

i) Immediate SSB formation and repair

Approximately 5,000 and 30,000 RT112 cells, for control and mirtoselect treatment, respectively, 20,000 RT4 cells for both control and mirtoselect treatment and 25,000 HT1376 cells for both control and mirtoselect treatment were seeded in 25 cm^2 culture flasks. For each experiment six culture flasks for the control and mirtoselect treated cells were prepared for each cell line, ultimately in triplicate (n=3 corresponding to 3 independent experiments). After 24 hours incubation to facilitate cell adherence, the growth media from each flask was removed. Cells designated for mirtoselect treatment, were treated with 50 µg/ml mirtoselect dissolved in the respective growth media. For control cells, respective complete growth media was used without any mirtoselect added. The mirtoselect dosing was carried daily for a period of seven days. At the end of day seven, the growth media from each treatment and control culture flasks was removed. Subsequently, the culture flasks were washed with PBS prior to the H_2O_2 treatment. The cells were treated with 50 µM H_2O_2 in serum free media on ice in the dark for 30 minutes. The H_2O_2 treated cells were then washed with PBS at the end of the treatment period. Following this, pre-warmed complete growth media (37 ºC) was added into the culture flasks of the respective cell lines and incubated at 37 ºC in 5 % CO_2 for 5, 15, 30, 45 and 60 minutes; the cells designated '0 minute' were immediately harvested (see next) and placed on ice. At the end of each time point, the cells were washed with PBS and then harvested using trypsin/EDTA. The cells were kept on ice prior to standard Comet assay analysis for immediate SSBs break formation and repair as described in **Chapter 3**, **Section 3.2.3**.

ii) Immediate oxidatively damaged base lesion formation and repair

Approximately 5,000 and 30,000 RT112 cells, for control and mirtoselect treatment, respectively, 20,000 RT4 cells for both control and mirtoselect treatment and 25,000 HT1376 cells for both control and mirtoselect treatment were seeded in 25 cm^2 culture flasks. For each experiment seven culture flasks for control and mitroselect treated cells were prepared for each cell line, ultimately in triplicate (n=3 corresponding to 3 independent experiments). After 24 hours incubation to facilitate cell adherence, the growth media from each flask was removed. Cells designated for mirtoselect treatment, were treated with 50 µg/ml mirtoselect dissolved in the respective growth media. For control cells, respective complete growth media was used without any mirtoselect added. The mirtoselect dosing was carried daily for a period of seven days. At the end of day seven, the growth media from each treatment and control culture flasks was removed. Subsequently, the culture flasks were washed with PBS prior to the H_2O_2 treatment. The cells were treated with 50 µM H_2O_2 in serum free media on ice in the dark for 30 minutes. The H_2O_2 treated cells were then washed with PBS at the end of treatment period. Following this, pre-warmed complete growth media (37 ºC) was added into the culture flasks of the respective cell lines and incubated at 37 ºC in 5 % CO_2 for 1, 3, 9, 15 and 24 hours; the cells designated '0 hour' were immediately harvested (see next) and held on ice (the start-time of the experiment was staggered to allow the cell harvesting to happen all at the same time). At the end of each time point, the cells were washed with PBS and then harvested using trypsin/EDTA. The cells were kept on ice prior to Comet assay analysis for both immediate SSBs break formation as described in **Chapter 3**, **Section 3.2.3**., plus oxidatively damaged purine DNA base lesions via the modified alkaline Comet assay protocol, as described below in **Section 4.2.2**.

4.2.2 Modified alkaline Comet assay protocol

The harvested cells were subjected to Comet assay analysis for oxidatively damaged purine DNA base lesions using the developed modified Comet assay protocol, consisting of the straightforward protocol, as described in **Sections 3.2.3.1 – 3.2.3.7**, with the following amendments. The harvested cells were used to prepare Comet assay slides as described in **Section 3.2.3.2** and the gel-embedded cells were then lysed and washed as described in **Sections 3.2.3.3 and 3.2.3.4**. Following the post-lysis wash with ice cold water for 5 minutes (see **Sections 3.2.3.4**), but prior to the unwinding/electrophoresis stages, the slides were subjected to a preconditioning wash with the enzyme reaction buffer (ERB) (see **Section 3.3.3**) for 5 minutes, and this was repeated three times. Next, 50 µl of 1/500 dilution (in ERB) of Fpg or hOGG1 or 50 µl ERB was added onto the gels and the gels was covered with 22 x 22 mm glass cover slips. The slides treated with Fpg or hOGG1 were then incubated for 30 or 45 minutes, respectively, at 37 ºC in a black humidified box (preventing light penetration whilst ensuring the LMP agarose gels is not hydrated) (**Figure 4.2**). After incubation, the slides were subjected to the DNA unwinding stage (see **Sections 3.2.3.4**) and from this point, the standard alkaline Comet assay protocols followed as described in **Sections 3.2.3.5** to **3.2.3.7**.

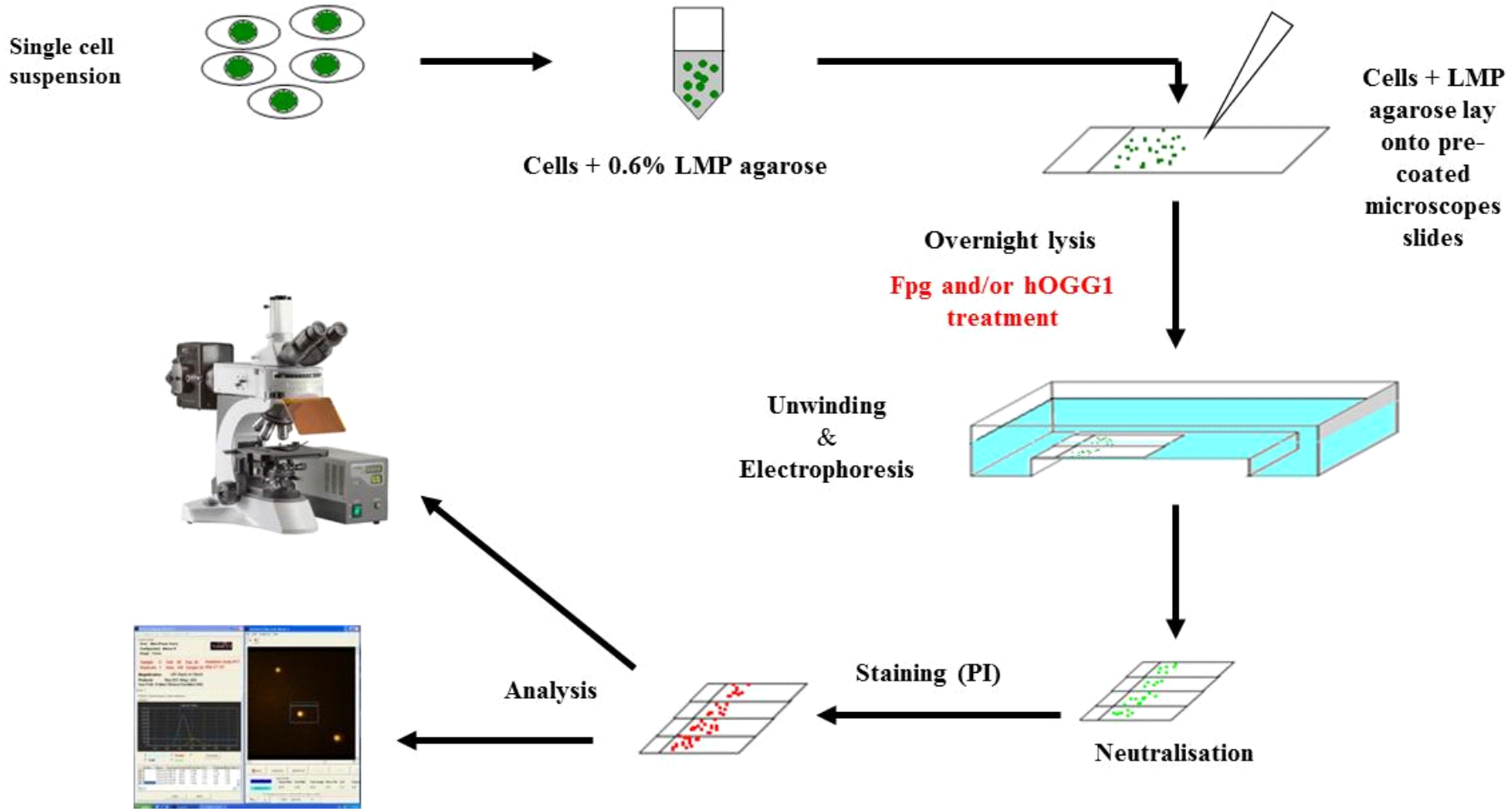


Figure 4.2: Schematic diagram of the modified alkaline Comet assay protocol. As for the standard protocol a single cell suspension is embedded in low melting point agarose on pre-coated slides and the embedded cells then lysed. However, the nucleoid bodies formed are then treated with the DNA glycosylase enzymes (Fpg or hOGG1). Following the endonuclease treatments the slides are subjected to unwinding and electrophoresis, neutralisation and staining and then analysis as in the standard protocol.

4.3 Results

A) The effect of mirtoselect on endogenous DNA lesion levels

Endogenous DNA lesion levels in RT112, RT4 and HT1376 cells treated with mirtoselect for seven days were determined using the developed modified alkaline Comet assay protocol. From **Figure 4.3 A**, overall there was a very low amount of endogenous base lesions detected in the RT112 bladder cancer cells as there was only approximately 1% and 2.5% additional % TD measured via hOGG1 and Fpg modified alkaline Comet assay, respectively, as compared to the control cells (exposed to ERB only). There was no significant difference between the measured amount of endogenous oxidised purine bases in the non-treated and mirtoselect treated cells as observed using the Fpg-Comet protocol. A similar observation was made using the hOGG1-Comet assay, where there was no significant difference on the amount of 8-oxoGua between the non-treated and mirtoselect treated cells. The level of tail DNA measured via the hOGG1 and Fpg Comet assay was higher than in the control cells, as expected. There were significant difference of the endogenous DNA lesion between the control and DNA glycosylase enzyme treated cells. Most probably this was due to the endogenous ROS, produced by the bladder cancer cell line under steady state conditions, contributing to a low amount of oxidised DNA base damage in the cells. In this case, mirtoselect did not interfere with the production of low amount of ROS. Wiseman and Halliwell have envisaged the possibility of minimal ROS production in the cells at the steady state condition (reviewed in Wiseman and Halliwell, 1996). However, **Figure 4.3 A** demonstrates that there was no evidence of a mirtoselect-mediated decrease in the endogenous DNA damage in the RT112 cells.

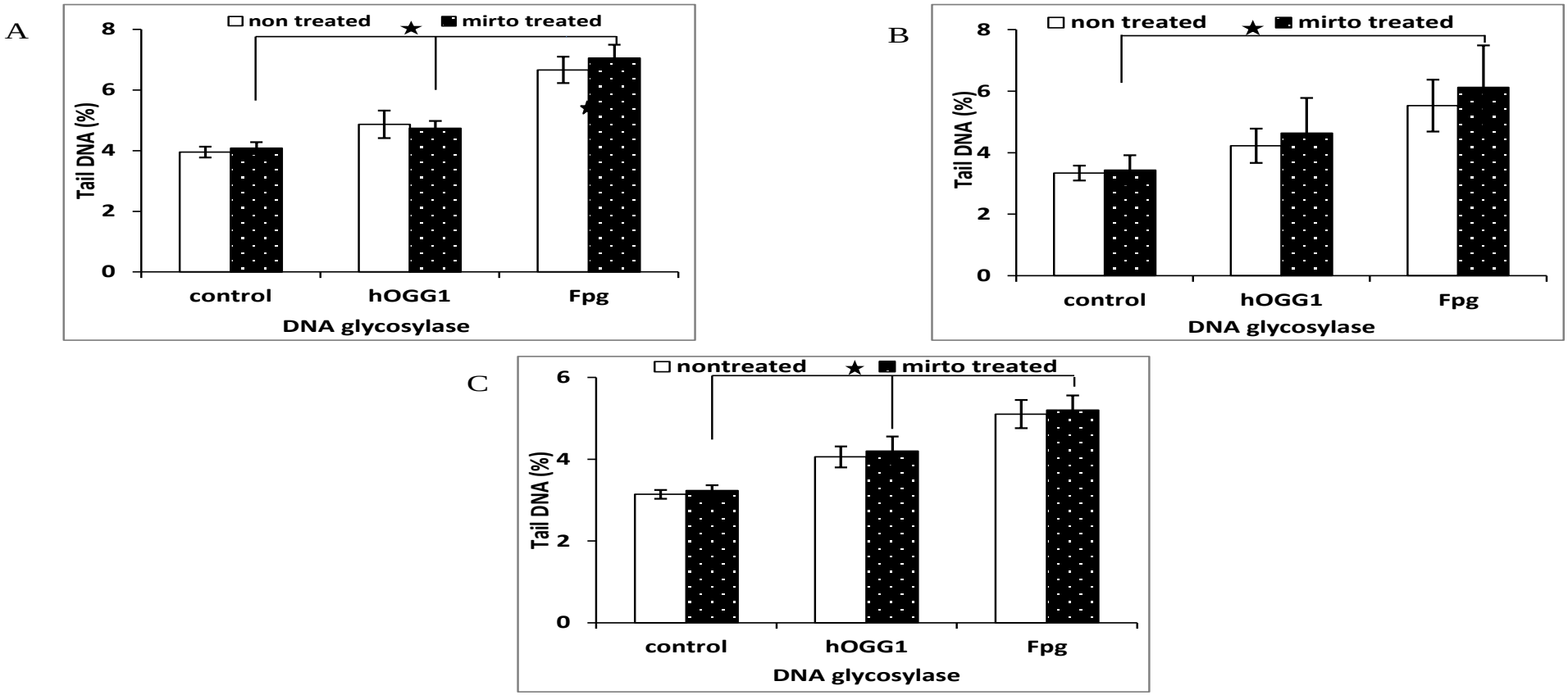


Figure 4.3: The effect of mirtoselect on the level of endogenous base lesions measured in RT112 (A), RT4 (B) and HT1376 (C) bladder cancer cell lines determined utilizing modified alkaline Comet assay methods. Results are the average of 3 independent experiments (mean ± SEM) (★ = $p<0.05$).

Similar to the RT112 cells, the amount of endogenous DNA base lesions present in both the RT4 and HT1376 bladder cancer cell lines treated with 50 µg/ml mirtoselect for seven days was determined by the Fpg and hOGG1 modified alkaline Comet assay (**Figure 4.3 B** & **C**). Again, there was a very low amount of endogenous DNA base lesions present in the control and mirtoselect treated RT4 and HT1376 cells; the amount of additional tail DNA revealed by the hOGG1and Fpg enzymes was only 1.1% and 2.5% TD, respectively, for RT4 cells when compared to control (**Figure 4.3 B**) and 1.0% and 2.5% TD, respectively, for HT1376 cells when compared to control (**Figure 4.3 C**). Also there was no significant difference between the amount of endogenous oxidised purine bases present in the non-treated and mirtoselect treated cells, as determined by the Fpg-Comet protocol. Similarly, there was no difference in the amount of 8-oxoGua between the non-treated and mirtoselect treated cells as determined by the hOGG1-Comet assay protocol. The amount of additional tail DNA measured in the hOGG1 and Fpg Comet assay was higher than the control cells, as expected. Similarly, non statitistical significance observed in RT112 also applied in RT4 and HT1376 bladder cancer cell line. Both **Figures 4.3 B**& **C** demonstrate that there was no evidence of a mirtoselect-mediated reduction in endogenous DNA damage in either the RT4 cells or HT1376 cells.

B) Effect of mirtoselect on SSBs repair capacity

The effect of mirtoselect on the repair capacity of the RT112, RT4 and HT1376 cells was investigated by exposing the mitroselect pre-treated and non-treated cells to 50 µM H_2O_2. The RT112, RT4 and HT1376 cells were sensitive to the H_2O_2 treatment with significant amounts of SSBs being induced by exposure to H_2O_2. Both non-treated control and the mitroselect pre-treated cells exhibited the typical characteristics of cells

repairing SSBs (**Figure 4.4 A, B & C**). There was an initial rapid decrease in single strand break levels during the first five minutes incubation, followed by a more modest repair activity for the remaining period, until 60 minutes where approximately 5% of TD remained. However, there was a significant difference ($p < 0.05$) in the amount of initial immediate SSBs induced by H_2O_2 observed between the mirtoselect pre-treated cells and the non-treated control cells (**Figure 4.4 A, B & C**).

H_2O_2 treatment managed to induce approximately 24% TD in the control cells whilst only 15% TD was induced in the mirtoselect pre-treated RT112 cells. However, the difference in the amount of initial SSB induced did not influence the repair kinetics of the RT112 cells as both cells showed near complete repair of the SSBs induced by 60mins. For the RT4 cells H_2O_2 treatment managed to induce approximately 24% TD in the non-treated control cells whilst only 15% TD was induced in the mirtoselect pre-treated RT112 cells; for the HT1376 cells H_2O_2 treatment managed to induce approximately 23% TD in the non-treated control cells whilst only 15% TD was induced in the mirtoselect pre-treated RT112 cells. However, for both sets of cells the difference in the amount of initial SSBs induced did not influence the repair kinetics of the cells as both cells showed near complete repair of the SSBs induced by 60mins

The difference in the amount of initial SSB induced is presumably due to a protective effect exhibited by mirtoselect and this observation will be further investigated and addressed in the following chapter.

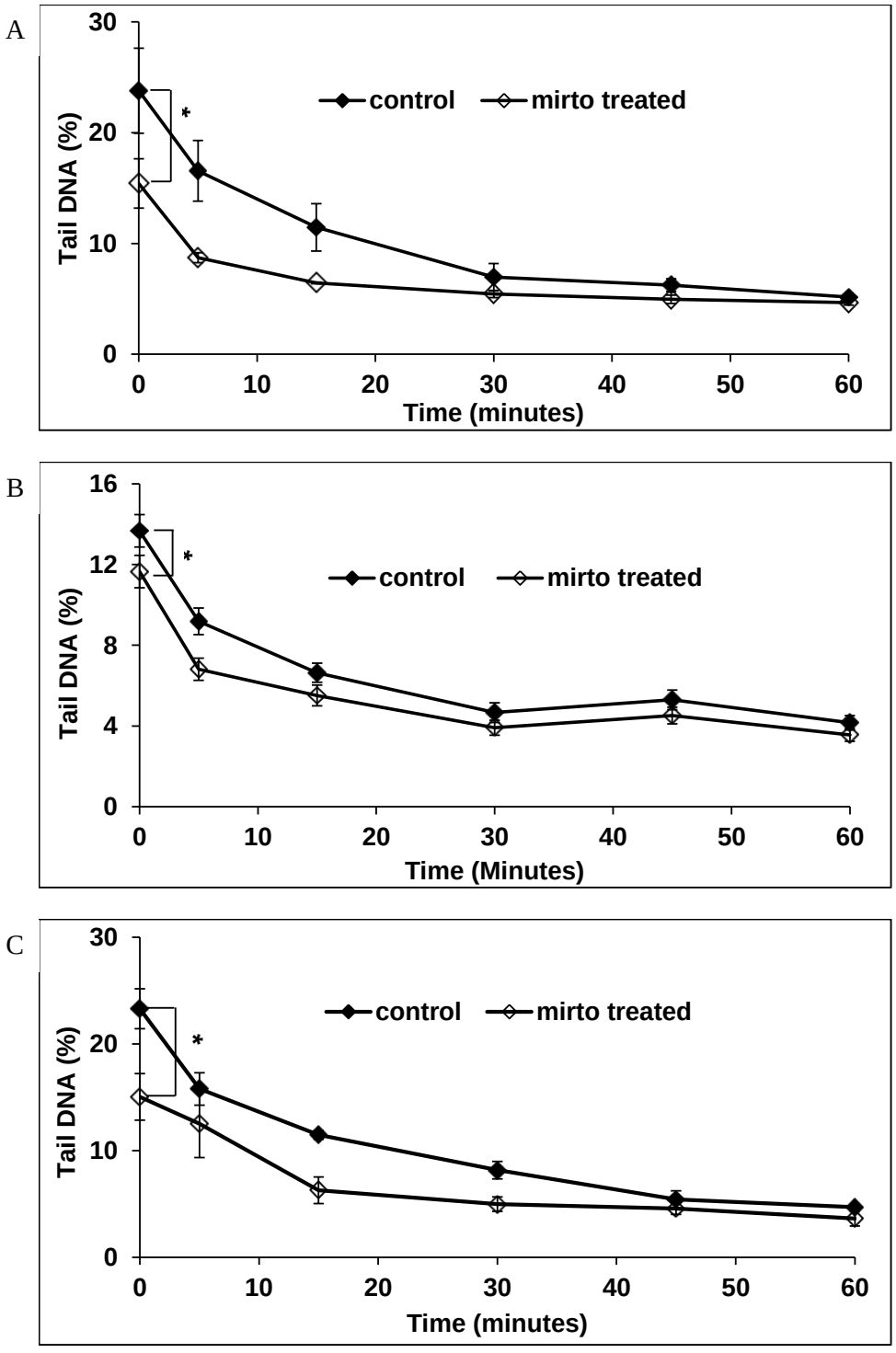


Figure 4.4: The SSB repair kinetics of (A) RT112, (B) RT4 and (C) HT1376 bladder cancer cells pre-treated with mirtoselect for 7 days and exposed to 50 µM H_2O_2 (RT112 & RT4) and 40 µM H_2O_2 (HT1376) determined via standard alkaline Comet assay methods. The cells were allowed to repair for 60 minutes at 37°C, 5% CO_2. In all the cell line used (RT112, RT4 and HT1376), the mirtoselect treated cells recorded lower amount of initial SSB (15%, 11% and 14% TD), whilst higher SSB were measured in control cells (25%, 14% and 23%) respectively. Results are the average of three independent experiments (± SEM) (* = $p < 0.05$).

C) The effect of mirtoselect on the formation and repair of exogenous DNA base lesions

i) Determining a timescale for oxidised DNA base lesion repair

There was no published information available concerning a likely timescale for the repair of oxidised DNA base lesions for the bladder cancer cell lines used in this study. Therefore, using RT112 cells as a candidate, typically representing the other cell lines used in this study, a pilot experiment was undertaken to determine the time required for virtually complete repair of DNA base lesions induced by exogenous H_2O_2. RT112 cells were exposed to 50 μM H_2O_2 on ice, in the dark. The cells were then allowed to repair the oxidatively damaged DNA for 1, 3, 6, 9, 15 and 24 hours (the start-time of the experiment was staggered to allow the cell harvesting to happen all at the same time). At the end of each time point, cells were washed with PBS and harvested using trypsin/EDTA. Cells from each time point were kept on ice prior to Comet assay analysis for both immediate SSB formation as described in **Chapter 3**, **Section 3.2.3** plus oxidatively damaged purine DNA base lesions via the modified alkaline Comet assay protocol, as described below in **Section 4.2.2**.

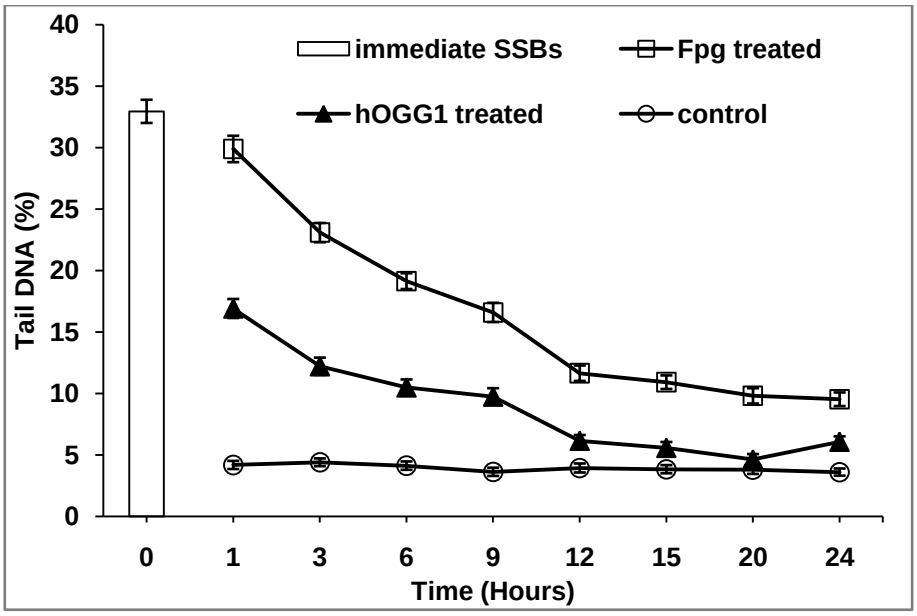


Figure 4.5: The effect of 50 μM H_2O_2 on the amount of immediate DNA SSBs and repair capacity of the RT112 cells determined via standard and modified alkaline Comet assay techniques. Result is from a single experiment consisting of triplicate incubations (mean ± SEM).

There was approximately 30% additional TD revealed as a result Fpg cleavage of enzyme sensitive sites and 17% additional TD revealed as a result hOGG1 cleavage one hour after the exogenous H_2O_2 treatment; there was a consistent amount of background damage in non-enzyme treated control cells (~ 5% TD) over 1 to 24 hours. **Figure 4.5** indicates that the amount of Fpg sensitive site was more than the hOGG1 sensitive sites, consistent with the respective substrate specificities of the two enzymes. As shown in **Figure 4.5** the timescale required for repair of the induced oxidised base lesions by RT112 cells was approximately 24 hours.

ii) The effect of mirtoselect on the exogenous DNA base lesion formation and repair capacity

For the RT112 cells, a proportion of the non-treated control and mirtoselect treated cells were set aside and used to assess the effect of mirtoselect on the level of immediate SSBs after the H_2O_2 treatment. For immediate SSB formation, the % TD

measured in the non-treated control cells was 25% whilst the immediate SSBs measured in the mirtoselect treated cells was significantly ($p < 0.05$) lower at approximately 17% (**Figure 4.6 A**). After 60 minutes incubation the amount of remaining Fpg and hOGG1sensitive sites induced by exogenous H_2O_2 was determined in both the non-treated control and mirtoselect pre-treated cells. A significantly ($p<0.05$) lower level of additional % TD Fpg sensitive sites (19%) was measured by the Fpg-Comet protocol for the mirtoselect treated cells as compared to the non-treated control cells (26%) one hour after the initial H_2O_2 treatment. A similar effect was also observed in the hOGG1 Comet data in which the amount of hOGG1 sensitive sites from the mirtoselect treated cells was lower (13%) than the amount of hOGG1 sensitive sites in the non-treated control cells (17%) one hour after the initial H_2O_2 treatment (**Figure 4.6 A**). After 3, 9, 15 and 24 hours of incubation the amount of Fpg and hOGG1 sensitive sites induced by the exogenous H_2O_2 was determined. After 3 and 9 hours incubation, the amount of Fpg and hOGG1 sensitive sites was still higher in the non-treated control cells compared with the mirtoselect pre-treated cells. However, after 15 and 24 hours incubation no real difference was observed between the non-treated control and the mirtoselect pre-treated cells and the %TD measured approached that of the background measures of the non-H_2O_2 treated control cells (**Figure 4.5**), indicating that the repair of oxidatively damaged DNA base lesions was nearly complete (**Figure 4.6 A**). Furthermore, the result indicated that mirtoselect has no effect on the repair kinetics of the respective DNA base lesions.

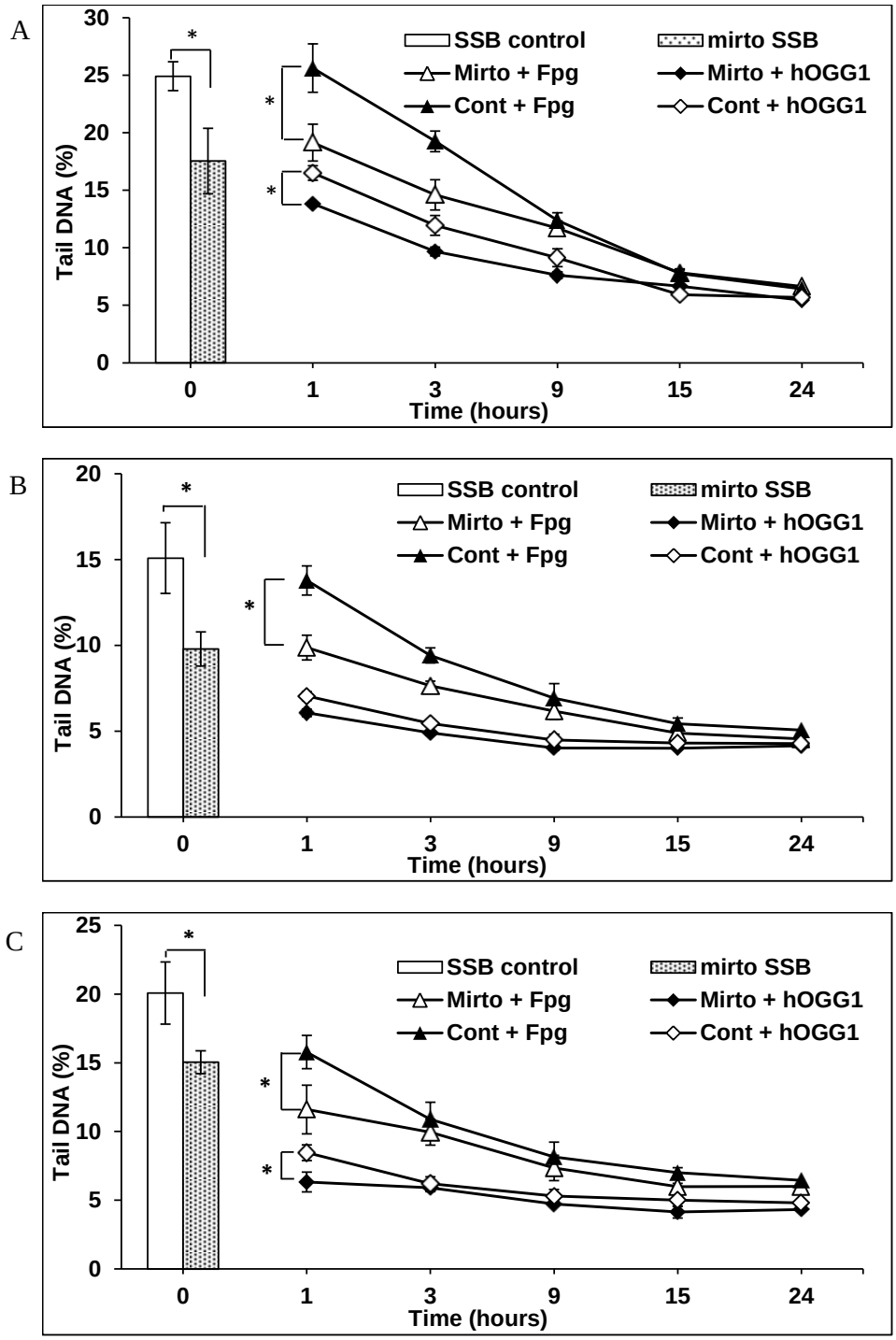


Figure 4.6: The effect of 50 µg/ml mirtoselect pre-treatment for seven days on the amount of immediate DNA SSBs and base lesions induced, plus the latter's repair, for RT112 (A), RT4 (B) and HT1376 (C) cells after treatment with either 50 µM H_2O_2 (RT112 and RT4) or 40 µM H_2O_2 (HT1376) determined by standard and modified alkaline Comet assay protocols. Results are the average of three independent experiments (± SEM). A significant difference between immediate DNA SSBs measured in the non-mirtoselect control and mirtoselect treated cells and between Fpg and hOGG1 sensitive sites in the control and mirtoselect treated cells is indicated (*$p < 0.05$).

Similar results were also observed for the RT4 and HT1376 cells (**Figure 4.6 B** & **C**). Again, significantly ($p < 0.05$) lower levels of immediate SSBs were measured in mirtoselect treated cells compared to the non-treated control cells, and the levels of Fpg and hOGG1sensitive sites one hour after the initial exogenous H_2O_2 treatment were again lower for the mirtoselect treated cells compared to the control cells. In the HT1376 cells levels of both Fpg and hOGG1sensitive sites were judged to be significantly ($p < 0.05$) lower in the mirtoselect treated cells compared to the non-treated control cells (**Figure 4.5 C**), however, for the RT4 cells only the level of Fpg sensitive sites were significantly ($p < 0.05$) lower in the mirtoselect treated cells compared to the non-treated control cells (**Figure 4.6 B**). Also, after 3 and 9 hours incubation, the amount of Fpg and hOGG1 sensitive sites was still higher in the non-treated control cells compared with the mirtoselect pre-treated cells. However, after 15 and 24 hours incubation no real difference was observed between the non-treated control and mirtoselect pre-treated cells as the % TD detected approached that of the background levels in non-H_2O_2 treated control cells (**Figure 4.5**), indicating that the repair of oxidatively damaged DNA base lesions was nearly complete. As for the RT112 cells, the results noted for the RT4 and HT1376 cells indicated that mirtoselect has no effect on the repair kinetics of the respective DNA base lesions.

4.4 Discussion

This part of the project exploited the hOGG1 and Fpg modified Comet assay protocols that were developed at an earlier stage of this study (**Chapter 3**). The DNA glycosylase enzymes used in these assays recognise various Fpg and hOGG1 sensitive lesions, cleaving the DNA at these lesion sites, so creating further 'additional' strands breaks, which are detected and measured as 'additional' % TD. With the inclusion of DNA glycosylase enzymes, the difference between the initial immediate single strand breaks, measured by standard alkaline Comet assay, and the new additional strand breaks, determined by the modified alkaline Comet assay, enables an assessment of the amount of endonuclease sensitive DNA base lesions present in the DNA of the bladder cancer cell lines. An example of this sort of study is the work of Collins and co-workers who determined the amount of strand breaks and pyrimidine oxidation in HeLa cells and lymphocytes by using an endonuclease III-comet procedure (Collins et al., 1995).

Oxidised DNA lesions have been implicated in many pathological processes, including mutagenesis, carcinogenesis and aging (Halliwell, 1989). Normal cellular metabolism results in the continuous generation of reactive oxygen species (ROS), such as superoxide radicals or non-radical hydrogen peroxide (Bowling and Beal, 1995). The hydroxyl radical is the most reactive toward biological molecules and produces multiple modifications in DNA, such as base and sugar damage, and DNA protein cross-links (Dizdaroglu, 1992). DNA damage is continuously ongoing, but under normal circumstances it is efficiently repaired (Demple and Harrison, 1994; Laval et al., 1998).

It is proposed that damage to DNA by ROS occurs naturally at a low steady-state level and is manifest as damaged DNA bases, for instance 8-oxoGua, which can be detected in nuclear DNA of human tissue and cells (Musarrat and Wani, 1994; Wiseman and Halliwell, 1996). The pattern of damage to the purine and pyrimidine

bases suggests that at least some of the damage occurs by $^{\bullet}OH$ attack, suggesting that $^{\bullet}OH$ is formed in the nucleus *in vivo* (Halliwell and Dizdaroglu, 1992). The omnipresent nature of the damage was further supported by the inability to acquire DNA samples with zero oxidative DNA damage (Collins et al., 1993; Halliwell and Dizdaroglu, 1992; Harris et al., 1994).

Immediate SSBs and oxidatively damaged DNA base lesions were successfully induced by the H_2O_2 in all three bladder cancer cell lines used in this study, as measured by the straightforward and modified alkaline Comet assay. It is proposed that H_2O_2 induces immediate DNA SSBs and oxidatively damaged DNA base lesion formation through $^{\bullet}OH$ generation. H_2O_2 reacts with the transitional metal ion to produce $^{\bullet}OH$ as described in the Fenton reaction (**equation 4.1**) (Termini, 2000).

$$H_2O_2 + M^{n+} \longrightarrow {}^{-}OH + {}^{\bullet}OH + M^{(n+1)+} \quad \textbf{(equation 4.1)}$$

M= metal ion, n= oxidation number

RT112, RT4 and HT1376 bladder cancer cell lines were treated with mirtoselect (50 µg/ml) for 7 days to investigate the ability of the constituent anthocyanins to protect against DNA damage. The results obtained using the hOGG1 and Fpg modified alkaline Comet assays showed there was no mirtoselect-mediated decrease of endogenous DNA base lesions in any of the three cell lines. In 1999, Pool-Zobel and co-workers studied the protective mechanisms of an anthocyanin extract from Black *choke* berry, elderberry, Macqui and Tintorera grapes as well as the pure compounds (cyanidin chloride, idaein chloride, cyanin chloride and vitamin C) which showed superior protective effects against H_2O_2-induced SSBs in human tumour HT29 clone19A cells. However, none of the compounds tested were able to reduce the endogenous oxidative

DNA damage when measured using ENDO III- and Fpg-Comet assays, which is consistent with the results observed here (Pool-Zobel et al., 1999).

Since there was no effect on endogenous DNA damage after treatment with mirtoselect, it may be proposed that mirtoselect does not interfere with endogenous free radicals activities in the particular bladder cancer cell lines studied. One possibility is that the available cellular antioxidant protection mechanisms are adequate to limit and deal with the endogenous DNA base damage without relying on the anthocyanin compounds to act as further antioxidants. Indeed, as the levels of endogenous oxidatively damaged DNA bases detected appear quite low (with both endonuclease-Comet protocols yielding no more than 3% increase in TD), this implies that the available cellular antioxidant protection mechanisms are sufficient. Furthermore, it is unlikely that the additional scavenging capacity of the added mitroselect will significantly impinge or impact upon the already high scavenging capacity of the cell.

Studies have shown many flavonoids to be effective antioxidants in a wide range of chemical oxidation systems, as demonstrated by their ability to scavenge peroxyl radicals, alkyl peroxyl radicals, superoxide hydroxyl radicals, and peroxynite in aqueous and organic environments (Duthie and Crozier, 2000). Recent studies have suggested that dietary flavonoids may protect against free radical–induced damage to DNA by a mechanism other than just direct free-radical scavenging (Ross and Kasum, 2002). Results from pulse radiolysis studies and a plasmid test system have shown that flavonoids can reduce the incidence of single-strand breaks in double-stranded DNA as well as residual base damage through fast chemical repair mechanisms (Anderson et al., 2000).

In this study, selected bladder cancer cell lines pre-treated with mirtoselect did not exhibit any differential effect with respect to their repair capacity towards H_2O_2-

induced DNA strand breaks. Generally, all the bladder cancer cell lines demonstrated typical repair kinetics for SSB repair, with an initial rapid decrease in SSB levels during the first 5-10 minutes of repair incubation, followed by a more modest repair activity for the remaining period, achieving virtually complete repair of the DNA strand breaks after 60 minutes. In a similar study by Collins and co-workers, HeLa cells incubated at 37 °C for one hour after H_2O_2 treatment showed the same level of breakage as untreated cells, indicating near complete repair over this timescale (Collins et al., 1995).

However, there was a significant difference in the amount of initial DNA strand breaks induced by the H_2O_2 between the mirtoselect pre-treated bladder cancer cells and the non-mitroselect treated control cells, even though the same concentration of H_2O_2 was used (note: for HT1376 cells the concentration of the exogenous H_2O_2 used was 40 μM) and the same treatment conditions were applied to all the bladder cancer cell lines. Significantly more DNA strand breaks were observed in the control cells than the cells that were pre-treated with mirtoselect. The differences observed indicate that mirtoselect possesses protective properties against the damaging effects of exogenously added H_2O_2. A similar observation was made by Lazze and co-workers, who, using rat hepatoma cells (MH1C1) and rat smooth muscle cells (SMC1) treated with different anthocyanin compounds, and showed significant reductions in immediate SSB formation, (Lazze et al., 2003). Human neuroblastoma cells (SH-SY5Y) and human promyelocytic cells (HL-60) pre-treated with anthocyanins from blackberry and boysenberry have also demonstrated a protective effect towards H_2O_2 induced SSB formation (Ghosh et al., 2006). The findings of these other researchers that flavonoid compounds can have protective effects against SSB formation supports the notion that anthocyanins present in mirtoselect would be able to mediate similar effects. The protective effects exhibited by anthocyanins are strongly associated with the chemical

structure of these compounds (Cao et al., 1997; Kong et al., 2003; RiceEvans et al., 1997) and this will be further discussed in the following chapter of this thesis.

The use of H_2O_2 to induce DNA damage in the bladder cancer cell lines in this study was appropriate as H_2O_2 has been shown to be one of the normal by-products of cellular metabolism. H_2O_2 is also able to diffuse freely across cell membranes. Horvathova and co-workers have demonstrated that SSBs were a major product detected by the alkaline Comet assay immediately after H_2O_2-treatment whilst alkali-labile sites representing only a minor fraction of the damage noted (Horvathova et al., 1998). The H_2O_2-induced DNA damage has also been shown to depend on the presence of redox active ions in HeLa cells (Jornot et al., 1998).

Compared to immediate SSB, oxidatively damaged DNA base lesions require longer repair times. DNA strand breaks in H_2O_2 treated Hela cells are rapidly lost after 1 hour incubation at 37 ºC and subsequently oxidised pyrimidines were successfully determined using ENDO III (Collins et al., 1995). Initially an attempt to measure the ENDO III sensitive sites in the cells was not successful as the sensitive sites was superimposed on an already high level of strand break (Collins et al., 1995). Normal lymphoblast cells (AG10111 and GM9387) have been shown to repair oxidised base lesions, induced by fluorescent light, efficiently after 6 hours of incubation (Lipinski et al., 1999). In a study by Collins and co-workers, lymphocytes were treated with 0.1 µM of a photosensitizer (Ro 19-8022) and 2 min irradiation (33 cm from a 1000 W halogen lamp, on ice) to induce 8-oxoGua in DNA, and required approximately 2 hours for repair to become apparent (Collins and Harrington, 2002). El-Zein and co-workers incubated lymphocytes cells at 37 ºC for 1 hour allowing the cells to recover after being treated with 60 µM H_2O_2 for 15 minutes at 4 ºC. Subsequently the cells were treated with hOGG1 for 40 minutes at 37 ºC in order to study the DNA repair capacity in

human peripheral blood lymphocytes amongs smokers (El-Zein et al., 2010). Indirectly, allowing the cells to recover for 1 hour prior to hOGG1 treatment reflecting the important of allowing the immediate SSB of the lymphocytes to be repaired before determining the oxidatively damaged DNA.

Until now, there were no data available regarding the timescale needed for the bladder cancer cell lines used in the present study to undertake repair of oxidatively damaged DNA base lesions *in vitro*. Based on the pilot experiment, presented in **Figure 4.5**, 24 hours is proposed as a suitable timescale to study the repair of induced oxidatively damaged DNA base lesions. Due to the labour intensive and time consuming nature of this study, the pilot experiment was only carried out in RT112 cells. On the basis that the studied cells are all from the same cancer site/site of origin the determined repair timescale (24 hours) was also applied to RT4 and HT1376 cells.

In addition to there being a significant difference in the amount of initial DNA strand breaks noted between the mirtoselect pre-treated and the non-treated control cells, the former also had a significantly lower amount of induced purine base damage compared with the non-mirtoselect treated control cells. This was the case for two of the cell lines but not the RT4 cells where the difference between the levels of Fpg sensitive sites was not judged significant. However, despite the non-significance of this result, there was still a lower level of 8-oxoGua/hOGG1 sensitive sites in the bladder cells pre-treated with mirtoselect compared with the non-mirtoselect treated control cells. Non-significant anthocyanin protection against oxidatively damaged DNA formation was also observed when rat hepatoma cells (MH1C1) and rat smooth muscle cells (SMC1) were pre-treated with anthocyanins exposed to *tert*-butyl-hydroperoxide (TBHP) (Lazze et al., 2003).

It is well established that the substrate specificity of Fpg is broader than that of hOGG1 (Krokan et al., 1997; Smith et al., 2006). This accounts for the results shown in **Figures 4.4 A, B** and **C** and Figures **4.6 A, B** and **C** in which the Fpg-Comet assay responses were generally higher than hOGG1-Comet responses.

As for the repair of induced SSBs, the repair kinetics of the induced oxidatively damaged DNA bases was not affected by pre-treatment of the cells with mirtoselect, with both non-treated control and mirtoselect pre-treated cells achieving near complete repair of induced DNA base damage by 15-24 hours.

The DNA damage detected in the form of SSBs and DNA base damage was presumably mediated by $^{\bullet}OH$ arising from the exogenous H_2O_2. Undoubtedly the protective effects against the H_2O_2-induced damage observed study came from the mirtoselect that was used to pre-treat the bladder cancer cell lines. These observations raise the question of how the anthocyanins mechanistically protect the DNA from the H_2O_2-induced damage?

Several mechanisms of action have been proposed as to how anthocyanins are able to protect the DNA from free radicals. Protection properties exhibited by the anthocyanins could be due to the ability of the compounds to scavenge free radicals (Delazar et al., 2010; Wang and Ballington, 2007). The other mechanism proposed stems from their ability to activate cellular antioxidant enzymes, especially those from the glutathione family (Masella et al., 2005; Shih et al., 2007). The protection properties of anthocyanins are also proposed to be associated with their ability to chelate redox active metal ions, reducing the availability of free metal ions to react with oxidising agents (in this instance H_2O_2) resulting in less free radicals being formed, so leading to less formation of oxidatively damaged DNA (Barbouti et al., 2001; Melidou et al., 2005). Therefore, further experiments were designed and undertaken to investigate the

mechanisms underlying mirtoselect mediated protection of of bladder cancer cell lines from H_2O_2-induced oxidatively damaged DNA.

4.5 Summary statement

The developed modified alkaline Comet assays (**Chapter 3**) were used to assess mirtoselect's effect on both endogenous and induced oxidatively damaged DNA levels in the three bladder cancer cell lines RT112, RT4 and HT1376. Using the modified assays it has been shown that pre-treatment of the bladder cancer cells with mirtoselect had no effect on detected levels of endogenous oxidatively damaged DNA nor did influence the repair kinetics of H_2O_2-induced immediate SSBs. However, mirtoselect pre-treatment did offer appreciable protective effects against both H_2O_2-induced immediate SSBs and oxidatively damaged DNA base lesions in all of the three bladder cancer cell lines. The differences observed in the levels of damage between the mirtoselect pre-treated and non-mirtoselect treated control cell supports the proposal that mitroselect contains potent antioxidants. The following Chapters describe studies undertaken to elucidate the mechanism of action exhibited by mirtoselect in protecting against oxidative stress.

Chapter 5.

Establishing a Protective Mechanism for Mirtoselect (1): The Effect of X-ray Irradiation on Mirtoselect Pre-treated Bladder Cancer Cell Lines *In Vitro*.

5.1 Introduction

5.1.1 DNA damage by ionising radiation

Radiation, passing through cells or tissues, produces chemical changes by depositing energy, causing ionisation and excitation of the molecules with which it interacts. Damage to cellular DNA can either occur by direct energy deposition in the DNA molecule (the 'direct' effect), or through 'indirect' attack on DNA by hydroxyl radicals generated from water radiolysis (Cadet et al., 2003; Ward, 1988). The $^{\bullet}$OH radicals cause base modifications by attack at sites of high electron density. Interactions with guanine preferentially occur at the C8 and C4 positions, producing 8-oxoGua or FaPyGua, respectively. For pyrimidines, the major product is thymine glycol (Cadet et al., 2003). In cells about two-thirds of X-ray or gamma-ray induced DNA damage is caused by indirect action; heavy particles, such as neutrons, act mostly by direct ionisation (Borek, 2004).

DNA damage, brought about by radiation exposure is, in the first instance, metal ion independent; this is in contrast to H_2O_2-induced DNA damage. This was confirmed by the fact that radiation-induced DNA damage could not be prevented by pre-incubating fibroblast cells with a concentration of deferoxamine mesylate (DFO) that efficiently abolishes H_2O_2-induced DNA damage (Duarte et al., 2007).

H_2O_2-induced DNA damage and ionising radiation-induced DNA damage share a high degree of similarity with regards to the final products generated; both H_2O_2 and radiation induce DNA SSBs, DSBs and oxidatively damaged DNA base lesions. This is a consequence of both insults causing oxidatively damaged DNA, both via the effects of $^{\bullet}$OH radicals. However, the requirement of metal ions for H_2O_2-induced DNA damage is unique to the latter and this will be exploited to investigate the mechanism of protection afforded by mirtoselect that was observed in **Chapter 4**. The basic

mechanisms of H_2O_2-induced DNA damage and ionising radiation-induced DNA damage are depicted in **Figure 5.1**.

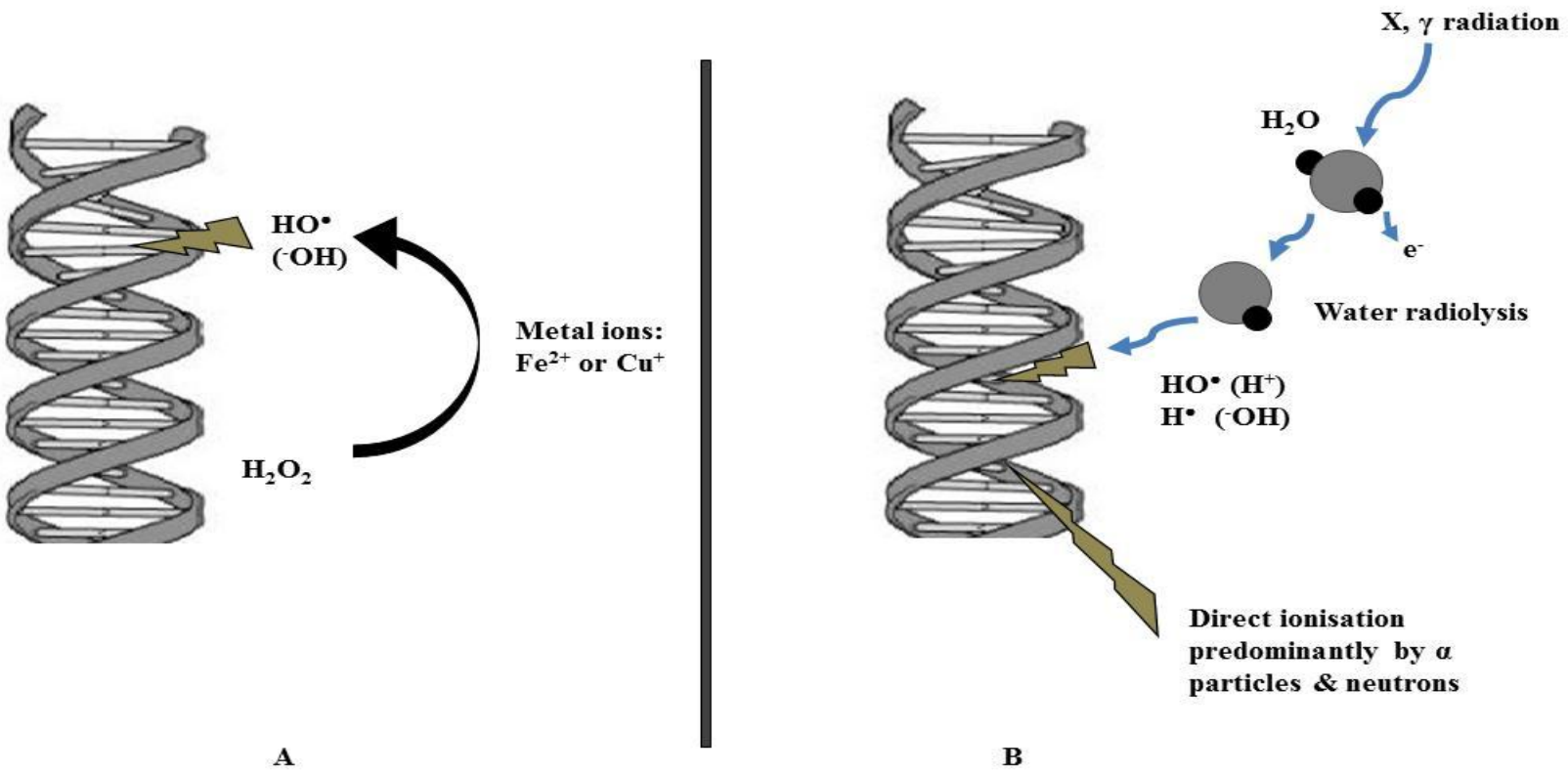


Figure 5.1: Schematic diagram depicting the basic mechanisms of H_2O_2-induced DNA damage and ionising radiation-induced DNA damage**. A** depicts the Fenton reaction in which H_2O_2 is reduced by free metal ions (most commonly either Fe^{2+} or Cu^{+}) producing hydroxyl radicals and hydroxyl anions; the latter are effectively buffered by cellular components and are considered innocuous (represented by parenthesis) whilst the former are highly reactive and attack the DNA. **B** depicts DNA damage inflicted by ionising radiation. Radiation can induce damage to cellular DNA via direct ionisation of the DNA or through the indirect effect whereby water ionisation (radiolysis) produces hydroxyl radicals which can inflict damage on the DNA. In cells, 2/3 of the DNA damage induced by ionising radiation arises through the formation of hydroxyl radicals via the indirect effect.

5.1.2 Anthocyanins as antioxidants

As previously shown in **Chapter 4**, the bladder cancer cell lines pre-treated with mirtoselect were protected against oxidatively damaged DNA induced by exposure to exogenous H_2O_2. Fewer immediate SSBs and oxidatively damaged DNA base lesions were observed in all three cell lines that were pre-treated with mirtoselect, compared to the non-treated control cells. Anthocyanins, described as very potent antioxidants, are able to protect DNA from free radicals (Kahkonen and Heinonen, 2003b; Sun et al., 2002; Zafra-Stone et al., 2007). The antioxidant effects of anthocyanins *in vitro* have been demonstrated using several cell culture systems including colon (Parry et al., 2006; Renis et al., 2008), endothelial(Bagchi et al., 2004) , liver (Meyers et al., 2003; Shih et al., 2007), breast (Olsson et al., 2004; Singletary et al., 2007), leukemic cells (Feng et al., 2007), and keratinocytes (Afaq et al., 2007). In these culture systems, anthocyanins exhibited multiple anti-toxic and anti-carcinogenic effects, for example, direct scavenging of ROS/increased radical scavenging capacity of cells and enhanced expression of Phase II detoxification enzymes (Wang and Stoner, 2008).

Potential mechanisms responsible for anthocyanin-mediated protection of DNA observed in the bladder cancer cell lines were briefly discussed in **Chapter 4**. The phenolic structure of anthocyanins is responsible for their antioxidant activity. The antioxidant activity of anthocyanins is largely due to their ability to scavenge ROS [such as superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), peroxides ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$)] (Wang and Jiao, 2000). However, these compounds may also function by chelating redox active metals in the cells (Kong et al., 2003). The radical scavenging (antioxidant) activity of anthocyanins is largely due to the presence of hydroxyl groups at position 3 of the C ring and also at the 3', 4' and 5' positions in the B ring of the molecule (Wang and Stoner, 2008).

As both the effects of H_2O_2-induced damage and ionising radiation-induced damage are mediated, to a major extent, via the formation of $^{\bullet}OH$ radicals, if $^{\bullet}OH$ radical scavenging is the principal mechanism by which mirtoselect protects against the exposure to exogenous H_2O_2, then mitroselect should protect against both H_2O_2-induced damage and ionising radiation-induced damage. Alternatively, if a different mechanism is responsible for the protective effects of mirtoselect, such as chelation of redox active metal ions, then the protection mediated by mirtoselect might be specific to H_2O_2-induced damage only. Hence, to determine if $^{\bullet}OH$ radical scavenging is the principal mechanism by which mirtoselect protects, experiments will be undertaken to determine the relative extent of protection mediated by mirtoselect towards ionising radiation-induced damage.

5.1.3 Outline of present study

Establishing a possible mechanism for the protection exerted by mirtoselect will be undertaken by comparing the possible protection observed towards radiation exposure *versus* that previously noted for exogenous H_2O_2 exposure. Bladder cancer cell lines will be treated daily with mirtoselect for a period of seven days, subsequently harvested and exposed to X-ray irradiation. The cells will then be analysed by the standard alkaline Comet assay to assess immediate SSBs as indices of immediate induced damage.

5.2 Materials and Methods

The chemicals and disposables used in the present study are the same as those stated in **Chapter 3**.

5.2.1 Cells and culture conditions

Approximately 5,000 and 30,000 RT112 cells for control and mirtoselect treatment respectively, 20,000 RT4 cells for both control and mirtoselect treatment and 25,000 HT1376 cells for both control and mirtoselect treatment were seeded in 25 cm^2 culture flasks. Control and treatment flasks of each bladder cancer cell line were prepared in duplicate. After 24 hours incubation to facilitate cell adherence, the growth media from each flask was removed. The treatment flasks were incubated with 50 µg/ml mirtoselect dissolved in the respective growth media whilst the control flasks were treated with the respective growth media alone. The mirtoselect dosings (and control 'dosings') were carried out daily for seven days. At the end of the seventh day, the growth media from each treatment and control culture flasks was removed, the cells washed with PBS and then harvested using trypsin/EDTA.

5.2.2 Radiation exposure

For radiation exposure, approximately 200,000 cells were suspended in serum free media in eppendorf tubes. The tubes were radiated with 4 and 8 Gy using a Pantak X-ray machine (dose rate of 1 Gy / min; 250 kV constant potential, 1.2 mm Cu; Pantak industrial X-ray machine). The irradiated cells were kept in the dark and on ice prior to slide preparation to prevent the cells from untoward physical damage and to minimise cellular repair activities that may take place after the radiation.

5.2.3 Alkaline Comet assay

The cells were analysed by the standard alkaline Comet assay, to assess immediate SSBs as indices of immediate induced damage, as described in **Chapter 3**.

5.2.4 Scoring and data analysis

Scoring of the Comet formation was carried utilising Komet software and epifluorescence microscope. Details of the scoring were given in **Section 3.2.3.7**.

5.3 Results

From **Figure 5.2,** clear evidence of a radiation dose dependent response for DNA damage was visible for both the non-mitroselect treated control and mirtoselect pre-treated cells. For all treatments, use of a higher dose (8 Gy) induced more DNA damage than the low dose (4 Gy), as measured by the Comet assay. The RT112 and RT4 cells were equally sensitive to damage when exposed to either 4 or 8 Gy, whilst there was a lower amount of DNA damage measured in the HT1376 cells. This contrasts with the relative damage sensitivity of the cells measured in **Chapter 3** following H_2O_2 exposure.

Several flavonoid compounds, for instance luteolin, quercitin, kaemferol, rutin, myricetin, epicathecin and genistein have been shown to exhibit protective effects against ionising radiation (Shimoi et al., 1994; Weiss and Landauer, 2003). Methanolic extracts of leaves of *Vernonia amygdalina* and *Hibiscus sabdariffa*, and vitamin C have also been demonstrated to possess protective effects against gamma radiation (Adaramoye et al., 2008). However, in this study, RT112, RT4 and HT1376 cells pre-treated with mirtoselect and exposed to X-ray irradiation at 4 and 8 Gy did not exhibit any evidence of protection, as depicted in **Figure 5.2**.

From **Figure 5.2**, there was ~ 4% background TD measured in both the non-mitroselect treated control and the mirtoselect pre-treated cells for each bladder cancer cell line analysed; for the RT112 cells, at 4 Gy, there was ~10% TD measured in non-mitroselect treated control cells and 11% TD in the mirtoselect pre-treated cells. Similarly, no real difference was observed in the RT4 cells, with ~10% TD measured in the non-mitroselect treated control cells and 11% in the mirtoselect pre-treated cells. Consistent with this lack of effect, in HT1376 cells, the amount of TD measured was approximately 8% in both the control and pre-treated cells. In summary, there was no

significant difference between the levels of damage measured for the non-mitroselect treated controls and mirtoselect pre-treated cells, when exposed to X-ray radiation at 4 Gy for all three cell lines.

Further analysis employing a higher dose of X-ray irradiation (8 Gy) also revealed no protection in the mirtoselect pre-treated cells (**Figure 5.2**). Higher amounts of DNA damage were measured following 8 Gy irradiation for all the bladder cancer cells used. In RT112, there was ~17% TD in the non-mitroselect treated control cells and ~18% TD measured in the mirtoselect pre-treated RT112 cells. In RT4, there was about 18% TD in the non-mitroselect treated control cells and ~19% TD in the mirtoselect pre-treated cells, whilst for HT1376 cells, there was ~ 12% TD measured in the non-mitroselect treated control cells and ~ 14% TD in the mirtoselect pre-treated cells.

Overall, exposure to mirtoselect failed to significantly alter the amount of tail DNA measured in either the irradiated or non-irradiated cells.

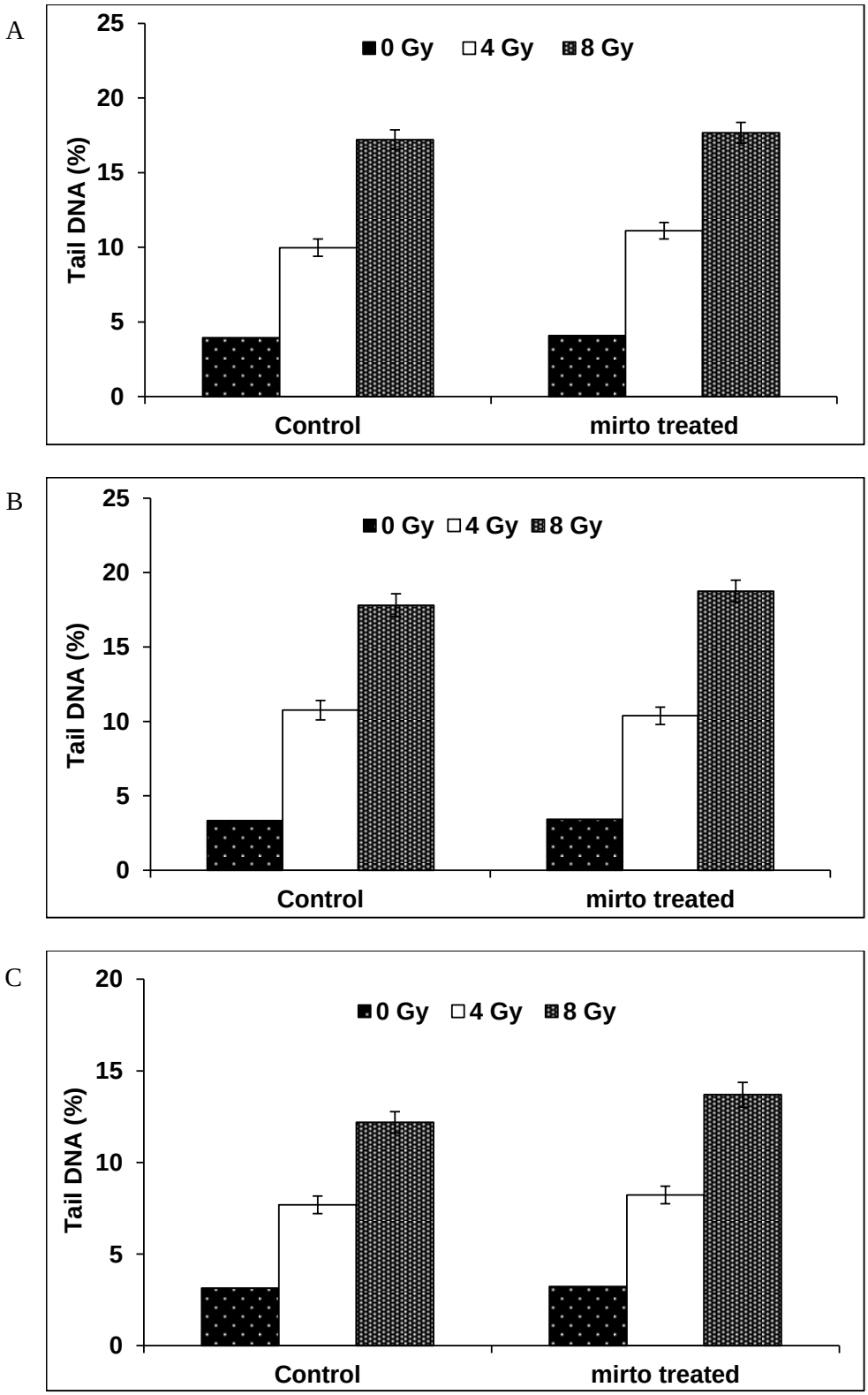


Figure 5.2: The effect of X-ray radiation on (A) RT112, (B) RT4 and (C) HT1376 bladder cancer cell lines (non-treated control & pre-treated with mirtoselect) determined via standard alkaline Comet assay methods. Results are the average of three independent experiments performed in triplicate incubations (mean±SEM).

5.4 Discussion

Several protection mechanisms for anthocyanin compounds against free radical attack on DNA have been proposed through numerous studies (Hou et al., 2004; Nijveldt et al., 2001; Stoner, 2008; Wang et al., 1997). These protection properties were briefly discussed in **Chapter 2**. The current study using X-ray irradiation was carried out as a simple but effective method to begin to differentiate the possible mechanisms of protection offered by mirtoselect against exogenous H_2O_2 exposure, as observed in **Chapter 4**.

Ionising radiation reacts with water molecules to produce hydroxyl radicals (vonSonntag., 1987). Radiation-induced hydroxyl radicals can react with both deoxyribose and bases in DNA to produce damage that includes strand breaks (Henle et al., 1995; Yamamoto et al., 1985) and base lesions (i.e. 8-oxoGua) (Dizdaroglu, 1985). The decision to utilize X-ray irradiation for this study can be considered an appropriate choice as it is well documented that Fe (II)-EDTA/H_2O_2 and ionising radiation generate oxidatively damaged DNA and strand breaks, which is consistent with the hypothesis that chemical intermediates of similar reactivity are involved in each system. These results are also consistent with previous studies in which the sequence specificity of DNA damage produced by γ-radiation was shown to be similar to that of Fe (II)-EDTA/H_2O_2 (Hayes et al., 1990; Price and Tullius, 1992). In both cases, the majority of the DNA damage can be attributed to hydroxyl radicals being generated (Kennedy et al., 1997).

This study has shown that pre-treatment of the three bladder cancer cell lines with mirtoselect was unable to protect them against formation of DNA SSBs induced by X-ray irradiation at 4 and 8 Gy. It is well documented that the majority of immediate cellular DNA damage (DNA strand breaks and oxidatively damaged DNA bases)

inflicted by ionising radiation, through the formation of free radicals (the indirect route), is via a metal ion independent route (Ward, 1988), hence the results of this study reflect the inability of anthocyanin compounds to scavenge hydroxyl radicals formed as a result of water radiolysis. If the anthocyanin compounds of mirtoselect significantly protect DNA via their free radical scavenging properties, there should have been lower amounts of DNA damage measured in the irradiated mirtoselect pre-treated bladder cancer cell lines, as compared to the irradiated non-mirtoselect treated control cells.

As mentioned in **Chapter 2**, anthocyanins are part of the major phenolic compounds found in plants known as flavonoids. The relationship of anthocyanidins (aglycones of anthocyanins) with other flavonoid compounds is shown in **Figure 5.3**. Flavonoids are formed in plants from the aromatic amino acids phenylalanine, tyrosine, and malonate (Harborne, 1986).

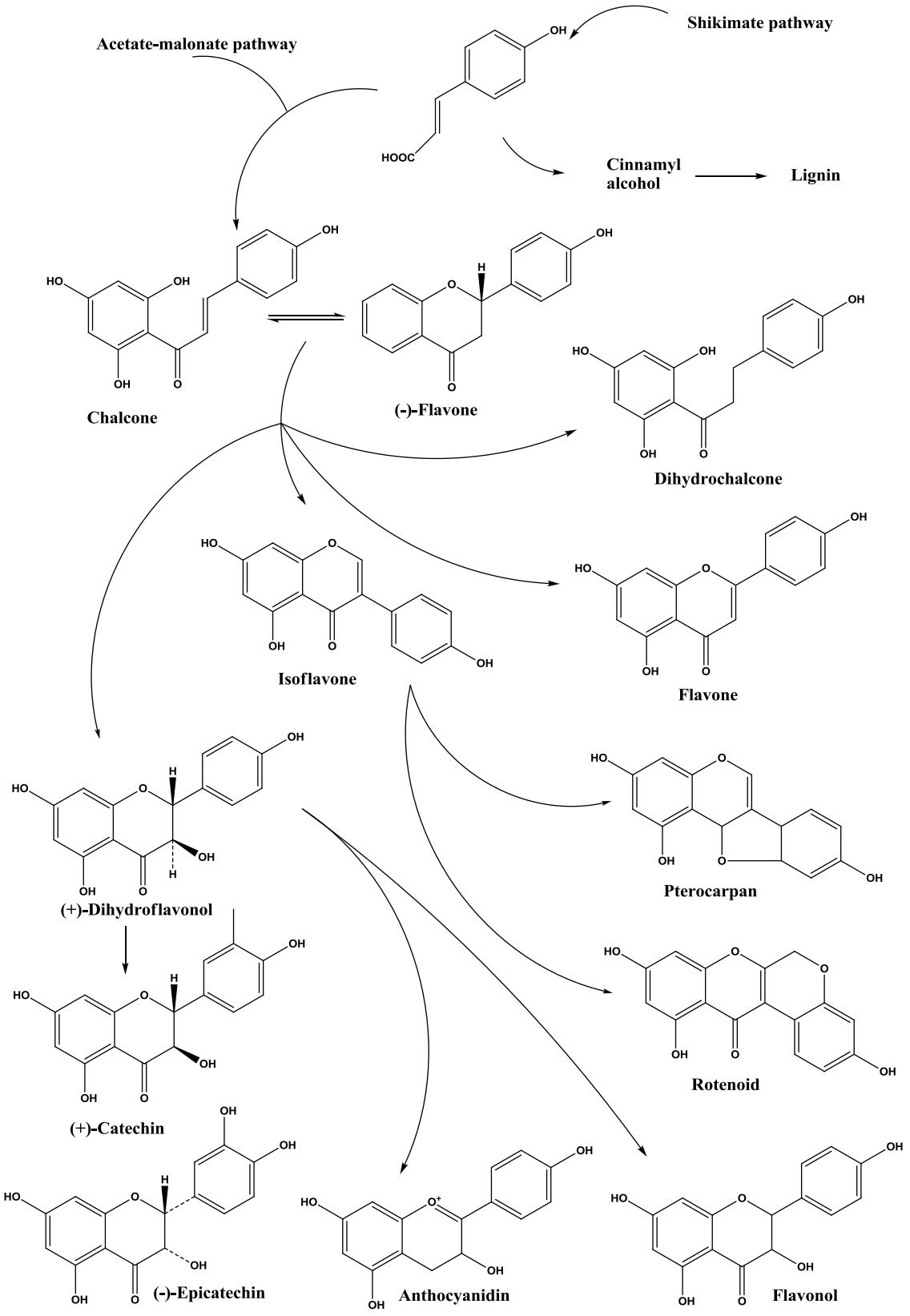


Figure 5.3:Inter-relationship of different flavonoid classes. Arrows indicate the principal biosynthetic routes in plants. (Adapted from Rice-Evans et al., 1997)

The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings: A, C and B (C_6-C_3-C_6). The carbon skeleton (C_6-C_3-C_6) consisting of the two aromatic rings linked by an aliphatic three carbon chain (**Figure 5.4**). This skeleton is made up of two distinct fragments: C_6-C_3 fragment that contains the B and C rings; and the C_6 fragments forming the A ring (Ranggana, 1987).

The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings. Among the many classes of flavonoids, those with potential biological activities are flavones, flavanones, isoflavones, flavonols, flavanonols, flavan-3-ols, and anthocyanins (**Figure 5.5**). Other flavonoid classes include biflavones, chalcones, aurones, and coumarins. Hydrolyzable tannins, proanthocyanidins (flavan-3-ol oligomers), caffeates, and lignans are all plant phenols, and they are usually classified separately, due to the fact that they usually exist as oligomer (Pietta, 2000).

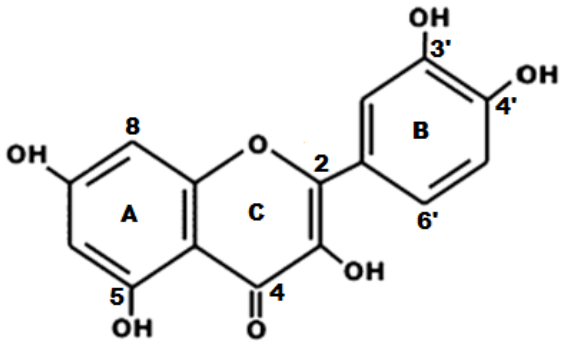


Figure 5.4: Basic flavonoid structure

Individual differences within each group result from the variation in number and arrangement of the hydroxyl groups as well as from the nature and extent of alkylation and/or glycosylation of these groups. The most commonly occurring flavones and

flavonols are those with dihydroxylation in the 3' and 4' positions of the B ring, and to a lesser extent, those with a lone B ring-hydroxyl group in the 4' position. The preferred glycosylation site on the flavonoids is the 3 position and less frequently the 7 position. Glucose is the most usual sugar residue but others, include galactose, rhamnose and xylose (RiceEvans et al., 1997).

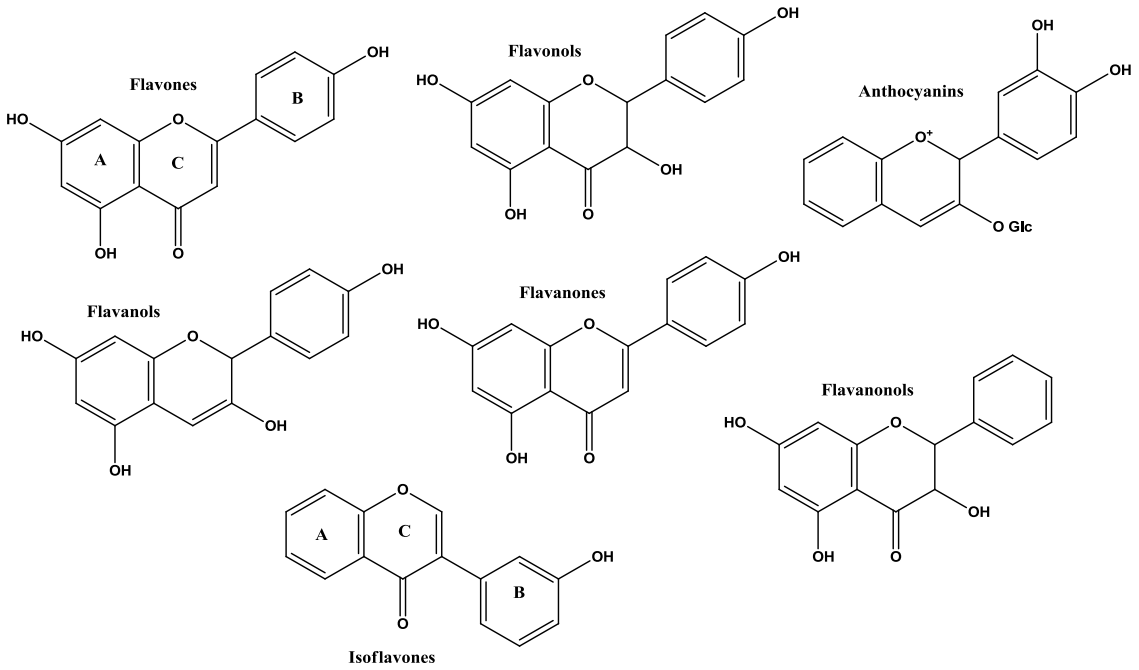


Figure 5.5: Structure of members of the flavonoid family

Luteolin, quercetin, kaempferol, rutin, myricetin, epicatechin and genistein have been demonstrated to possess protective effects against γ-ray irradiation in *in vivo* systems (Shimoi et al., 1994). Luteolin is from the flavone family; quercetin, kaempferol, rutin, myricetin are from flavonol family; epicatechin is from flavan-3ol family and genistein is from isoflavone family. These compounds share high similarity in the hydroxyl positioning at the 3, 5, 7, 2', 3', 4' and 5' positions of the A and B rings (**Table 5.1**). The difference between the above mentioned flavonoid compounds and anthocyanins is that these flavonoids do not carry a glycoside moiety at carbon 3 of ring C, whilst anthocyanins do have a glycoside moiety at this position (**Table 5.1**).

Table 5.1: Flavonoids and hydroxyl substituents

Name	Substituent						
	3	**5**	**7**	**2'**	**3'**	**4'**	**5'**
Luteolin		OH	OH		OH	OH	
Quercitin	OH	OH	OH		OH	OH	
Kaemferol	OH	OH	OH			OH	
Rutin	O-Rut	OH	OH		OH	OH	
Myricetin	OH	OH	OH		OH	OH	OH
Epicatechin	OH	OH	OH		OH	OH	
Genistein		OH	OH			OH	
***Cyanidin glycoside**	glc	OH	OH		OH	OH	H
***Delphinidin glycoside**	glc	OH	OH		OH	OH	OH
***Petunidin glycoside**	glc	OH	OH		OH	OH	OCH_3

glc: glycosides. * = present in mirtoselect

Rut: Rutinoside

It was proposed that major determinants for radical-scavenging capability of the flavonoids are based on the following factors; (i) the presence of a catechol group in the B ring (hydroxyl groups at 3' and 4'), which has better electron donating properties and is a radical target, and (ii) a 2, 3- double bond conjugated with the 4-oxo group, which is responsible for electron delocalization (Pietta, 2000). The existence of a 3 hydroxyl group and a double bond at C2 and C3 in the flavonoids also contributes towards the antioxidant activity of these compounds. Van Acker and co-workers demonstrated that quercetin, which possesses a catechol moiety, a 3-hydroxyl group and a double bond at C2 and C3 in the heterocyclic ring, is a very strong antioxidant (van Acker et al., 1998; van Acker et al., 1996b) with high radical-scavenging activity. Additional hydroxyl or

methoxyl groups at positions 3, 5 and 7 of rings A and C seem to have less impact on the antioxidant property of the compound (van Acker et al., 1996b).

Several anthocyanins present in mirtoselect (cyanidin-3-glycoside, delphinidin-3-glycoside and petunidin-3-glycoside) also possess a hydroxyl moiety at carbon 5 and 7 and at the 3' and 4' positions of the B ring, similar to some of the flavonoids mentioned above, but carry a glycosides moiety at carbon 3 of the C ring (**Table 5.1**). Glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones (Pietta, 2000), however, the reason for this has not yet been conclusively identified A study carried out substituting the groups of anthocyanin compounds has shown that degree of glycosylation to be highly correlated with their reactivity and pigment stability via altering the electron distribution on the molecule (Wrolstad et al., 2005).

Voltammetry study was carried out to determine the electron-donating abilities of flavonoids. In general, the voltammetric techniques involve the application of a potential (*E*) to an electrode and the monitoring of the resulting current (*i*) flowing the electrochemical cells. The applied potential forces a change in the concentration of an electroactive species at the electrode surface by electrochemically reducing or oxidizing it (Kounaves, 1997). Van acker and co-workers utilised this system to differentiate between flavonoid compounds with free radical scavenging properties by determining the half peak oxidation potential (*E*p/2), which measures the ease of reduction or oxidation (van Acker et al., 1998; van Acker et al., 1996a). However, not all the flavonoids tested by van Acker and co-workers followed the expected/established criteria. Generally, flavonoids without glycosides and anthocyanidins (aglycone of anthocyanins) with catechol structures, exhibiting less than 0.2 half-peak oxidation potential ($Ep_1/2$) have been demonstrated to have free radical scavenging activities (van

Acker *et al*., 1998). Van Acker and co-workers also suggested that Fe^{2+} chelation only plays a role in the inhibition of lipid peroxidation by less active scavengers (van Acker et al., 1996a). A study of anthocyanins from grapes (*Vitis vinifera*) with glycosides at carbon 3 of the C rings and carbon 5 of the A rings under acidic (pH 3.5) and neutral (pH 7.0) conditions, exhibited different responses with respect to their oxidative potential. Anthocyanins more easily lost an electron from the catechol moiety of carbons 3' and 4' in the C ring at pH 7 ($Ep_1/2 < 0.2$) than under acidic conditions ($Ep_1/2 >0.\ 2$). Anthocyanins have also been shown to lose an electron from the hydroxyl group on carbons 5 and 7 of ring A, but exhibited higher oxidation potential ($Ep_2/2 > 0.2$) at either acidic or neutral conditions (**Table 5.2**) (Janeiro and Brett, 2007).

Table 5.2: Different pulse voltammetry for anthocyanins oxidation from grape (Janeiro and Brett, 2007)

Anthocyanins	**pH**	***E*p$_1$/2 (Volts)**	***E*p$_2$/2 (Volts)**
	3.5	0.27	0.44
	7.0	0.19	0.25
	3.5	0.27	0.49
	7.0	0.19	0.32
	3.5	0.25	0.44
	7.0	0.16	0.25
	3.5	0.25	0.49
	7.0	0.16	0.32
	3.5	0.20	0.44
	7.0	0.09	0.25
	3.5	0.20	0.44
	7.0	0.11	0.30
Petunidin chloride	3.5	0.22	0.45
	7.0	0.14	0.315

Therefore, considering the 'rule of thumb', proposed by van Acker and co-workers (1996) and data from Janeiro and Brett (2007), anthocyanins could be considered to be good free radical scavengers (van Acker et al., 1996b; Janeiro and Brett, 2007). However, results of the current study showed that the antioxidant property of anthocyanins in mirtoselect was not due to free radical scavenging.

One possible reason for the discrepancy was that the mirtoselect may not in fact have been present at the time of irradiation; the mirtoselect containing media was removed and the cells washed and harvested prior to their irradiation in media (this was to mimic the circumstances and conditions of the exogenous H_2O_2 exposure (**Chapter 4**). Therefore, the intracellular mitroselect may have been washed out of the cells prior to irradiation. However, in another experiment, undertaken as part of a parallel PhD study undertaken in the group (Higgins, 2011), irradiation of cells in mirtoselect containing media also failed to offer any protection against DNA damage.

It is not easy to differentiate between free radical scavenging properties and metal chelation properties of flavonoid compounds. Many of the free radical scavenging properties of several flavonoid compound quoted in this study, were obtained from direct chemical analysis of the compound without any intervention with biological materials (cells). However, cellular studies using flavonoids *in vitro* have shown chelation of redox active metals ions to be a more important antioxidant mechanism (Melidou et al., 2005).

In this study, there was clear evidence that anthocyanins failed to protect bladder cancer cells from DNA damage induced by the X-ray irradiation. This has ruled out the possible mechanism of protection by anthocyanins, proposed in **Chapter 4**, as being due to their free radical scavenging properties. Therefore, subsequent work involved

investigating the role of free metal ion chelation in the antioxidant activity of mirtoselect.

5.5 Summary statement

This study has shown that the antioxidant effect of mirtoselect observed in the bladder cancer cell lines when exposed to the exogenous H_2O_2 was not due to the free radical scavenging properties of the anthocyanins. There was no significant difference between the amount of DNA damage measured in the irradiated mirtoselect pre-treated and non-mitroselect treated control cells. Therefore, further experiments were devised and undertaken to investigate the possible chelation of free metal ions as the protective mechanism responsible.

Chapter 6.

Establishing a Protective Mechanism for Mirtoselect (2): Determining the Effect of Mirtoselect on the Free Labile Iron Pool (LIP) in Bladder Cancer Cell Lines *In Vitro*.

6.1 Introduction

6.1.1 The cellular labile iron pool (LIP)

Mirtoselect has been shown to protect bladder cancer cell lines against exogenous H_2O_2 exposure (**Chapter 4**). However mirtoselect was unable to protect the same bladder cancer cells, when exposed to X-ray irradiation at 4 and 8 Gy (**Chapter 5**). Two mechanisms by which antioxidants can mediate their protective role have been proposed; these are free radical scavenging and metal ion chelation. The first mechanism, free radical scavenging, can happen through either H-atom transfer, in which a free radical removes a hydrogen atom from the antioxidant, or a one-electron transfer mechanism, in which the antioxidant donates an electron to the free radical. The antioxidant radical/product, arising from both reactions must be stable enough to prevent or delay further radical chain reactions.

A second antioxidant mechanism, not so well studied, is based on the ability of some of these compounds to chelate transition metals ions (particularly iron and copper). This gives rise to stable complexes that 'confine' the metal ions, preventing them from participating in free radical generation (Jovanovic, 1998). During the Fenton reaction (Brown et al., 1998; van Acker et al., 1998), hydroxyl radicals are produced from hydrogen peroxide by the latter's reduction by redox active metal ions of a low oxidation state refer to **equation 4.1.**

Metal-chelating compounds remove/sequester the metal ions and can alter their redox potentials rendering them inactive. Moreover, the use of natural metal chelators such as flavonoids may be more favourable than other synthetic chelators, which may present some problems of toxicity. For example, deferoxamine, used in the treatment of ß-thalassemia patients often causes pain and swelling at the site of injection (Wong and Richardson, 2003). Flavonoids, with their multiple hydroxyl groups and a carbonyl

group on the C ring, have several available sites for metal complexation (Leopoldini et al., 2006). A study carried out on the flavonoid quercetin (**Figure 6.1**), , showed it was able to form a complex with Al (III) and Pb (II), (Cornard and Merlin, 2002; Cornard et al., 1997), with the preferential coordination sites of quercetin being the 3-hydroxy moiety and the catechol group for aluminium and lead ions, respectively (Leopoldini et al., 2006).

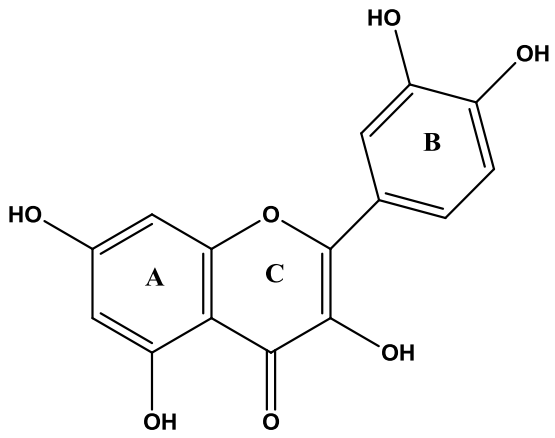


Figure 6.1: Chemical structure of quercetin.

Andjelković and co-workers have demonstrated the structure dependency of the metal ion binding sites of seven phenolic acids and hydroxytyrosol by comparing phenolic acids bearing either galloyl or catechol groups with phenolic acids without these groups (Andjelkovic et al., 2006). As reported earlier (RiceEvans et al., 1997; RiceEvans et al., 1996), of the three possible metal chelating moieties in flavonoids (the catechol moiety in ring B, the 3-hydroxyl and 4-oxo groups in the heterocyclic C ring, and the 4-oxo and 5-hydroxyl groups between the heterocyclic C ring and the A ring), it is the 3′, 4′-dihydroxy group on the B-ring which is the structural feature most responsible for metal ion chelation by phenolic acids. The activity of this group is due

to its electron-donating abilities. Phenolic compound lacking this moiety exhibit no metal chelation properties (Andjelkovic et al., 2006).

It has been well established that H_2O_2 is able to move freely across cell membranes. Indeed, it can mediate effects in cells or tissues nearby to its site of generation, acting in a paracrine fashion, similar to nitric oxide (Bienert et al., 2006). In this study, when the bladder cancer cell lines (either pre-treated or non-pre-treated with mirtoselect) were exposed to exogenous H_2O_2, the H_2O_2 crossed the cell membrane then passed across the nuclear membrane and reacted with the redox active ions, mainly iron and/or copper, that were in the DNA milieu. This reaction produces $^{\bullet}OH$ which, in turn, yields the DNA damage noted in **Chapter 5**. The location of redox-active ions is of utmost importance for the ultimate effect, because $^{\bullet}OH$, due to its extreme reactivity, interacts exclusively in the vicinity of the bound metal (Chevion, 1988). Formation of $^{\bullet}OH$ close to DNA results in it being damaged, leading to a wide variety of lesions, including base modifications, single and double strand breaks, and sister chromatid exchanges (Barbouti et al., 2001; Jornot et al., 1998).

The issue of which are the actual redox active ions that participate in the formation of $^{\bullet}OH$ from H_2O_2 has been addressed in several studies. Barbouti and co-workers have shown that under normal conditions, intracellular redox active iron, not copper, participated in H_2O_2-induced single strand break formation in cellular DNA of Jurkat cells (Barbouti et al., 2001). Their finding was further supported by Rae and co-workers, whose research on the requirement of a copper chaperone for superoxide dismutase enzymes found that intracellular free copper is limited to less than one free copper ion per cell (Rae et al., 1999). Recently, it has been documented that intracellular copper ions did not participate in the formation oxidatively damaged DNA in *Escherichia coli* (Macomber et al., 2007).

Iron is a ubiquitous metal in cells, present in the structure of many enzymes and proteins (Meneghini, 1997) and is therefore essential for life. A small 'transit' pool of cellular iron is believed to catalyse the generation of highly reactive oxygen species within mammalian cells. This 'transit' pool links the cellular iron uptake, via transferrin, with iron storage in proteins (i.e. ferritin) and the synthesis of iron-containing proteins (haem and non-haem iron proteins) (Halliwell, 1989; Young, 1994). Iron belonging to this 'transit' pool is thought to be in steady-state equilibrium, bound to low-molecular-weight compounds (such as ATP, citrate or phosphate) or loosely attached to proteins (Halliwell, 1989). It can be defined as 'chelatable' because chelating molecules can be used for its detection.

Cellular chelatable iron is generally referred to as being cytosolic (Breuer et al., 1995; Epsztejn et al., 1997; Halliwell, 1989), although there is also evidence for the existence of chelatable iron in other metabolic compartments of the cell. For instance, in mitochondria, iron is incorporated into protoporphyrin IX during haem synthesis. A 'transit' pool of chelatable iron must therefore be assumed to exist within these organelles and attempts to measure it have been made with isolated mitochondria (Ceccarelli et al., 1995; Foury and Cazzalini, 1997; Tangeras et al., 1980). Some attention has also been given to redox-active iron within lysosomes (Brunk et al., 1995; Nilsson et al., 1997), which has been shown to be responsible for oxidative cell injury under different pathological conditions (Nankivell et al., 1994; Ollinger and Brunk, 1995; Ollinger and Roberg, 1997). There is also evidence of the existence of chelatable, redox-active iron within mammalian nuclei, in which, by iron-dependent mechanisms, hydroxyl-radical-mediated DNA damage has been shown to occur under several pathological conditions (Halliwell, 1989; Meneghini, 1997). For this to take place, DNA-associated chelatable Fe^{2+} would have to be present (Petrat et al., 2001).

The role of LIP in the induction of oxidatively damaged DNA was unclear, until Petrat and co-workers demonstrated the existence of a LIP pool in cell nuclei (Petrat et al., 2001). Subsequent studies have shown a significant correlation between cellular LIP level and the yield of 8-oxoGua, a typical marker of reactive oxygen species-induced DNA damage, in human lymphocytes (Gackowski et al., 2002). A strong correlation has also been found between the LIP level and DNA strand breaks, estimated by the Comet assay, in cells treated with H_2O_2 (Kruszewski, 2003). Chelating of intracellular iron with desferrioxamine prevents the formation of DNA strand breaks and cytotoxicity induced by H_2O_2 to nuclear (Barbouti et al., 2001; Kruszewski et al., 1995; Mello and Meneghini, 1991) and mitochondrial DNA (Itoh et al., 1994). Up regulation of the heavy subunit of ferritin (H-FT) expression resulted in a decrease in the LIP, reduced oxidative stress responses (Epsztejn et al., 1999) and protected cells against H_2O_2-induced cyto- and genotoxicity (Cozzi et al., 2000). On the other hand, lowering of the cellular FT level accomplished by antisense-mediated synthesis inhibition resulted in higher LIP level (Kakhlon et al., 2001) and an enhanced susceptibility to oxidative stress (Lin and Girotti, 1998).

Taking into account that only iron ions participate in the formation of $^{\bullet}OH$ from H_2O_2, a study of whether the anthocyanins in mirtoselect are able to chelate the free redox active iron ions in the bladder cancer cell lines was proposed. Petrat and co-workers have successfully demonstrated the presence of a chelatable iron pool in isolated rat hepatocytes using a fluorescent probe; Phen Green SK (Petrat et al., 1999). Duarte and Jones, 2007 have used the method of Petrat and co-workers (Petrat et al., 2001; Petrat et al., 1999) to measure the intracellular labile iron pool (LIP) in human diploid fibroblast cells to determine the role of vitamin C in H_2O_2-induced damage and iron homeostasis (Duarte and Jones, 2007).

6.1.2 Measurement of LIP by fluorescent probes

The cellular labile iron pool (LIP) is defined as a pool of redox-active iron complexes. It was first suggested by Jacobs (Jacobs, 1977) as an intermediate or transitory pool between extracellular iron and cellular iron associated with proteins. Operationally it is defined as a cell chelatable pool that comprises both ionic forms of iron (Fe^{+2} and Fe^{+3}) associated with a diverse population of ligands such as organic anions (phosphates and carboxylates), polypeptides, and surface components of membranes (e.g., phospholipid head groups). Thus, the broadest definition of LIP implies that it consists of chemical forms that can potentially participate in redox-cycling but can be scavenged by permeant chelators. The latter property forms the basis for the quantification of cellular LIP, which, in quiescent conditions, represents only a minor fraction (<5%) of the total cell iron (50–100 μM) (Epsztejn et al., 1999). Although that fraction can change dynamically following biochemical stimuli, the resulting changes are usually transitory and homeostatic in nature. However, in extreme cases of iron overload or deprivation, the changes might exceed the cell's homeostatic capacity, thus compromising its integrity (Kakhlon and Cabantchik, 2002).

Great effort has been devoted to developing suitable methods for determining the concentration of intracellular chelatable iron, however, only a few methods have been described (Baliga et al., 1993; Cairo et al., 1995; Sergent et al., 1995). Most are unable to exploit small amounts of biological material and are prone to false readings arising from tissue processing (*e.g.*, proteolysis). Subsequently, the use of fluorescent dyes in combination with digital fluorescence microscopy has become increasingly important for the detection of intracellular ions. One method using the fluorescent dye calcein (**Figure 6.2**), has been described for the assessment of the chelatable iron pool in cultured erythroleukemia K562 cells (Breuer et al., 1995). Cell-disruptive methods

such as homogenization or lysis followed by ultrafiltration, high performance liquid chromatography, electron paramagnetic resonance spectroscopy, affinity chromatography, or spectrophotometry of iron-chelator complexes, require relatively large amounts of tissue and are prone to false readings. Because the chelatable iron pool is considered to amount to only 0.2% to 3.0% of the total cellular iron content (Baliga et al., 1993; Linder CM, 1983; Ollinger and Roberg, 1997), a suitable method must not only be highly sensitive to chelatable iron, and be able to differentiate exactly between chelatable and tightly protein-bound iron ions, but it also must strictly avoid iron release from proteins into the chelatable pool. In tissue homogenates and cell lysates, mobilization of iron from high-molecular-weight sources (*e.g.*, proteins like ferritin), *e.g.*, via proteolysis, is always a risk (Petrat et al., 1999).

Petrat and co-workers have used the fluorescent probes calcein, fluorescein desferrioxamine **(**FL-DFO), phen green SK (PG SK), and phen green FL (PG FL) to assess the chelatable iron pool of isolated rat hepatocytes. Because methods based on non-invasive fluorescence microscopy use living cells, disturbance from any pre-treatment of biological samples is minimized (Petrat et al., 1999).

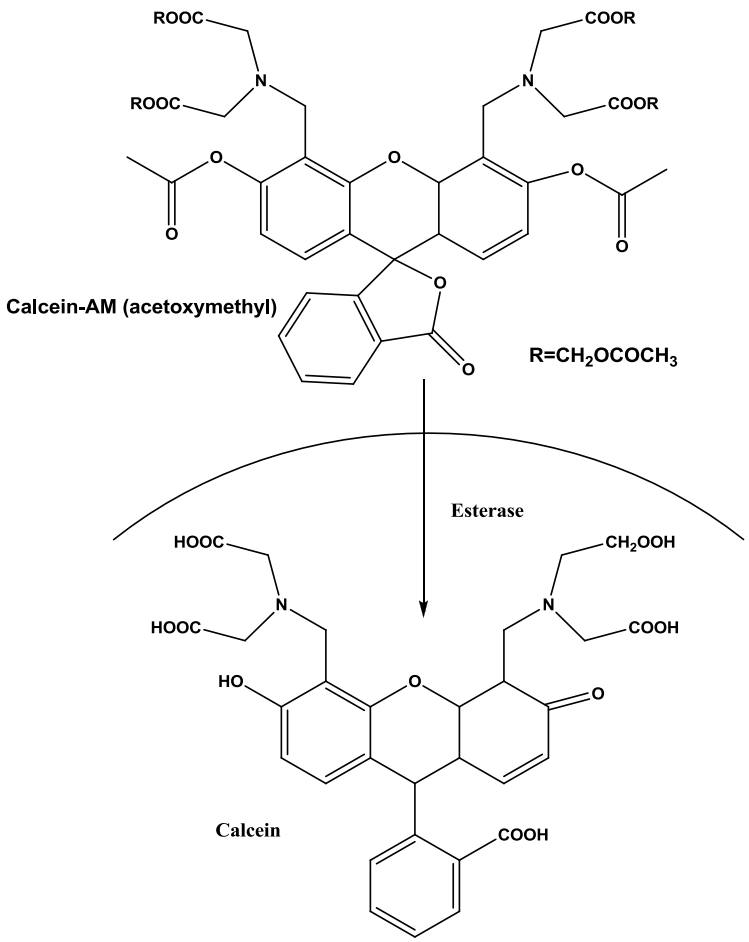


Figure 6.2: Chemical structure of Calcein-AM (non-fluorescent form) and calcein (fluorescent form)

Fluorescent indicators can be used to quantify ion concentrations and to evaluate ion speciation (Tsien et al., 1982). They can also be used to study the kinetics of complex formation and dissociation because it is possible to follow the re-equilibration process through time by measuring changes in fluorescence. PG SK is from a family of photoinduced electron transfer (PET) sensors in which the metal ion is bound by a specific ligand covalently attached to a fluorophore (de Silva et al., 1996), in this case phenanthroline and a fluorescein derivative (**Figure 6.3**).

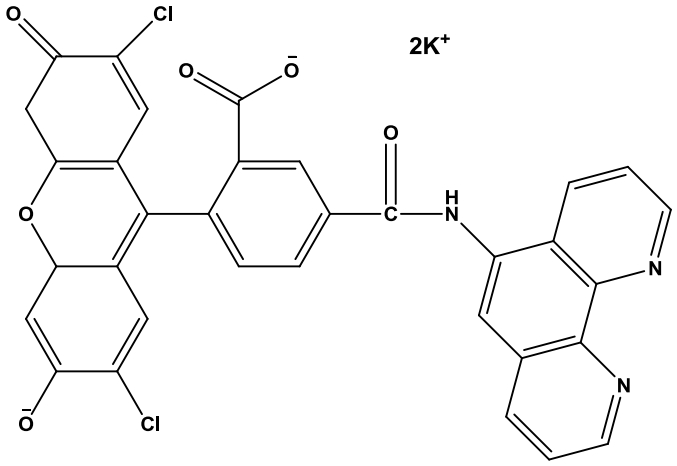


Figure 6.3: Chemical structure of Phen Green SK

6.1.3 Fluorescent measurement by PG SK

A more recent approach to estimate the LIP assumes it to be composed of relatively low-affinity iron complexes in which iron is in dynamic equilibrium and amenable to chelation (Epsztejn et al., 1997). This approach relies on chelators that can function as cellular iron sensors without affecting cell integrity, A given chelator would be considered suitable as a cell iron sensor, namely capable of reporting the amount of iron in the LIP, if it could: (i) be loaded into a cell and retained therein without affecting its integrity; (ii) induce a minor shift in the dynamic equilibrium of iron toward the chelator complex; and, (iii) undergo a swift, reversible, and detectable spectroscopic change (Epsztejn et al., 1999). Targeting the probe to a particular cell compartment is a desirable feature that can be achieved by conferring additional properties upon the molecules (Cabantchik Z.I., 2001). The available repertoire of iron sensors that fulfils those criteria is still limited (Cabantchik Z.I., 2001; Esposito et al., 2002; Petrat et al., 2001; Petrat et al., 1999; Zahrebelski et al., 1995) comprising essentially EDTA-like and phenanthroline molecules tagged with fluorescent moieties as metal reporter devices.

PG SK (**Figure 6.3**), a phenanthroline-based fluorescent probe for Fe^{+2}, was also used to estimate the intracellular chelatable iron as the major component of the LIP (Petrat et al., 2001; Petrat et al., 1999) following the principles established for calcein-AM (CAL). This probe is generally more sensitive for detecting iron than CAL due to the high (3:1) stoichiometry of probe:metal binding and relatively high iron-binding affinity for Fe^{+2}, the soluble form of the metal. However, once formed, the Fe^{+2}-PG SK complexes are more difficult to dissociate by competing Fe^{+2} chelators in excess. This hampers somewhat the quantification of the probe-associated metal (*i.e.* the quenched fluorescence signal) and limits the use of the probe for dynamic tracing of the metal. At this point it is not clear whether or not PG SK detection of labile Fe^{+2} in cells depends also or even primarily on the cellular reductive capacity for converting labile Fe^{+3} to Fe^{+2} (Kakhlon and Cabantchik, 2002) Thus, even though most of the LIP might be comprised of Fe^{+3} complexes, PG SK binding of Fe^{+2} could trigger the reduction of Fe^{+3} from labile sources and scavenge most or all the metal from the LIP. This combined property of chelator-evoked change in the oxidation state of the metal and the oxido-reductive properties of the cell minimizes the intrinsic differences between CAL and PG SK as cell probes for specific oxidation states of Fe. The probes, although both FL derivatives, differ in their response to pH, as PG SK shows relatively sharper pH dependence of fluorescence in the pH range 6.0–7.5 (Kakhlon and Cabantchik, 2002).

The fluorescence-based methods have enabled the monitoring of changes in the LIP in living cells over a wide range of iron concentrations. **Figure 6.4** exemplifies the use of PG SK for the detection of iron influx in cells exposed to transferrin or ferrous ammonium sulfate (FAS). The amount of iron taken up is determined from the increment in LIP (Δ LIP), which is revealed by addition of the permeant iron chelator 2, 2-bipyridyl (BIP).

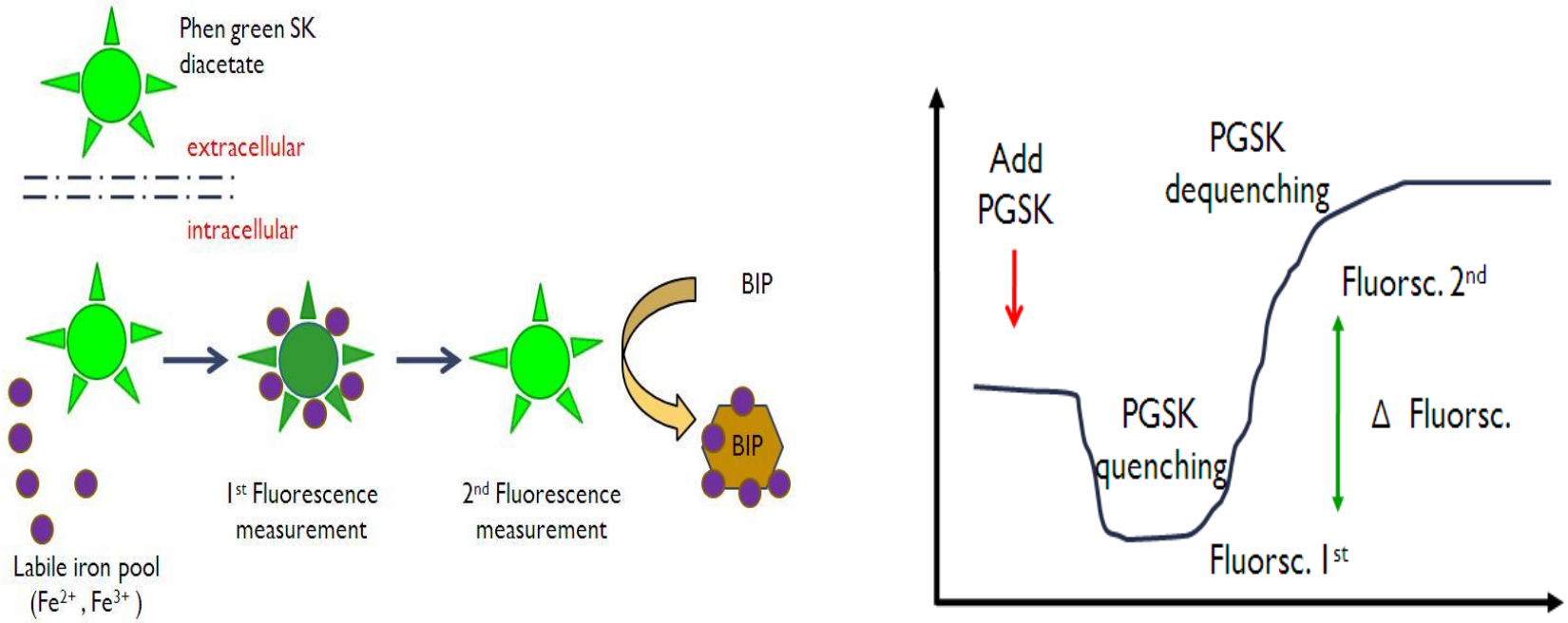


Figure 6.4: The phen green SK (PG SK) method for measuring the labile iron pool. (A) Cells are loaded with the PG SK diacetate. PG SK diacetate is non-fluorescent and membrane permeable. Upon entry to cells, PG SK diacetate is hydrolyzed to give the fluorescent PG SK, which is quenched upon binding of iron. BIP, a permeant strong iron chelator, evokes fluorescence dequenching (ΔF) by binding PG SK-bound iron in a manner proportional to PG SK-bound iron [PG SK-Fe]. (Adapted from Kakhlon and Cabantchik , 2002)

6.1.4 Outline of the present study

Measurements of LIP levels in cultured cells have indicated that levels are maintained within a relatively narrow range of concentrations (Epsztejn et al., 1997; Esposito et al., 2002). It is assumed these concentrations are maintained homeostatically for cells to meet the metabolic demands for iron whilst minimizing its potential engagement in ROS formation.

The aim of this part of the study is to determine the effect of mirtoselect on the free labile iron pool (LIP) in bladder cancer cell lines *in vitro* as a possible protective mechanism for mirtoselect towards DNA oxidation from exogenous peroxide treatment. Bladder cancer cell lines used in this study will be pre-treated with two different concentrations of mirtoselect. Subsequently, the pre-treated cells will be flooded with PG SK solution, prepared according to protocols used by Duarte and Jones (Duarte and Jones, 2007). The first reading of fluorescent intensity will be carried out after the addition of the PG SK.

After the first fluorescence reading, the PG SK solution will be removed and 2, 2-BIP solution will be added into the pre-treated cells. After 15 minutes incubation the second fluorescencee intensity reading will be carried out.

Controls consisting of non-mirtoselect treated cells incubated with deferoxamine mesylate (DFO) were prepared and subjected to similar analysis as carried out on the mirtoselect treated cells.

6.2 Materials and Methods

Growth media for the bladder cancer cell lines were as described before in **Chapter 2**. McCoy's 5A and DMEM without phenol red were purchased from Fischer scientific (Loughborough, UK). EMEM without phenol red and PG SK were purchased from Invitrogen (Paisley, UK). Deferoxamine mesylate (DFO) and 2,2-Bipyridyl (BIP) were purchased from Sigma (Poole, UK) and black 96 well plates were purchased from Perkin Elmer (Shelton, USA).

6.2.1 Cells and culture conditions

Similar growth conditions, to those described in **Chapter 2,** were applied for each cell line.

6.2.2 Labile iron pool measurement

Relative changes in the intracellular LIP were measured using an adaptation of the method of Duarte and Jones (Duarte and Jones, 2007). Approximately 10,000 cells/well of RT112, RT 4 and HT1376 were seeded in black 96 well plates in quadruplicate. After 24 hours incubation to allow cell adherence, the cells were washed with PBS solution and treated with 50 or 100 μg/ml mirtoselect or DFO dissolved in respective complete growth medium for 4 hours. The cells were then incubated in humidified 5% CO_2 at 37 ºC. After incubation with the appropriate treatment, the cells were washed with pre-warmed (37 °C) PBS and incubated with 10 μM PG SK diacetate in the appropriate growth medium without serum and without phenol-red for 10 min at 37 °C. Cells were then washed 3 times with pre-warmed growth media. Fluorescence of the metal indicator PG SK (excitation maximum 485 nm, emission maximum 520 nm) was then measured just before and 15 min after the addition of the highly permeable

chelator 2,2′-Bipyridyl (2,2′-BIP, 5 mM) in a FLUOstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany) and expressed as arbitrary fluorescence units. The increase in fluorescence produced by the chelator is proportional to the size of the intracellular redox active iron. The size of the intracellular redox active iron pool under different treatments was compared using the equation below, where F_1 and F_2 equate to fluorescence before and after the addition of the chelator, respectively, as described by Darbari *et al*., 2003:

Fractional increase in fluorescence (ΔF) = $(F_2 - F_1)/F_2$.

6.2.3 Data analysis

Data are expressed as mean values ± standard deviation for eight replicates from two independent experiments. Mean values of experimental data were compared with the baseline data of the control cells by the non-parametric Kruskal-Wallis test (Statgraphics centurion version VX; Herndon, USA) wherever appropriate. Significance was accepted at a 5 % significance level ($p<0.05$).

6.3 Results

Adapting protocols described by Petrat and co-workers enabled Duarte and Jones to successfully demonstrate that ascorbic acid enhanced H_2O_2-induced damage and cell death by an iron-dependant process (Duarte and Jones, 2007). The method described by Duarte and Jones, was slightly modified to suit the present requirement of measuring the ability of mirtoselect to chelate free labile iron in the RT112, RT4 and HT1376 bladder cancer cells. Instead of 12 hours used by Duarte and Jones to treat the human diploid fibroblast cells with vitamin C (Duarte and Jones, 2007), selected bladder cancer cell lines used in this study was treated for 4 hours with mirtoselect. This method is based on quenching the fluorescence of the transition metal indicator PG SK by intracellular iron and its subsequent dequenching on addition of a strong membrane-permeable iron chelator, 2,2′-BIP. PG SK is capable of removing iron that is complexed with organic cellular iron chelators (citrate, phosphate, ATP) (Petrat et al., 2001).

The LIP detection method, adapted from Duarte and Jones, was successfully used to demonstrate the ability of anthocyanins to chelate free redox active iron in the RT112, RT4 and HT1376 cells after the mirtoselect pre-treatment (**Figure 6.5**). After the addition of PG SK, mirtoselect pre-treated cells responded to the addition of 2,2′-BIP with a relative decrease in the fluorescence signal, Δ fluorescence, due to a lower level of iron release from PG SK, which indicates lower redox active iron present.

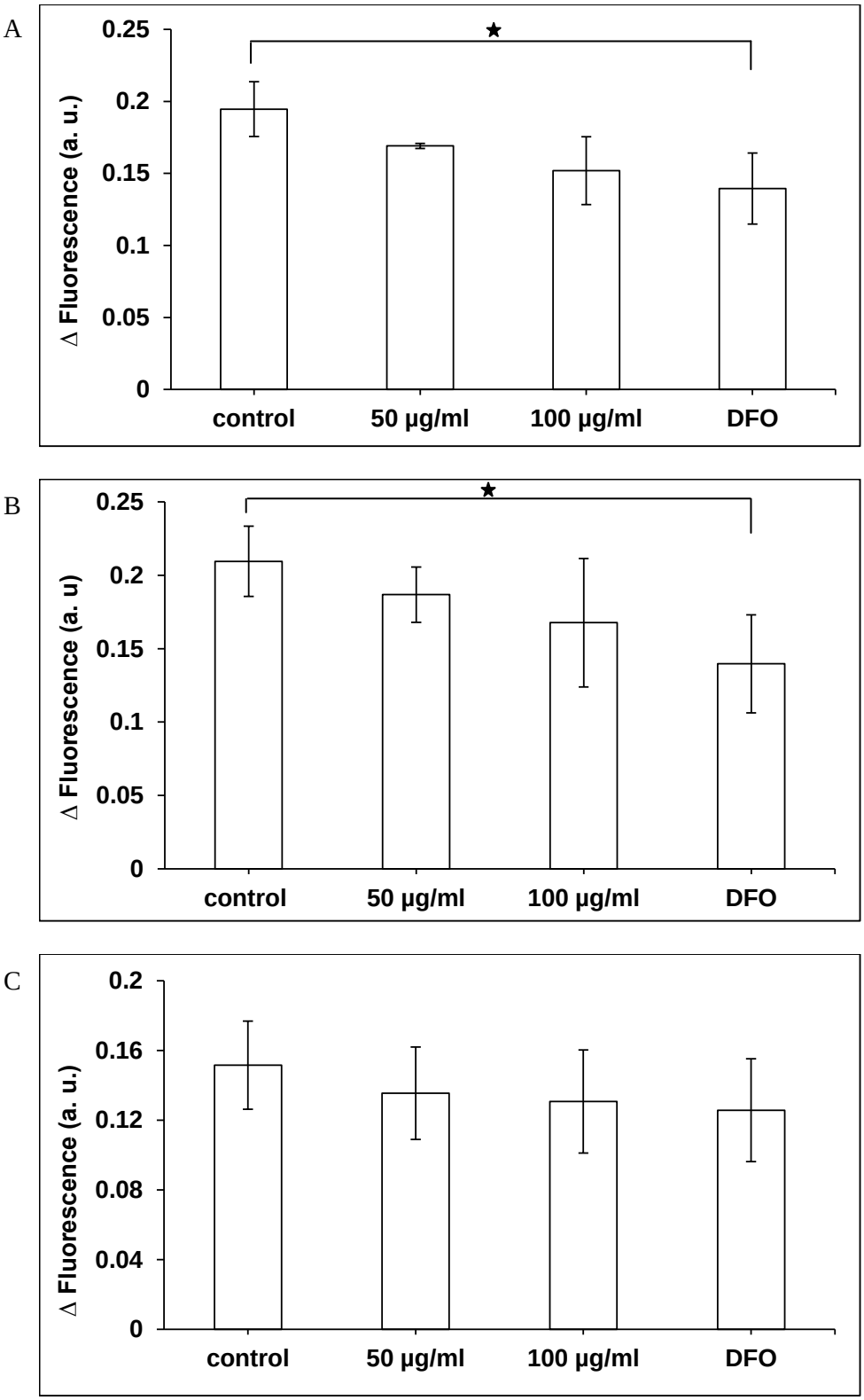


Figure 6.5: Effect of 50 and 100 µg/ml mirtoselect and 300 µM DFO on the relative levels of LIP measured in the (A) RT112, (B) RT4 and (C) HT1376 bladder cancer cell line. Results are the mean ± SD of two independent experiments carried out in quadruplicate incubations. (★= $p < 0.05$).

The results also indicated that the ability of anthocyanins to chelate the LIP was related to the concentration of mirtoselect. From **Figure 6.5 A**, there was approximately 0.17 Δ fluorescence (arb. units) measured after the RT112 cells line were treated with 50 µg/ml mirtoselect whilst the Δ fluorescence was slightly lower (0.15) when the cells were treated with 100 µg/ml mirtoselect. There was approximately 0.14 Δ fluorescence measured in the DFO treated RT112 cells. However, there was no significant difference between the fluorescence intensity measured at different mirtoselect concentrations used in this study and the control RT112 cells. However, there was also only a non-significant reduction in fluorescence relative to control RT112 cells with DFO, a potent synthetic iron chelator used as a positive control

In RT4 cells, the fluorescence signal was approximately 0.18 units measured after the cells were treated with 50 µg/ml mirtoselect, whilst the fluorescence intensity was slightly lower (0.17) for the cells pre-treated with 100 µg/ml mirtoselect (**Figure 6.5 B**). There was approximately 0.14 fluorescence units measured in the DFO treated RT4 cells. However, again, there was no significant difference between the fluorescence measured at different mirtoselect concentrations used in this study and the control RT4 cells, but there was a significant difference between the DFO treated and control RT4 cells.

In HT1376 cells, the measured fluorescence was approximately 0.14 for the HT1376 cells treated with 50 µg/ml mirtoselect whilst the fluorescence intensity was slightly lower (0.13 AU) for the cells pre-treated with 100 µg/ml mirtoselect (**Figure 6.5 C**). The fluorescence (~ 12.7 AU) measured in the HT1376 DFO treated cells was slightly lower than the HT1376 cells incubated with 100 µg/ml mirtoselect. There was no significant difference between the results acquired for the mitroselect treated cells

and the control HT1376 cells nor was there a significant difference between the DFO treated and control cells.

Overall, the results indicated that the possibility of anthocyanins to chelate the LIP was related to the concentration of mirtoselect used in the treatment (i.e. a dose dependant pattern observed). The consistent observation in each cell line data may imply the possible credible mechanism of action for mirtoselect is associated with free metal ion chelation property. .

6.4 Discussion

Investigating the potential role of anthocyanins as protective metal ion chelators requires a sensitive and suitable method for the assessment of chelatable metal ions. In 1999, Petrat and co-workers established a non-disruptive method that could be used to measure chelatable iron pools in adherent hepatocytes cells *in vitro* using a fluorescence probe known as phen green SK. Phen green SK was found to be superior to the conventional probe calcein-AM and other fluorescent probes, for instance PG FL and FL DFO (Petrat et al., 1999). In 2007, Duarte and Jones successfully adapted this method to demonstrate the ability of ascorbic acid to modulate iron metabolism and enhance iron-dependant damage in human fibroblasts (Duarte and Jones, 2007). A similar approach was used in the present effort to assess the ability of anthocyanins to chelate the labile iron pool in bladder cancer cell lines.

Whilst there was no statistically significant difference observed in the ability of mirtoselect to chelate redox active iron in each of the three bladder cancer cell lines used, there was a clear dose dependant effect on the amount of chelatable redox active iron present after treatment with the different mirtoselect concentrations employed. There was, however, a significant difference between the fluorescence recorded in the control and DFO treated cells for the RT112 and RT4 cell lines, but not for the HT1376 cells.

When the decision was made to undertake these experiments to measure the ability of mirtoselect to chelate redox active iron in the bladder cancer cell lines, a number of key preliminary elements needed when setting up/optimising the experimental protocols, were not taken into account. This is primarily because this PhD work was approaching its deadline. The principle protocol was taken from Duarte and Jones, 2007 and modified to suit the requirement of this study. There could be several

factors that may contribute to the non-significant nature of the results obtained from this study:

1. There was no optimisation step taken to evaluate the suitability of the PG SK concentration used in the bladder cancer cell lines. The PG SK concentration was taken directly from Duarte and Jones (Duarte and Jones, 2007). The optimization of the PG SK concentration is important to ensure adequate amount of PG SK was used. Avoiding the unsatisfactory signal measured if too low PG SK was used and/or over expressed of fluorescent measured if too much PG SK was used.
2. There was no optimisation carried out on the incubation time needed for PG SK and BIP treatment. The incubation times used in this study were again taken directly from Petrat et al., 1999 and Duarte and Jones, 2007.
3. In all the previous experiments, the mirtoselect pre-treatment was carried for seven days, with daily dosing. However, in this study it was not possible to treat the bladder cancer cells for seven days as the 96 well plates used would not be able to hold/culture the cells for the seven days. The mirtoselect pre-treatment was only done for four hours before the fluorometry analysis was carried out. There was a probability that four hours treatment may not be sufficient for the majority of the anthocyanins to get across the cell membranes and render their maximal activity.
4. This test was carried out in 96 well plates, which have limited space when control, 50 & 100 µg/ml mirtoselect treated and DFO treated cells were studied together. The best design was achieved by arranging the control and different treatments on the same 96 well plates, so all the different treatments can be carried out at the same time, with minimal technical errors; however,

this allowed only a single replicate of each different treatment per plate. Due to the time constraints, the experiment was carried out only twice. Hence, even though the data (**Figure 6.5**) showed positive activities with respect to iron chelation, there was no significant difference between the mirtoselect treatments and the controls. Taking into account the total replicates of the study (n = 4), there could be low statistical power issues. Therefore, there is a probability of type II statistical errors occurring. This statistical error takes place when there is a failure to reject the null hyphotesis (H_0) even though it is not true. This could happen when small numbers of samples are used or if the data acquired are quite variable.

Nevertheless, this study has shown that mirtoselect was able to chelate the redox active iron in the bladder cancer cell lines used even though the results did not show any significant difference. A prerequisite for the protective action of individual flavonoids was their ability to penetrate through the cell membrane and be present in the interior of cells. Several flavonoids (7,8-dihydroxyflavone, galangin, eriodictyol, 3-hydroxyflavone, 5-hydroxyflavone, 7-hydroxyflavone, apigenin, and luteolin) (**Figure 6.6**) that share specific structural characteristics were used to pre-treat Jurkat cells prior to exposure to H_2O_2, and were shown to offer significant protection against DNA damage (Melidou et al., 2005). To determine whether the observed protective capacities of flavonoids were due to the iron chelation properties, Melidou and co-workers embarked on an experiment utilising iron saturated complexes. If the protection offered against cellular DNA damage was due to chelation of intracellular iron, then iron-saturated flavonoid complexes should be ineffective. To test their hypothesis, Jurkat cells were pre-incubated for 15 min with 30 μM flavonoids (luteolin, galangin, or

quercetin), which were previously mixed with increasing concentrations of Fe^{2+} in the form of $Fe(NH_4)_2(SO_4)_2$ (ranging from 0 to 60 μM). It was shown that the protection offered by these flavonoids was gradually decreased as the ratio of $[Fe^{2+}]$/[flavonoid] increased. The results obtained strongly support the idea of intracellular binding of redox-active iron as being the basis for the protective capacity of flavonoids. Based on these results, it is plausible to assume that this mechanism of prevention of hydroxyl radical generation, at sites where redox-active iron is present, is a much more effective strategy for DNA protection than hydroxyl radical scavenging (Melidou et al., 2005).

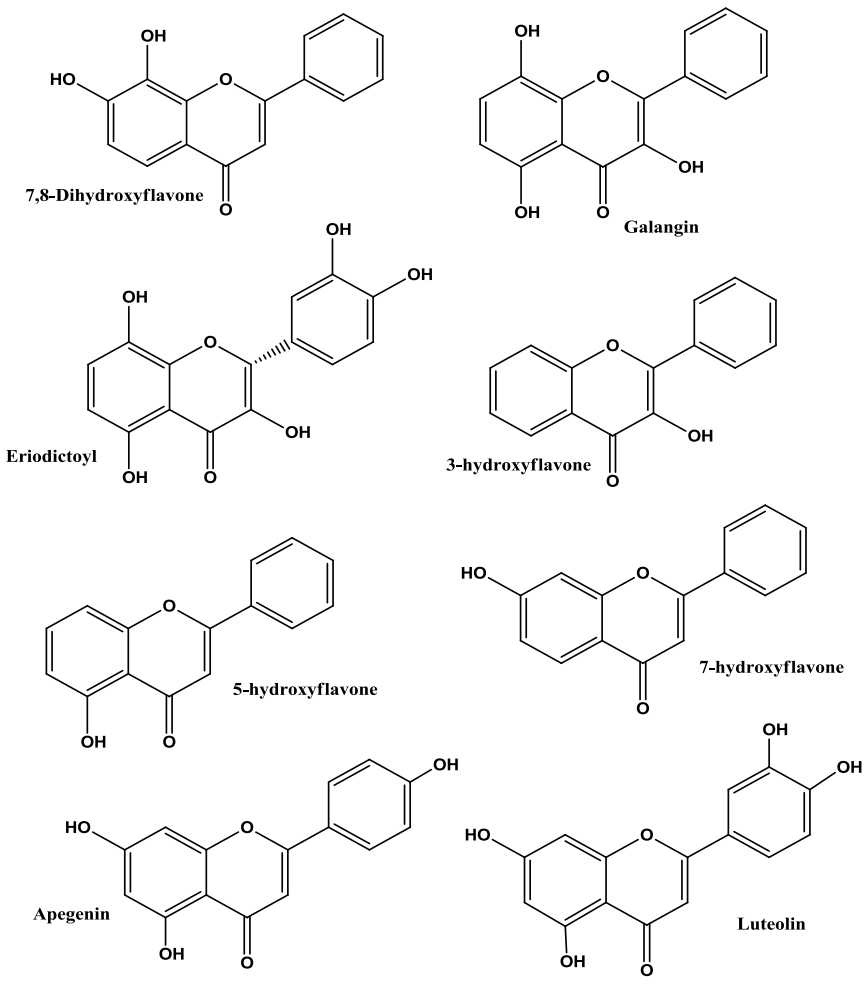


Figure 6.6: Structure of flavonoid compounds used in the study by Melidou and co-workers.

Information about the actual location of intracellular redox-active iron is limited. Although these iron ions are often regarded as bound to low molecular-weight substances, it is probable that most of them are loosely attached to macromolecules such as proteins, DNA, RNA, and carbohydrates (Melidou et al., 2005). Recently a number of publications have demonstrated that a relatively high amount of redox-active iron is localized in the endosomal and lysosomal cell compartments. It was proposed that, this iron plays a crucial role as a mediator of H_2O_2-induced DNA damage and apoptosis (Doulias et al., 2003; Doulias et al., 2005; Kurz et al., 2004; Persson et al., 2003; Radisky and Kaplan, 1998; Tenopoulou et al., 2005; Yu et al., 2003). The role of LIP in the induction of oxidative DNA damage became clearer with the demonstration by Petrat and co-workers (Petrat et al., 2001) of the existence of a LIP pool in cell nucleus and the subsequent observation of a significant correlation between cellular LIP level and the yield of 8-oxoGua in human lymphocytes (Gackowski et al., 2002).

It has been demonstrated that the main structural requirements for optimal protective capacity of individual flavonoids were either the presence of two hydroxyl groups at carbon 3' and 4'of the ring B or a hydroxyl and an oxo group at proximal carbon positions (Melidou et al., 2005). The proposed binding sites for trace metals to flavonoid molecules are the catechol moiety in the B ring, the 3-hydroxyl and 4-oxo groups in the heterocyclic C ring, and the 4-oxo and 5-hydroxyl groups between the heterocyclic C ring and the A ring (**Figure 6.7**). However, a major contribution to metal chelation is the catechol moiety, as exemplified by the more pronounced bathochromic shift (a shift of a spectral band to longer wavelengths as a result of substitution in a molecule or a change in the conditions) produced by chelation of copper to quercetin compared to that of kaempferol (similar in structure to quercetin except that it lacks the catechol group in the B ring) (Sestili et al., 2002; van Acker et al., 1996a).

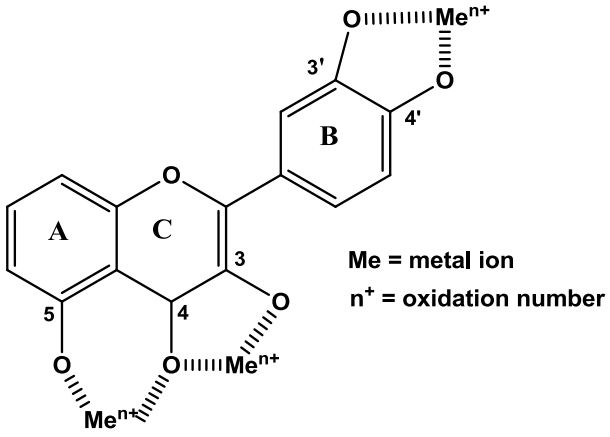


Figure 6.7: Proposed metal ion binding sites on the flavonoids molecules featuring 3' and 4' catechol position and 3-hydroxyl and 4-oxo groups in the heterocyclic ring, and the 4-oxo and 5-hydroxyl groups between the heterocyclic C ring and the A ring (adapted from Pietta et al., 2000).

Similar to van Acker and co-workers (van Acker et al., 1996b), it was also proposed that, the presence of the C2 and C3 double bond increased the protective ability by more than 1 order of magnitude, as indicated by comparing luteolin with eriodictyol and quercetin with taxifolin. Because these compounds have identical chemical structures except for the absence of the C2 and C3 double bond in the latter, it was established that a broad conjugating system greatly enhances the protective capacities of the respective flavonoids (Melidou et al., 2005).

The combination of a hydroxyl group at position 3 with an oxo group at position 4 of the C ring was also shown to create a functional group that made the respective flavonoids protective as observed in 3-hydroxyflavone, which could decrease H_2O_2-induced DNA damage. The presence of an additional OH group at position 5 further enhanced the effectiveness of protection, as indicated by comparing 3-hydroxyflavone and galangin (Melidou et al., 2005). On the other hand, the bare combination of 5-OH with 4-oxo groups in the absence of the 3-OH moiety does not offer any further DNA

protection although it is known to contribute toward increasing the antioxidant capacity of the corresponding flavonoids (Rice Evans et al., 1996). It was also demonstrated that all flavonols (which contain 4-oxo and 3-hydroxyl groups), except kaempferol and morin, exerted relatively high protective potentials (Melidou et al., 2005). Englemann and co-workers have established binding constants for different moieties of flavonoid molecules (3-hydroxyflavone (flavonol), 5,7-dihydroxyflavone (chrysin), and 3',4'-dihydroxyflavone). This study showed that ferric complexation favours a catechol moiety (3' and 4' hydroxyl) (Engelmann et al., 2005). Based on these binding constants it was predicted that the catechol and 4-oxo-3-hydroxy (but not 4-oxo-5-hydroxy) moieties of flavonoids may be important as iron chelators in living cells (Melidou et al., 2005).

For the flavonoids present in mirtoselect to exhibit efficient protection against H_2O_2-induced DNA damage, it is proposed they must possess the following two properties: (a) are able to pass through the plasma membrane and (b) are able to remove loosely bound redox-active iron from specific intracellular locations. Cyanidin-3-glycosides, petunidin-3-glycosides and delphidin-3-glycosides are among the anthocyanin compounds present in mirtoselect, with structures having the catechol at position 3' and 4' of the B ring, associated with binding of redox active iron (Silva et al., 2002). Anthocyanins have also been shown to fulfil the first requirement in order to be able to offer efficient protection against H_2O_2-induced DNA damage. Anthocyanins, for instance, cyanidin 3-sambubioside and cyanidin 3-glucoside were detected in the plasma of elderly women in its glycate form (Cao et al., 2001; Cao and Prior, 1999). Recently, a study carried out by Cooke and co-workers also demonstrated that anthocyanins are able to cross the cell membranes. They have managed to recover about 71% of cyanidin-3-glucoside from human plasma after oral administration (Cooke et al.,

2006). The glycosylation of flavonoids reduces their free radical scavenging activity when compared to the corresponding aglycones (Shahidi et al., 1992). However, Pool-Zobel and co-workers have demonstrated, through the Ferric Reducing Ability (FRA) test, that the antioxidative capacity of the anthocyanin fraction from *Aronia melanocarpa* may be a reflection of the compounds' potential to chelate $FeCl_3$, as well as to scavenge free radicals. Interestingly, whatever the mechanisms, the activities are not only due to the aglycone (cyanidin) but more so to the corresponding glycosides, cyanin, and idaein (Pool-Zobel et al., 1999). Therefore, the existence of a glycoside moiety in position 3 of the C ring did not totally diminish the antioxidant activity of the anthocyanins.

6.5 Summary statement

The present study has demonstrated that constituents of mirtoselect were able to reduce/lower the labile iron pool present in the bladder cancer cells used in this study; this effect is most probably mediated via the chelation of the labile iron. The non-significance of the results observed does not discount the ability of the anthocyanins to mediate this effect as there was a clear and consistent dose-reponse noted for all three cell lines tested, with the effect being of a similar magnitude and in the same direction as that noted for the the DFO treated cells (the effective positive control). There are further factors that deem the mirtoselect anthocyanins as being the agents responsible for chelation of redox active iron ions; anthocyanins have been shown to be able to pass cell membranes in its glycate form, and there are anthocyanin compounds that possessed the catechol moiety that has been shown to be important in the chelation of metal ions.

In conclusion, results from this study suggest that mirtoselect seems to exhibit its antioxidant property via metal chelation, rather than free radical scavenging activities. Eventhough the results from this study showed no siginificant different between the different mirtoselect concentration (100 and 50 µg/ml) used there was a consistent pattern observed from the data acquired.

Chapter 7.

General Discussion.

7.1 Summary of the overall results

To help understand and conclude this study, it is worth briefly summarising the key results obtained.

In **Chapter 2** the standardised anthocyanin extract, mirtoselect, was shown to act in an anti-proliferative manner towards the three bladder cancer cell lines studied *in vitro*, in a dose dependent fashion. There was strong inhibition of proliferation observed in both the RT112 and RT4 cells pre-treated with 75 µg/ml and 100 µg/ml mirtoselect, whilst the HT1376 cells exhibited only moderate inhibition. The noted dose dependency of the anti-proliferative effects of mirtoselect provided the optimal concentration (50 µg/ml mirtoselect) to be utilised in the subsequent antioxidant experiments.

The investigation of mirtoselect as an antioxidant was made possible through the development of the modified alkaline Comet assay protocol (**Chapter 3**). Central to this development was the preparation of the oxidatively damaged DNA base lesion-containing "substrate cells". These "substrate cells" were utilised in studies aimed at optimising the amount of Fpg and hOGG1 needed to maximally reveal oxidatively damaged DNA base lesions. This was succesfully determined utilising two different enzyme concentrations (Fpg: 14.66 and 29.33 U/ml, hOGG1: 1.60 and 3.2 U/ml) of the commercially supplied enzymes, with the Fpg (29.33 U/ml) and hOGG1 (3.2 U/ml)being chosen for use in the subsequent antioxidant investigations. The observation of a greater response for Fpg compared to hOGG1 is in agreement with the known substrate specificities of the respective enzymes.

In an initial investigation of the antioxidant capacity of mirtoselect, the modified alkaline Comet assay protocol was used to determine the effect of mirtoselect pre-treatment on the amount of endogenous Fpg and hOGG1sensitive sites present in RT112, RT4 and HT1376 cells. Similar to previous studies conducted by other

researchers, anthocyanins did not alter the level of Fpg and hOGG1sensitive sites in any of the bladder cancer cells studied. However, in subsequent experiments which involved exposure to exogenous H_2O_2, anthocyanins exhibited a significant degree of protection in all three bladder cancer cell lines. Whilst the anthocyanins managed to protect the cells from the exogenous oxidising agent, the repair capacity towards the induced oxidatively damaged DNA base lesions or SSBs remained unchanged when compared to the non-mirtoselect treated control cells (**Chapter 4**).

The protective effect exhibited by mirtoselect raised the question as to the likely mechanism(s) responsible for the reduction of DNA damage detected in this study. It is well established that anthocyanin compounds present in mirtoselect are classified as strong antioxidants. However, the exact mechanism(s) of action engaged by these compounds has not been ascertained, but is thought to include free radical scavenging and/or chelation of redox active metal-ions. Therefore, subsequent experiments were devised and undertaken to determine whether the antioxidant properties of anthocyanins were due to free radical scavenging and/or redox active metal-ion chelation.

Following on from the protective effects exhibited by the mirtoselect pre-treatment of cells noted in **Chapter 4**, an experiment was conducted employing X-ray irradiation to elucidate the mechanism of action exerted by anthocyanins (**Chapter 5**). In cells, ionising radiation is known to cause immediate DNA damage mostly via hydroxyl radical formation, but through a mechanism that is independent of metal ions. Therefore, comparison of the level of DNA damage induced by X-ray irradiation with that caused by H_2O_2 exposure in mirtoselect pre-treated cells enabled the evaluation of free radical scavenging as the mechanism responsible for the protective effect of mirtoselect. As mirtoselect failed to alter the yield of immediate radiation-induced damage, this indicated that free radical scavenging was not the primary mechanism

responsible for the protective effect. It was therefore, next suggested that the protection conferred was through redox active metal-ion chelation. This proposal was supported by studies assessing the effect of mirtoselect pre-treatment on the labile iron pool (LIP) in bladder cancer cells (**Chapter 7**). Through the use a phenanthroline-based fluorescent probe for Fe^{+2}, the ability of mirtoselect to chelate redox active ions present in the bladder cancer cells was demonstrated. Whilst the data from this study were not statistically significant, the observation of mirtoselect reducing/lowering the LIP level was found to be both dose dependant and consistent across all three cell lines studied.

7.2 Discussion

In both the UK and USA, from the time of first diagnosis till death, bladder cancer has been deemed the most expensive cancer to treat (Lotan et al., 2009; Sangar et al., 2005). In the UK, bladder cancer contributes a significant financial burden to the NHS (Mostafid et al., 2009; Sangar et al., 2005). In addition to this, despite advances in the treatment of bladder cancer in recent years, the rate of bladder cancer incidence in the UK has not changed for almost two decades (Deweerd et al., 1982; Mostafid et al., 2009). Consequently, it has been suggested that a whole new approach to the management of bladder cancer should be formulated so that the NHS financial burden can be reduced (Sangar et al., 2005). Whilst there are still opportunities for 'conventional' bladder cancer treatments to be further improved (Choudhury et al., 2011), it is an appropriate time for alternative options, such as bladder cancer chemoprevention, to be properly and systematically considered.

The nature of bladder cancer, with its long latency period of approximately 25 years (from undifferentiated urothelial cells to fully cancerous cells) (O Shaughnessy et al., 2002) makes it a highly appropriate candidate for cancer chemoprevention. Other criteria that make this cancer site a good model for clinical/academic study in a prevention setting, is the relative ease of access for tumour sampling and observation.

It is proposed that the initiation of the bladder cancer is highly associated with exposure to aromatic amines, for instance 4-ABP, that can lead to DNA damage in the form of adducts. However, in addition to this, cellular metabolism of these compounds can generate harmful ROS and these also contribute to the damage and mutation burden experienced by the DNA of urothelial cells. Since the initiation of bladder cancer is associated with exposure to ROS, a strong antioxidant compound that can protect the

DNA of urothelial cells could be considered an appropriate agent for bladder cancer chemoprevention.

A study by Cooke and co-workers showed that mirtoselect significantly lowered the formation of intestinal adenomas in the *Apc*^{Min} mouse. Modified and unmodified anthocyanin compounds were also found at the analytical detection limit in the plasma and at quantifiable levels in the intestinal mucosa and urine. Anthocyanin glucuronide and methyl metabolites were identified in intestinal tissue and urine. Total anthocyanin levels in mice ingesting mirtoselect were 8.1 l µg/g , in the intestinal mucosa, and 12.3 µg/ml in the urine (Cooke et al., 2006). Taking into consideration that anthocyanins constitute **25** % of what is present in the mirtoselect obtained from Indena S.p.A, the amount of anthocyanins present in the urine (12.3 µg/ml) equates to a dose of 50 µg/ml mirtoselect, and there are several studies published that have demonstrated that 50 µg/ml anthocyanins as an appropriate concentration to deliver substantial impact of antioxidant property (Joseph, 2004; Malik et al., 2003; Nizamutdinova et al., 2009; Tsoyi et al., 2008; Vareed et al., 2006; Yi et al., 2005). Potentially higher concentrations of mirtoselect can be delivered to the human bladder through ingestion of higher doses or else direct administration into the bladder. Therefore an investigation was also carried out to determine the effect of mirtoselect at concentrations up to 100 µg/ml.

Mirtoselect at 75 and 100 µg/ml significantly inhibited RT112 and RT4 cell proliferation *in vitro* whilst substantial inhibition was observed for the HT1376 cells. ARE, in particular berry extracts, have been widely investigated for their potential anti-proliferative properties against a variety of cancer cells, and some have shown promising results. For instance, strawberry and raspberry extracts strongly inhibited proliferation of human colon cancer cells (CaCo-2) (McDougall et al., 2008) and

bilberry extracts have been shown to be effective against human leukaemia cells (HL-60) (Katsube et al., 2003). Additionally, blueberry and cranberry extracts suppress proliferation of human breast cancer cells (MCF7) and colon carcinoma cells (HT-29) (Seeram et al., 2006). However, the mechanism(s) underlying the anti-proliferative properties of these extracts is still not fully understood. ARE of açai at 100 and 200 µg/ml causes DNA fragmentation suggesting the anti-proliferative effect of ARE against C-6 glioma cells is caused by inducing cell apoptosis (Hogan et al., 2010). The açai extract was previously reported to induce apoptosis in human leukaemia cells (HL-60) by activating caspase-3 (Del Pozo-Insfran et al., 2006). Cyanidin, an agylcone of anthocyanins, has been shown to induce a dose-dependent growth inhibitory effect in fibroblasts with a significant reduction of cells in S phase, whilst delphinidin, another anthocyanidin inhibited proliferation of fibroblasts via accumulation of cells in the S phase, suggesting a block in the transition of S to G_2 phase. On the other hand, treatment of uterine carcinoma and colon adenocarcinoma cells with delphinidin leads to a reduction of cells in G_1 phase and appearance of a fraction of cells with hypodiploid DNA content, thus demonstrating an apoptotic effect (Lazze et al., 2004). In a parallel project to this study also conducted within the group, treatment of the same bladder cancer cells, as used here, showed no effect of mirtoselect on the distribution of cells within the cell cycle (Higgins, 2011).

Investigating the capability of mirtoselect to protect the bladder cancer cell lines from oxidatively damaged DNA required an effective method to measure the occurrence of oxidatively damaged DNA, such as 8-oxoGua. There are a number of analytical techniques that can be used to assess DNA damage and adduct formation and every technique has its own advantages and disadvantages (Himmelstein et al., 2009). High pressure liquid chromatography coupled with tandem mass spectrometry (HPLC-

MS/MS) and single cell gel electrophoresis, more commonly known as the Comet assay (Collins et al., 2008), are amongst the most popular and now routinely used methods to detect oxidatively damaged DNA. The earlier use of gas chromatography-mass spectrometry (GC-MS) enabled structural identification, but the results obtained were always suspect, due to the possibility of artefactual DNA oxidation during sample preparation; this technique also requires a relatively high amount of DNA (up to 500 µg) (Guetens et al., 2002). On the other hand, HPLC-MS/MS is able to provide structural elucidation without such extensive sample preparation (so limiting artefactual DNA oxidation), however, this technique requires costly specialised equipment and it still requires relatively large amounts of material, *ca.* 10 - 100 µg of DNA (Farmer and Singh, 2008; Singh and Farmer, 2006). The Comet assay is a straightforward, sensitive and relatively cheap way of determining the amount of DNA damage in cells and the technique is considered to be largely free of artefactual DNA oxidation. Whilst the assay measures DNA strand break damage, the specificity of this technique with respect to measuring oxidatively damaged DNA can be increased by the inclusion of specific DNA glycosylase enzymes which recognise and cleave the DNA strands at the site of specific DNA base lesions. This technique requires only a few thousand cells per sample, equivalent to less than 1 µg of DNA. This low material requirement is of great benefit in allowing this technique to be applied to the analysis of clinical samples/biopsies (Collins, 2004; Collins et al., 2008; Singh et al., 1988; Speit et al., 2004). However, this technique is prone to both inter and intra experimental variation (Forchhammer et al., 2010; Johansson et al., 2010; Moller et al., 2010) and studies are underway to develop standard materials to help reduce this variation. Indeed, a recent study to develop and introduce the use of *bone fide* internal standard materials resulted

in significant reductions for both inter-and intra- experiment variation (Zainol et al., 2009).

Taking into consideration all of the available techniques, it was decided that assessment of the antioxidant properties of mirtoselect, in the selected bladder cancer cell lines (RT112, RT4 and HT1376), would be investigated using the alkaline Comet assay, with the inclusion of Fpg and hOGG1 enzymes to specifically assess oxidatively damaged DNA. Optimisation of the amount of DNA glycosylase to be employed was a factor of the utmost importance, since if too low an amount of enzyme was used, this would lead to an underestimation of the amount of oxidatively damaged DNA present, whilst excessive amounts would lead to indiscriminate cutting of the DNA, so overestimating the damage present. Optimisation of the amount of glycosylase enzyme levels required started with the preparation of oxidatively damaged DNA base lesion-containing "substrate cells". These were prepared by optimising the H_2O_2 concentration and treatment, then the time needed for complete SSB repair. Treatment of each bladder cancer cell line with 50 µM H_2O_2 (RT112 and RT4) or 40 µM H_2O_2 (HT1376), followed by repair incubation for 60 minutes produced the requisite oxidatively damaged DNA base lesion-containing "substrate cells". Subsequently, the optimised enzyme concentrations for Fpg and hOGG1 were determined as being Fpg: 14.66 and 29.33 U/ml, hOGG1: 1.60 and 3.2 U/ml of the initial commercially available stock. Smith and co-workers, used 1/3,000, 1/300 and 1/1,000 dilutions of Fpg, ENDO III and hOGG1, respectively, in their study to determine the specificity of hOGG1 (Smith et al., 2006). Several Comet assay researchers have obtained Fpg enzyme from Professor Andrew Collins (Oslo University, Norway) and have used a 1/3000 dilution in many of their Comet assay studies. Commercially produced DNA glycosylase enzymes, as used in this study, which are available on the open market are expected to be more dilute

than the one produced on a non-commercial basis by Professor Collins (personal communication with Professor Andrew Collins).

Success in establishing the modified alkaline Comet assay protocols, with the inclusion of Fpg (29.33 U/ml) and hOGG1 (3.2 U/ml), enabled utilisation of this technique to assess the effect of mirtoselect on oxidatively damaged DNA in the selected bladder cancer cell lines (RT112, RT4 and HT1376).

Prior to assessing the antioxidant properties of mirtoselect, a study was carried out to determine whether mirtoselect was able to influence the SSB repair capacity of the bladder cancer cell lines. The results acquired suggested that mirtoselect did not play any role in changing the SSB repair kinetics in these particular cells. The two flavonoids quercetin and rutin have been shown to decrease the amount of DNA damage induced by H_2O_2 in V79 cells but these compounds did not increase the rate of repair of DNA strand breaks in Caco-2, Hep-G2 and V79 cell lines (Aherne and O'Brien, 2000). Pool-Zobel and co-workers further demonstrated that anthocyanin fractions from *Aronia melanocarpa* fruits were able to prevent H_2O_2 induced SSB DNA damage in HT29 clone 19A colon cells (Pool-Zobel et al., 1999). Similar to these reports, in the present study, even though there was no effect on the repair capacity of the bladder cancer cells pre-treated with mirtoselect, there were significantly lower amounts of immediate DNA SSBs induced following pre-treatment with mirtoselect (see below).

Treating the bladder cancer cell lines with 50 µg/ml mirtoselect for seven days, did not affect the level of endogenous oxidatively damaged DNA base lesions measured in any of the three bladder cancer cell lines. A similar observation was made by Pool-Zobel and co-workers, in which the anthocyanin fractions from *Aronia melanocarpa*, concentrates of elderberry, macqui, and tintorera grapes, as well as cyanidin, idaein

(cyanidin-3-galactoside) and cyanin (cyanidin-3,5-diglucoside) did not alter the steady state-level of oxidatively damaged DNA bases in HT29 clone 19A, human colon tumour cell line (Pool-Zobel et al., 1999). It is believed that damage to DNA by ROS occurs naturally at low steady-state levels and is manifest as modified bases, for instance 8-oxoGua lesions, which are detected in nuclear DNA from human tissue and cells (Musarrat and Wani, 1994; Wiseman and Halliwell, 1996). The pattern of damage observed on the purine and pyrimidine bases suggests that at least some of the damage occurs by $^{\bullet}OH$ attack, suggesting that $^{\bullet}OH$ is formed in the nucleus *in vivo* (Halliwell and Dizdaroglu, 1992).

When the exogenous oxidising agent, H_2O_2, was used to determine the effect of mirtoselect on the level of induced oxidatively damaged DNA bases in the bladder cancer cells, interesting results were observed for all cell lines used in this study. Lower amounts of immediate DNA strand breaks plus lower levels of immediate oxidatively damaged purine base lesions were observed, as revealed by the Fpg and hOGG1 Comet assays for all the cells that was pre-treated with mirtoselect for seven days, compared to the corresponding untreated control cells. Recently, Spormann and co-workers reported a reduction in oxidatively damaged DNA measured by Fpg Comet assay in haemodialysis patients consuming anthocyanin/polyphenolic-rich juice made from red grapes (40%), blackberries (20%), sour cherries (15%), blackcurrant (15%) and elderberries(10%) (Spormann et al., 2008). A similar study of the flavonoids catechin and epicatechin revealed they protected against *N*-nitrosamines and benzo(*a*)pyrene-induced DNA damage, measured as strand breaks and oxidized purines/pyrimidines, in human hepatoma cells (Hep-G2) (Delgado et al., 2008).

Whilst there was a significant antioxidant effect observed in all the bladder cancer cell lines used in this study, mirtoselect did not influence the repair kinetics of

the oxidatively damaged DNA bases in any of the cell lines. The observed antioxidant effect prompted an investigation into the mechanism of action by which mirtoselect protects the pre-treated cells. There is no doubt that TEAC, DPPH test, ORAC (Melidou et al., 2005; Milardovic et al., 2006; Pistelli et al., 2006) and many other types of analysis have contributed significantly towards the elucidation of the antioxidant properties of many flavonoid compounds, but it is possible that the mechanism of action identified using such systems was not accurate due to the absent of intact live cells (Melidou et al., 2005).

Elucidation of mirtoselect's mechanisms of action in cells was carried out by comparing the protective effect observed when the pre-treated bladder cancer cell lines were exposed to exogenous H_2O_2 or to X-ray irradiation. Radiation causes changes in the cells or tissues through which it passes by depositing energy and causing ionisation and excitation of the molecules with which it interacts. In cells ionising radiation is known to cause immediate DNA damage, mostly through indirect attack on DNA by hydroxyl radicals generated from water radiolysis (Ward, 1988). Radiation-induced damage is therefore an iron ion independent whilst H_2O_2-induced DNA damage is dependent on the presence of redox active transition iron ions. Exposing the control and mirtoselect pre-treated cells to 4 and 8 Gy failed to exhibit any protective effect, compared to when the cells were exposed to exogenous H_2O_2. Therefore, the protective effect exhibited by mirtoselect was not a consequence of the constituent anthocyanins scavenging hydroxyl free radicals, but may be due to its ability to chelate redox active iron ions.

The iron ion chelation properties of mirtoselect were proven through the lower measurement of LIP in the bladder cancer cells pre-treated with 50 and 100 µg/ml mirtoselect. The difference in results obtained between the test and control samples did

not reach statistical significance , however, the ability of mirtoselect to reduce LIP levels was dose dependant and consistent across all three cell lines studied. Pool-Zobel and co-workers and Melidou and co-workers also suggested that the mechanism of action of the flavonoid and anthocyanin compounds used in their respective studies was due to chelation of transition iron ions (Melidou et al., 2005; Pool-Zobel et al., 1999). The ability of anthocyanins to exert their antioxidant and other therapeutic properties is strongly associated with their chemical structure. Systematic efforts to correlate the structure of flavonoids with their ability to prevent damage of cellular components in intact cells exposed to oxidative stress are currently lacking, and the molecular mechanisms underlying the protective actions of flavonoids in cells under conditions of oxidative stress are poorly understood and need further investigation (Melidou et al., 2005; van Acker et al., 1996b).

Mirtoselect has undoubtedly exhibited some potent antioxidant properties towards the human bladder cancer cells studied here *in vitro*. The assessment carried out has demonstrated the ability of mirtoselect to reduce oxidatively damaged DNA formation in the bladder cancer cell lines. This, together with other supporting data warrants future consideration of mirtoselect as a candidate for development as a bladder cancer chemopreventive agent

7.3 Future works

There are several factors that need further attention before claiming that mirtoselect, or the constituent anthocyanins, be developed as an agent for bladder cancer chemoprevention. For instance, pharmacological and pharmaceutical issues including stability, bioavailability and ease of formulation of the constituent anthocyanins need to be addressed before it can be considered for further drug development.

Future *in vitro* work towards determining the possible protective effects against exogenous H_2O_2 exposure by the individual constituent anthocyanin compounds present in mirtoselect, especially compounds bearing catechol moieties on their B ring, should be carried out to further delineate the protective effects observed in this study. To further understand structure-activity relationship for anthocyanins, with regard to the antioxidant properties exhibited, studies undertaken at different pH (to establish the contribution of oxonium ions) and the effect of glycoside moiety attachments should be properly and systematically investigated. In **Chapter 6** the results failed to show any significant difference between two mirtoselect concentrations used to determine the ability of mirtoselect to chelate iron ions. It will be better if further experiments can be undertaken taking into account all the factors raised in the discussion of **Chapter 6**. This study should be further supported by running further Comet assay experiments showing the effect of exogenous H_2O_2 exposure after DFO treatment of the bladder cancer cells. This study will confirm the involvement of transition iron ions in the formation of DNA damage by H_2O_2 exposure; a similar study carried by other researchers on different cell lines showed that H_2O_2 exposure of DFO pre-treated cells did not produce any damage (Duarte and Jones, 2007).

Petrat and co-workers have shown the existence of LIP in the nucleus (Petrat et al., 2001). A study could be designed to track the movement of the anthocyanin compound in the cells, perhaps by using anthocyanins with fluorescent tags. The protective effect observed and the proposed mechanism of action of anthocyanins in mirtoselect through transition iron ion chelation would be further substantiated if the tagged anthocyanins were detected in the nucleus of the pre-treated cells.

Another important aspect that needs to be comprehensively investigated is the effect of anthocyanins on normal and pre-lesion urothelial cells. Southgate and co-workers have developed an efficient technique to culture normal human bladder epithelial cells with the cells managing to grow and be cultured for a finite period of time (Southgate et al., 1994; Southgate J, 2002). There is also the possibility of studying the effect of anthocyanins on precancerous bladder cells. Genetically manipulated human precancerous urothelial cells have been created using simian virus (SV-40) and a high-risk human papilloma virus (HPV) (Crallan et al., 2006). Employing the techniques developed by Southgate and co-workers, Varley and co-workers have successfully engineered squamous differentiated cells, similar to normal human urothelial cells, by manipulating the peroxisome proliferator activated receptor γ (PPARγ) (Varley et al., 2004a; Varley et al., 2004b).

Thomasset also raises the issue regarding the possible biological potency of the major metabolites of anthocyanins observed in plasma and urine; currently little is known regarding the biological effects of metabolites compared to the parent anthocyanins. In addition, development of improved analytical methods for anthocyanins would increase the accuracy of the determination of the pharmacokinetics of these agents in future studies (Thomasset et al., 2009).

Anthocyanins have also been demonstrated to participate in the regulation of cellular oxidant defence mechanisms. Anthocyanin-enriched bilberry extract has been shown to modulate pre- or post-translational levels of the oxidative stress defence enzymes heme-oxygenase-1 and glutathione *S*-transferase-pi in cultured human retinal pigment epithelial cells (ARPE-19) (Milbury et al., 2007). In the future, the effect of mirtoselect on the modulation of glutathione enzymes and other oxidative stress defence enzymes in the bladder cancer cell lines could be considered.

In conclusion, the antioxidant properties observed after pre-treatment of bladder cancer cell line (RT112, RT4 and HT1376) with 50 µg/ml mirtoselect have been shown to occur seemingly due to the metal ion chelation properties of mirtoselect, this being presumably mediated by anthocyanin compounds contained in mirtoselect. The results obtained have fulfilled the aims and objectives set for this study and overall the findings contribute useful scientific information for the further study and possible development of anthocyanins as potential bladder cancer chemopreventive agents.

Appendix

List of Publications and Communication at Scientific Meetings

1. Samantha Carrera ,Petra J. de Verdier, Zahid Khan, Bo Zhao , Alka Mahale, Karen J. Bowman, **Muri Zainol** , George D. D. Jones, Sam W. Lee, Stuart A. Aaronson and Salvador Macip (2010) **Protection of cells in physiological oxygen tensions against DNA damage-induced apoptosis**. The Journal of Biological Chemistry; April, **285**: 13658-13665.

2. **Murizal Zainol**, Julia Stoute, Gabriela M. Almeida, Alexander Rapp, Karen J. Bowman, George D. D. Jones and ECVAG (2009) **Introducing a true internal standard for the Comet assay to minimize intra- and inter-experiment variability in measures of DNA damage and repair**. Nucleic Acids Res; December, 37(22): e150-e158.

3. **Murizal Zainol**, William P Steward, Karen Brown & George DD Jones (2009) **Chemoprevention of Anthocyanins on Bladder Cancer Cells.** Presentation at the 32nd Annual Meeting of UKEMS, University of Leeds, Leeds, UK.

4. **Murizal Zainol**, William P Steward, Karen Brown & George DD Jones (2008**) Assessing oxidative DNA damage in bladder cancer cells using the modified alkaline Comet assay.** Poster at the NCRI Cancer Conference, International Convention Centre, Birmingham, UK.

5. **Murizal Zainol**, William P Steward, J Kilian Mellon, Karen Brown & George DD Jones (2008). **Development of the Comet Assay to Assess Oxidative DNA Damage in Bladder Cancer Cells.** UKEMS/NordEMS/MEG/IGG Joint Conference, University of Northumbria, Newcastle upon Tyne, UK.

6. Murizal Zainol, William P Steward, J Kilian Mellon, Karen Brown & George DD Jones (2008). **Development of the Comet Assay to Assess Oxidative DNA Damage in Bladder Cancer Cells.** Poster at the BAUS Section of Academic Urology Annual Scientific Meeting, London. UK.

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