

**EVALUATION OF THE ALKALINE COMET ASSAY AS A  
PREDICTIVE TEST OF INVASIVE BLADDER CANCER  
TREATMENT: PREDICTION OF BLADDER CANCER CELL  
RADIOSENSITIVITY AND CHEMOSENSITIVITY**

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at the University of Leicester

By

MANAR MONEEF AL-MONEEF B.Sc. (Hons).  
Department of Oncology  
University of Leicester

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# **EVALUATION OF THE ALKALINE COMET ASSAY AS A PREDICTIVE TEST OF INVASIVE BLADDER CANCER TREATMENT: PREDICTION OF BLADDER CANCER CELL RADIOSENSITIVITY AND CHEMOSENSITIVITY**

**Manar Moneef AL-Moneef**

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## **ABSTRACT**

This thesis investigates the alkaline comet assay (ACA) as a potentially rapid, prognostic test in the treatment of muscle invasive bladder cancer. In the UK, the two main treatment options for invasive bladder cancer are radiotherapy (RT) or cystectomy. However, ~50% of patients undergoing RT fail to respond, and these patients are disadvantaged by the absence of predictive information regarding their tumour's radiosensitivity. Furthermore, in the continuing search for effective local control with improved survival, chemotherapeutic agents have been used in conjunction with RT and cystectomy. However, in the treatment of some patients, local control is achieved without the use of cytotoxic chemotherapy and as such these patients do not benefit from this additional treatment. If tumour cell radiosensitivity and chemosensitivity could be predicted in advance, it may be possible to improve control rates by selecting patients for the most appropriate treatment. In the present study we are evaluating ACA as a rapid predictive measure of bladder cancer cell radiosensitivity and chemosensitivity.

Using a panel of six human bladder cancer cell lines of differing radio- and chemosensitivities, the relationship of cell survival and viability to the formation and repair of both radiogenic and chemotherapeutic drug-induced DNA damage, as determined by ACA, was assessed. For all the cell lines studied the ACA measures of initial DNA damage formation correlate with cell killing/loss of cell viability, with a greater comet response/damage level being noted in the sensitive cells. Measures of repair, also correlate with cell survival and viability, though to a lesser degree.

Finally, in a preliminary study, epithelial cells isolated from human bladder cancer biopsies also reveal a range of radio-responses as determined by ACA, which is taken to reflect actual variation in tumour cell radiosensitivity.

Overall, these studies demonstrate ACA to be a good predictor of bladder cancer cell radiosensitivity and chemosensitivity.

*This Thesis Is Dedicated To My Parents  
Sameera and Moneef Al-Moneef,  
With Much Love and Thanks For Everything.*

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***CHAPTER ONE***

***GENERAL INTRODUCTION***

## **1.1 INTRODUCTION**

This thesis is concerned with an investigation of the alkaline comet assay (ACA) as a rapid predictive test in determining the radiosensitivity and chemosensitivity of bladder cancer cell lines, with particular regard to the role that this assay may play in bladder cancer treatment in the future. The first part of this General Introduction describes the causative factors and the mechanisms behind the development of bladder cancer. The second part deals with the treatment of bladder cancer at different stages of the disease, in particular, the possible treatment options for muscle invasive bladder cancer. Finally, this Chapter ends with a description of the aims for the study, that is, the evaluation of the alkaline comet assay as a predictive test of bladder cancer cell radiosensitivity and chemosensitivity.

## **1.2 CANCER**

Cancer can be defined generally as a disease in which there is uncontrolled multiplication and spread within the body of abnormal cells (Rang *et al.*, 1990). Cancer cells have three characteristics not seen in normal cells. These are uncontrolled proliferation, invasiveness and the capacity to metastasise, none of which are subject to the regulatory processes seen in normal cells. One of the major reasons that cancer cells undergo uncontrolled proliferation appears to be that they develop the ability to produce and respond to their own growth factors (see Section 1.2.3).

### **1.2.1 INCIDENCE OF CANCER**

Cancer is one of the leading causes of death worldwide. According to the world health organisation (WHO), cardiovascular disease is the leading cause of death accounting for 30% of deaths per year, followed by infectious disease (18%) and then cancer (13%). Records suggest that cancer caused 7 million deaths in the year 2000 (WHO annual review). However, a decline in the mortality rate of cancer has been observed in Europe and the USA. This might be due to the improved screening, early diagnosis and improvement in treatment, compared with undeveloped countries, as the number of new cases and mortality rate increases worldwide. The risk for specific cancers

varies with different populations and maps have been constructed that identify populations at varying risk for specific malignancies. The differences in cancer prevalence suggest that perhaps 65% to 70% of cancers in Europe and the USA are due to factors in the environment (Doll *et al.*, 1981), including diet, tobacco, chemicals, radiation and high exposure to sunlight.

Breast cancer is the most common cancer in women accounting for 30% of all cancer cases, followed by lung cancer (13%), colon and rectum cancer (11%), cervical cancer (6%), ovarian cancer plus lymphoma's (4%) and bladder cancer (3%). However, in men prostate cancer is the most common cancer accounting for 43% of all new cancer cases, followed by lung cancer (13%), colon and rectum cancer (8%), and bladder cancer (5%). Lung cancer is the leading cause of cancer death in both males and females accounting for 32% and 25% of all cancer deaths respectively. In women breast cancer is second (17%), followed by colon and rectum cancers (10%), ovarian cancer (5%) and cervical cancer (2%) (Landis *et al.*, 1998). Additional leading sites of cancer mortality in men are prostate cancer (14%), colon and rectum cancer (9%) and bladder cancer (5%) (Parker *et al.*, 1997).

## 1.2.2 CARCINOGENESIS

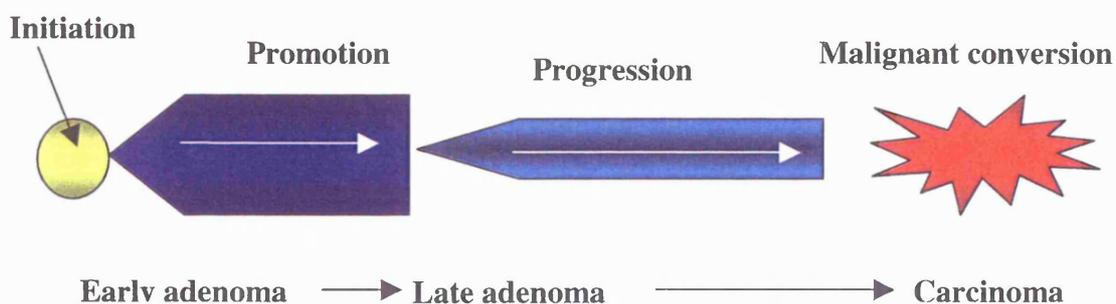
Our environment has been described as a “sea of carcinogens” awash with a variety of chemicals and, to a lesser extent, with oncogenic viruses and high energy radiation, all of which may contribute significantly to cancer incidence in humans. Carcinogenesis is a multistage process initiated and promoted by a variety of exogenous or endogenous processes. The ability of a carcinogen to induce tumour formation is intrinsic to the nature of the carcinogen and its potential ability to interact with a host and modify its genomic deoxyribonucleic acid (DNA).

Carcinogenesis is often characterised by four sequential stages: initiation, promotion, progression and malignant conversion (Figure 1.1). In this process initiation occurs when a carcinogen interacts with DNA, producing strand breaks or, more often, an altered nucleotide called an adduct. Then, if the genome is replicated before repair enzymes can correct the damage, a DNA polymerase may mis-repair the damaged sequence and permanently fix a heritable error in the genome. For adducts, this entails

the insertion of an incorrect nucleotide in the DNA strand opposite the damage. The vast majority of such misincorporations are probably of no hindrance to the cell. However, if the alteration occurs in a sequence that encodes a growth regulatory protein, this may provide the cell with selective growth advantage and allow exposure to a class of compounds known as promoters. Promoters are believed to select or stimulate proliferation of initiated cells to form multiple benign tumours or hyperplastic lesions. This represents the second stage of carcinogenesis.

Finally, a second genetic event (or series of events) is proposed that delivers some permanent advantage to initiated cells and increases the probability that the cell will become neoplastic. This stage, called progression, provides the impetus for conversion from benign adenomas to an infiltrative and finally metastasising neoplasm. These alterations could arise from additional exposure to a carcinogen. Regardless of the mechanism, the outcome is an irreversible change in the cell that allows expression of the neoplastic phenotype. In light of this theory, a complete carcinogen is an agent capable, through single or multiple exposures, of providing an initiating insult, promoting a selective growth advantage for altered cells and generating lesions during progression that result in malignant conversion.

Carcinogenesis is caused by either a processes inherited within the cell known as endogenous carcinogenesis or from exposures to exogenous agents.



**Figure 1.1.** Carcinogenesis, a multistage process, begins with the genetic event of initiation followed by selective expansion of altered cells during promotion to form early adenomas. With a second genetic event, a small number of promoted adenomas progress to form late adenomas, some of which may then undergo malignant conversion (McKinnell *et al.*, 1998).

### **1.2.2.1 Endogenous Carcinogenesis**

The mechanism of carcinogenesis might be completely internal, especially for certain embryonal tumours that arise in the paediatric population, where direct exposure to exogenous chemicals is more limited. Endogenous processes might account for certain events in carcinogenesis, for example, the cellular processes involved in DNA synthesis and repair are inherently mutagenic and therefore can pose a carcinogenic risk.

Additionally, the multistep nature of carcinogenesis and the aneuploidy common in tumour cells during the latter stages of tumourigenesis has led to speculation of there being a mutator phenotype (this is a genetic sequence, which when mutated could increase the rate of spontaneous mutations throughout the genome). Another potential source of endogenous transforming mutations may be from certain biochemical processes. For example, respiration and phagocytosis generate significant quantities of oxygen free radicals and are estimated to yield 10,000 alterations in DNA per human cell per day (Richter *et al.*, 1988), though this may be somewhat over estimated (Helbock *et al.*, 1998).

Conceivably, therefore, carcinogenesis is caused by processes inherent in cells and not necessarily from exposures to exogenous agents. That is not to say that occupational exposures to carcinogens do not accelerate tumourigenesis. There is no question that exogenous chemicals can significantly increase the risk of cancer.

### **1.2.2.2 Exogenous Carcinogenesis**

The exposure to exogenous carcinogens may contribute significantly to increase the incidence of cancer in humans. Identifying and reducing the etiological agents from the environment could significantly reduce tumour incidence. Hundreds of occupational and environmental carcinogens have been identified, including a variety of organic substances, chemicals, viruses and radiations.

Numerous chemical carcinogens have been identified that ultimately act directly on DNA, causing damage to the DNA. These classes of carcinogens are known as

genotoxic carcinogens. Polycyclic aromatic hydrocarbons (PAH) have been studied extensively as metabolism-dependent chemical carcinogens and have proven invaluable in elucidating the mechanisms of carcinogen activation. They can be found in tobacco, whisky, grilled meat and to some extent in petrol. Aromatic amines have been identified as a major class of chemical carcinogens through their use in industries; they can be found in rubber and chemical dye manufacturing. Industrial alkylating agents account for up to 95% of various carcinogenic chemicals. These agents differ in the type of residue transferred to create a DNA adduct. They are either intrinsically reactive with DNA or can be metabolically activated to a DNA reactive form. These so-called electrophiles bond with the electron-sharing atoms of the DNA nucleotides, such as ring nitrogen or exocyclic oxygen atoms, to form stable altered nucleotides or adducts (Hemminki *et al.*, 1994). Because of their ability to react with DNA and cause misincorporations, many carcinogens are also mutagenic (Shelby and Zeiger 1990).

Other carcinogens, however, appear to be nongenotoxic; that is, they do not act directly on the DNA, they exert their carcinogenic effect in other ways. Asbestos, for example, induces large chromosomal alterations but no point mutations. Pure promoters like phenobarbital are non-transforming and non-mutagenic by themselves but can enhance tumorigenicity several fold in combination with weakly transforming doses of carcinogen (Yaurand *et al.*, 1986).

Although it is agreed that, of the exogenous agents, chemicals are responsible for the greater share of cancer, chronic exposure to ultraviolet light or ionising radiation may increase the risk of some cancers especially skin cancer, leukemias and melanomas. Other forms of carcinogenesis are through viral participation. Viral participation in carcinogenesis is a 'rare' occurrence. However, viral studies have provided an important area of research in carcinogenesis, perhaps the best example has been demonstrated for human papilloma viruses (HPV) and cervical cancer (Riou *et al.*, 1990).

Although many carcinogens exhibit mutagenic activity, as just described, not all carcinogens have been shown to be mutagenic, nor all mutagens carcinogenic. After a carcinogen has been taken up, processed, and transported to its target (*eg.* the DNA),

the carcinogenic process may be further modified by a number of dynamic processes inherent to the host. Such properties as DNA repair processes, cellular proliferation and hormonal status enhance the carcinogenicity by effecting the fixation and maintenance of a carcinogenic lesion.

The interaction of a carcinogen with DNA may result in a hydroxyl, alkyl, aralkyl or arylamine-modified nucleotide, which can cause mis-incorporation of the wrong nucleotide in the opposite strand during DNA replication. Alternatively, intercalation of a ring compound into the DNA strand can cause frameshift mutations, and DNA strand breaks that may cause sequence additions or deletions. The fixation of the mutation in the genome depends on a dynamic equilibrium between cell replication and DNA repair processes. Prior to repair, normal DNA replication can produce mismatches where DNA adducts occur, generating point mutations. These mutations can be prevented/ lessened if the amounts of DNA damage do not exceed the ability of the cell to repair. Cells defective in DNA repair tend to accumulate excess DNA damage, leading to apoptosis.

### **1.2.3 CANCER ASSOCIATED GENES**

Over the past 20 years, hundreds of dominant transforming genes have been identified. These genes encode proteins involved in signal transduction or cell cycle regulation. When overexpressed or structurally altered, these oncogenic proteins selectively induce the proliferation of cells that express them. More recently, studies of families predisposed to specific types of cancers have yielded what is at present a small but important group of recessive tumour suppressor genes. Loss of suppressor function by deletion or point mutation of both gene copies allows cells to proliferate unregulated or with reduced restraints. The discovery of both oncogenes and suppressor genes has proved pivotal in our understanding of the mechanisms of carcinogenesis.

#### **1.2.3.1 Oncogenes**

An oncogene is an altered form of a normal cellular gene called a proto-oncogene. It encodes a regulatory protein with dominant transforming properties; that is, a single copy of the altered sequence can transform a cell and the normal sequence of the

second allele cannot block its transforming ability. The most frequent observed mechanism of oncogene activation involves point mutations and chromosomal rearrangements. A point mutation is a mutation at a specific nucleotide in a specific sequence in the DNA. Point mutations are associated with some adenocarcinomas of the colon (Forrester *et al.*, 1987), pancreatic adenocarcinomas (Almoguera *et al.*, 1988) and ovarian tumours (Berchuk *et al.*, 1990). Chromosomal rearrangements provide another mechanism for oncogene activation and may be a common pathway for induction of leukaemias in humans (Nowell and Hungerford 1960).

### **1.2.3.2 Tumour Suppressor Genes**

More than a dozen tumour suppressor genes have been identified. These genes encode inhibitory proteins whose function is lost in cancer. These proteins are responsible for regulating the growth of the cell in a negative and recessive manner (both gene copies of a diploid cell must be lost before a biological effect is seen). The most common mechanism of tumour suppressor gene inactivation is by point mutation or deletion of a specific chromosomal region that contains a suppressor gene. The loss of the suppressor gene function occurs in the germline, allowing it to be passed to subsequent generations. One of the most frequently altered genes found in human tumours is the p53 suppressor gene. Its loss is associated with breast cancer, brain cancer, bladder cancer, osteosarcomas and leukaemia (Malkin *et al.*, 1990 & 1993).

### **1.2.4 CANCER TREATMENT**

Curing cancer remains an often-elusive goal. The choice of specific therapy depends on many factors. Histology, stage, location and size of the tumour may be the key factors in determining the best treatment. Three established strategies are commonly used to eradicate cancer cells in the patient; surgery, radiotherapy and chemotherapy. Each of these strategies is called a “modality” and the term “conventional” refers to the therapy accepted as the best available standard treatment. Because they’re different strategies, each modality is associated with specific risks and side effects.

#### **1.2.4.1 Surgery**

Surgery is the oldest modality of cancer treatment; physical removal of the malignant tissue from the affected site is the foundation of surgery. Surgery is most effective in the treatment of localised primary tumours (McKinnell *et al.*, 1996), but has a limited role in disease management once the cancer has spread beyond the primary location.

#### **1.2.4.2 Radiation Therapy**

Radiation therapy, “radiotherapy”, is a second modality in cancer treatment. During radiotherapy, malignant cells are exposed to ionising radiation from either an external or implanted radiation source. The resulting radiation-induced damage causes the death of the cell when it tries to divide, leading to a gradual reduction in tumour mass. Radiotherapy is most effective in treating localised disease, such as localised lymphomas, cancer of the tongue and oral cavity, cancer of the cervix and cancer of the prostate (Bast *et al.*, 1996). Radiotherapy can cure selected early stage cancers and can thus sometimes be used as a single modality. However, for invasive and metastatic tumours, radiotherapy alone does not play a major curative role in disease management.

#### **1.2.4.3 Chemotherapy**

Chemotherapy is the third modality in cancer treatment. Cytotoxic chemotherapy is designed to kill proliferating cells, so it is another type of cytoreductive therapy. Cytotoxic chemotherapy works best against cells actively progressing through the cell cycle and these drugs are generally less effective against the same cells in a quiescent state. Like radiotherapy, chemotherapy eradicates dividing cells in renewing tissues, and the anticancer drug acts as a cytoreductant in both tumour tissue and normal tissue. Chemotherapy is a systemic therapy and although this means toxicity will be more extensive, systemic therapy is the only conventional modality that potentially treats every malignant cell of a metastatic cancer (McKinnell *et al.*, 1998).

#### **1.2.4.4 Alternative Therapies and Combined Therapies**

Several other strategies are currently applied in cancer treatment. Biotherapy, immunotherapy and hormonal therapy are three strategies used to modify the biology of the malignancy so its growth can be controlled. A high quality of life can be maintained and the patient can live a normal life span without risk of death from cancer or its complications. These therapies may be effective against tumours that do not respond to conventional cytotoxic modalities.

In the treatment of cancer, single modalities are often used successfully for early detected cancers and localised tumours. However, patients with intermediate stage or high grades of disease are disadvantaged by tumour recurrence and so suffer a significantly higher mortality rate. During the past two decades, major improvement in cancer treatment has been achieved through the use of combined modalities, which has significantly reduced the morbidity and mortality associated with each treatment. Curing cancer patients requires the combined use of effective local, regional and systemic therapies, which is the rationale behind multimodality therapy. Combined modalities are being used to tackle increasing numbers of malignancies with the hope of improving the quality of life and reducing mortality rate.

### **1.3 BLADDER CANCER**

Bladder cancer is the fourth most common cancer among men and the eighth most common cancer among women. An estimated 53,200 new cases and 12,500 bladder cancer deaths are expected to occur in the USA, and 10,000 cases are diagnosed in the UK annually leading to 5,000 deaths per year (Greenlee *et al.*, 2000). Internationally, incidence rates of bladder cancer vary almost 10-fold. Highest rates occur in Western Europe and North America, whilst relatively low rates are found in Eastern Europe and Asia (Parkin *et al.*, 1999). Cancer of the bladder occurs primarily in older white men; the incidence rate among black men is about 50% of that among whites. The male: female ratio is at least 3:1, approaching 4:1 in whites. Two thirds of cases occur among persons aged 65 and older. Bladder cancer incidence continues to increase worldwide by 5-10% every 5 years. Despite the fact that bladder cancer is one of the

most investigated tumours, only a small percentage of the disease can be explained by recognised risk factors (Bailey *et al.*, 1999).

### **1.3.1 AETIOLOGY AND RISK FACTORS**

A number of factors have been implicated in the development of bladder cancer and most relate to environmental carcinogens found in industrialised society.

#### **1.3.1.1 Cigarette Smoking**

Smoking is recognised as the number one cause of cancer in industrialised countries, with a two- to five-fold increase in the risk of bladder cancer being due to smoking (Morrison *et al.*, 1984). Smokers have a higher incidence of bladder tumour recurrences, as well as a higher proportion of tumours of higher stage and grade than do non-smokers. The correlation between smoking and cancer is reportedly higher with bladder cancer than with lung cancer (Brian *et al.*, 2001).

#### **1.3.1.2 Occupational Exposure**

In the 1950s, occupational exposure to two aromatic amines, 2-naphthylamine and benzidine in the British dyestuff and rubber industries, were found to be associated with bladder cancer (Case *et al.*, 1954). The mean time from start of exposure to death was 25 years, with the greatest risk being to those workers who started work before age 25. Subsequent studies suggested that exposure to 4-aminobiphenyl and two curing agents that are structural analogues of benzidine, 4,4'-methylene-dianiline and 4,4'-methylene-bis {2-chloroaniline}, may also increase the risk of bladder cancer (Schulte *et al.*, 1986). Aromatic amines are detoxified by N-acetylation. The N-acetyl transferase enzyme in the liver is polymorphic, displaying a slow and a rapid acetylator phenotype. The slow acetylator phenotype is associated with an increased risk of bladder cancer (Evans *et al.*, 1984).

A number of other occupational exposures have a potential association with bladder cancer. Workers in the leather industry are at increased risk of bladder cancer, but the carcinogen has not yet been identified (Cole *et al.*, 1972). Many other carcinogens have been identified such as benzene, dioxane and methylene chloride.

### **1.3.1.3 Chromosomal Changes**

Numerous genetic alterations have been associated with the development and evolution of bladder cancer. The most commonly seen abnormalities are found on chromosomes 1, 5, 7, 9, 11 and 17. Abnormalities in chromosome 9 are common in all stages and grades of bladder cancer. Several studies suggest that p53 mutation is common in bladder cancer and might play a critical role in tumour progression. Mutated p53 species in the nucleus of tumour cells might predict for relapse and impaired survival in patients who had cystectomy as a treatment for bladder cancer (Uchida *et al.*, 1995).

The retinoblastoma (Rb) gene located on chromosome segment 14q 13 is another tumour suppressor gene that plays a potential role in bladder cancer. Alterations in the Rb gene are seen in less than 50% of cases analysed, but studies indicate that inactivation is associated with disease progression. Expression of epidermal growth factor (EGF) and EGF receptor (EGFR) correlates with increasing grade and stage of the tumour (Smith *et al.*, 1989). This suggests a role in bladder cancer that should be evaluated further.

### **1.3.1.4 Dietary Factors**

Caffeine has been implicated in bladder cancer but the relationship has been hard to define due to the widespread use of caffeine, as well as its association with a variety of other known carcinogens, such as smoking. The artificial sweetener saccharin was banned based on animal studies suggesting that it is a bladder carcinogen, but the studies have not clearly demonstrated the relationship between artificial sweeteners and bladder cancer (Morrison *et al.*, 1982).

### **1.3.1.5 Other Factors**

Other documented risk factors include a history of schistosomiasis, heavy consumption of phenacetin-containing analgesics and cyclophosphamide chemotherapy. Exposure to *Schistosoma haematobium* infection is associated with a predominantly increased risk of squamous cell carcinoma of the bladder (Newling *et al.*, 1990). Heavy consumption of phenacetin-containing analgesics were linked to

cancer of the renal pelvis, ureter, and the bladder (McCredie *et al.*, 1983). Cyclophosphamide, an alkylating agent, has been linked to an increased risk of bladder cancer (Travis *et al.*, 1995).

### **1.3.2 BENIGN VERSUS MALIGNANT BLADDER TUMOURS**

Papillomas are considered to be benign tumours in the bladder; they closely resemble the normal urothelium. Although they have more than the normal seven layers of urothelium, they show normal nuclear polarity in more than 95% of the tumour and no or only slight pleomorphism. Benign tumours generally lack the intrinsic features that produce the lethal potential to malignant tumours. The vast majority of benign tumours cause little or no damage to the host. However, if the benign tumours continue to grow within the bladder, they may cause a problem by pressing on the surrounding organs (Stevin *et al.*, 1996).

A malignant bladder tumour consists of cancer cells which have the ability to proliferate and spread beyond the bladder if not treated. The cells of malignant tumours have the intrinsic ability to kill the host. The vast majority of malignant cells are less well differentiated than their benign counterparts; some are so poorly differentiated that they defy histopathological identification. Malignant tumours of the bladder persist as superficial or muscle invasive bladder cancers, with the latter having a high chance of metastatic spread.

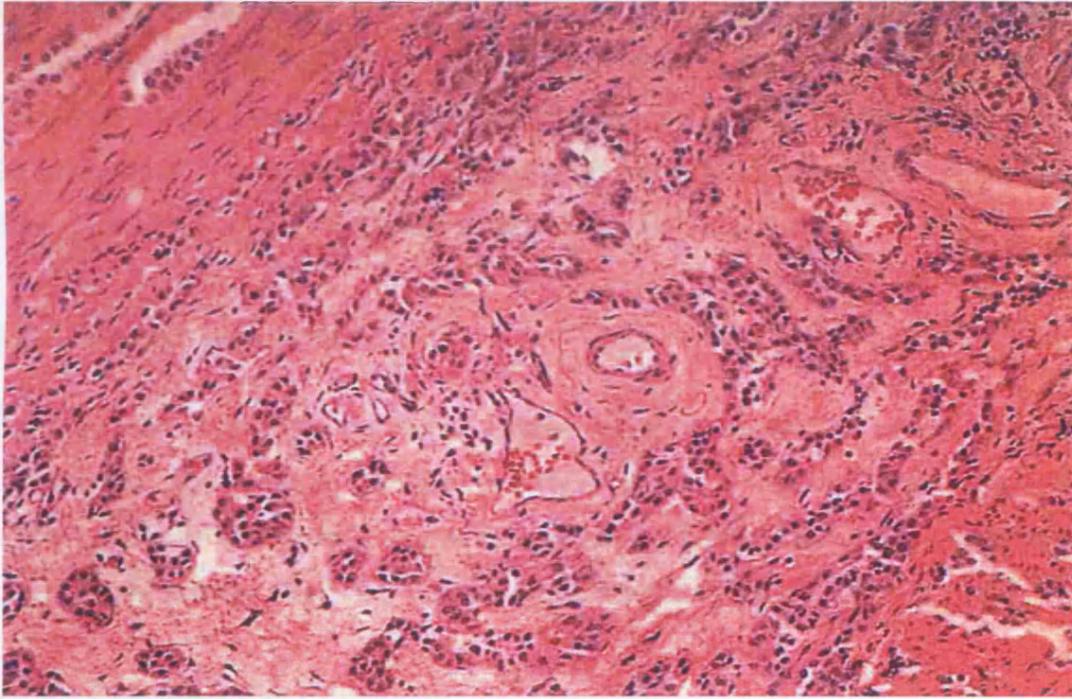
### **1.3.3 HISTOLOGY**

The importance of histological examination in the diagnosis of bladder cancer is vital to the management of all patients with the disease. Histological examination reveals there are different types of carcinoma in the bladder.

#### **1.3.3.1 Transitional Cell Carcinoma**

Transitional cell carcinoma (TCC), derived from the transitional epithelium, accounts for almost 90% of bladder cancer seen in industrialised countries such as USA and the UK (Figure 1.2). There are three major types of transitional cell carcinoma: superficial, which account for 70% of cases, invasive, which account for 25%-30% of

cases and an additional and important type seen in about 10% of cases is carcinoma *in situ* (CIS).



**Figure 1.2.** A bladder section showing transitional cells carcinoma, nets and cords of infiltrating lesions are present within smooth muscle bundles (Skarin *et al.*, 1996).

### 1.3.3.1.1 Superficial Bladder Cancer

Superficial bladder cancer is a malignant bladder cancer, consisting of proliferating tissue that does not invade the bladder wall. Most superficial tumours lie between the inner epithelium wall of the bladder and the lamina propria. For superficial bladder cancer, grade is an important predictor of recurrence and progression. Pathologic grade I, II and III (low, intermediate, high) is based on the number of mitoses, presence of nuclear abnormalities, and cellular atypia. High-grade tumours show loss of polarisation of the nuclei and moderate to prominent pleomorphism. These tumours can be resected successfully without cystectomy (see Section 1.3.6.1) (Presti *et al.*, 1991).

#### **1.3.3.1.2 Muscle Invasive Bladder Cancer**

Invasive bladder cancer accounts for up to 30% of malignant bladder cancer cases. The tumour invades the bladder wall and metastasises to other parts of the body. Bladder cancer can spread by invasion through the bladder wall, or via involvement of the lymph nodes. Usually lymphatic spread of malignant cells occurs much earlier than metastatic spread. Muscle invasive disease, however, is usually high grade and depth of invasion is an important prognostic factor for outcome.

#### **1.3.3.1.3 Carcinoma *In situ***

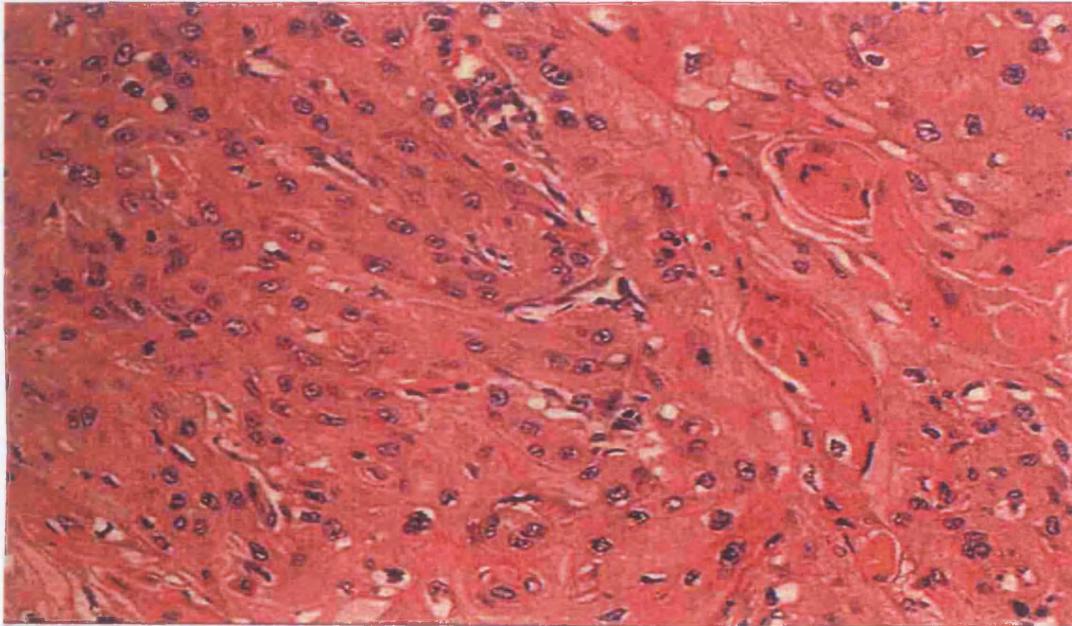
Carcinoma *in situ* (CIS) is defined as a non-invasive, high grade flat cancer confined to the epithelium. It can be localised or diffuse and it may occur in association with either superficial or muscle-invasive transitional cell carcinoma. On the other hand, carcinoma *in situ* can also occur without a concurrent exophytic tumour.

#### **1.3.3.2 Squamous Cell Carcinoma**

Squamous cell carcinoma affects the squamous epithelium and it accounts for up to 5 - 10% of bladder cancer in the UK (Figure 1.3). Squamous cell cancer is associated with chronic infection (e.g. schistosomiasis) and they tend to grow in large masses with a high degree of necrosis. This disease is seen in countries where *Schistosoma* infection is endemic such as Egypt, where 70% of bladder cancers are of squamous cell carcinoma type.

#### **1.3.3.3 Adenocarcinoma**

Adenocarcinoma is a rare type of bladder carcinoma, accounting for about 2% of cases. Approximately 30-35% of these are urachal in origin and location, while the remainder are associated with bladder exstrophy or are non-urachal in origin. Adenocarcinoma is usually solitary, high grade and ulcerative. They are indistinguishable histologically from adenocarcinoma of the colon or rectum and clinical determination of the source of origin is difficult. Many of the patients with adenocarcinoma have poor prognosis, due to the advanced stage at the time of diagnosis (Newling *et al.*, 1990).



**Figure 1.3.** A bladder section showing well-differentiated squamous cell carcinoma. Sheets of polygonal keratinising cells with intercellular bridges produce extracellular keratin and form pearls (Skarin *et al.*, 1996).

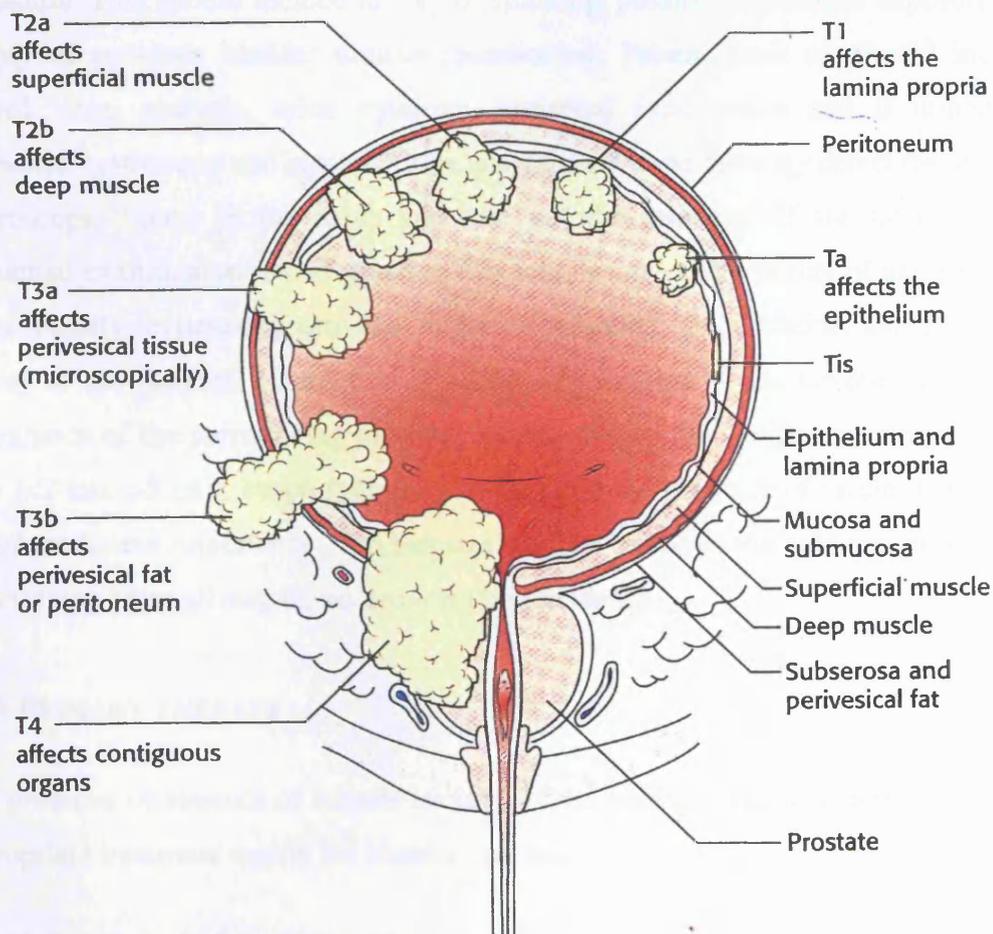
#### **1.3.3.4 Undifferentiated Carcinomas**

Undifferentiated carcinomas are small cell carcinomas that have a very high nuclear to cytoplasmic ratio, and usually form sheets or nests of cells. Field changes of a premalignant nature are often found in association with undifferentiated carcinoma of the bladder, and range from atypia (which indicates that an increased number of cell layers is present, with loss of polarity of a still intact umbrella layer), to mild or severe dysplasia (which indicate an increase in the size of the nuclei that are basally located and exhibit loss of the usual polarity). The recognition of such changes is important in determining the prognosis of patients, relative to the future risk of recurrence or progression.

#### **1.3.4 STAGING AND GRADING**

Staging and grading of bladder tumours are key factors in determining the appropriate treatment option for each patient. Tumour staging describes the size of the primary lesion, degree of invasion, lymph node involvement and metastases. Tumour nodes metastases (TNM) staging is the system most commonly used. Prognosis for all stages is highly dependent on the accurate clinical staging. The staging system has four

major sites in the bladder (Figure 1.4). T<sub>1</sub> affects the inner epithelium, T<sub>2</sub> affect the muscle, T<sub>3</sub> affect the perivesical fat and finally T<sub>4</sub> affects contiguous organs (Rubin *et al.*, 1992). Tumour grading indicates the degree of malignancy. Grading of transitional cell carcinoma is assessed from I to III. Grade I applies to tumours with the least degree of cellular anaplasia compatible with a diagnosis of malignancy. Grade III applies to tumours with the most severe degree of cellular anaplasia. The criteria given for anaplasia are increased cellularity, nuclear crowding, altered cell polarity, irregular cell size, nuclear pleomorphism, altered chromatin pattern, displaced or abnormal mitotic figures and giant cells. Grade is inseparably linked with stage. However, whilst neither grade only nor stage can predict the rate of recurrence or degree of invasion, both are important in determining the prognosis and survival of bladder tumours.



**Figure 1.4.** Staging of bladder cancer (Bailey 1999).

### **1.3.5 CLINICAL PRESENTATION AND INVESTIGATION**

Hematuria is the most frequent presenting clinical symptom of bladder cancer (80% of all cases). It may be associated with irritative symptoms but is often painless and episodic. Hematuria is typically present through urination, 17% of patients present with severe bleeding and clot formation, and 20% present with dysuria (increased frequency and urgency to pass urine). Some asymptomatic patients proven to have bladder cancer are found to have microscopic hematuria or less frequently pyuria. Bladder tumours can cause strangury (pain on straining to urinate), urinary retention, urethral obstruction, sepsis and life threatening hematuria, though the latter is very rare (Mommmsen *et al.*, 1983).

A thorough history should be obtained from all patients presenting with painless hematuria. This should include history of smoking, possible carcinogen exposure and history of previous bladder tumour resectioning. Patient work up should include voided urine analysis, urine cytology, bimanual examination and if tumour is suspected cystoscopy and biopsy. Urine analysis and urine cytology detect the level of microscopic blood in the urine and rule out the infection of the urinary tract. Bimanual examination is performed to evaluate the size and mobility of any palpable mass. Finally, cystoscopy provides direct visualisation of the bladder and facilitates biopsy of the bladder. Number, size, shape, and location of the tumours as well as appearance of the surrounding mucosa, urethra, and urethral orifice are documented. Size (<2 cm, >5 cm), shape (papillary or flat) and the presence of carcinoma *in situ* are of predictive importance. The biopsies are then collected via cold cup or hot wire resectioning from all suspicious areas within the bladder.

### **1.3.6 PRIMARY THERAPY**

The presence or absence of muscle invasion is the principle factor in determining the appropriate treatment option for bladder cancer.

#### **1.3.6.1 Treatment of Superficial Bladder Cancer**

Patients with superficial transitional cell carcinoma of bladder, who account for 70% of all newly diagnosed bladder cancer cases, are treated by transurethral resection of

the bladder tumour (TURBT). The appropriate treatment regimen may also include intravesical therapy for selected patients. Decision concerning the use of adjuvant therapy is based primarily on the probability of disease progression and not on the probability of recurrence. Essentially 60% of patients with superficial transitional cell carcinoma will eventually experience recurrent disease after TURBT (Heney *et al.*, 1983). Progression to invasive disease after TURBT alone occurs in 20%-30% of patients who suffer recurrence and is closely associated with tumour type and tumour grade (Fitzpatrick *et al.*, 1986).

Intravesicular therapy involves the instillation of a therapeutic agent into the bladder. Chemotherapy is currently the most effective intravesicular agent for the treatment of superficial TCC of the bladder. Intravesicular chemotherapy has shown a decrease in the short-term tumour recurrence rate by 50%, but this has not lead to a decrease in long-term progression of disease or an increase in survival rates (Fitzpatrick *et al.*, 1986). Chemotherapy has been used in TURBT in an attempt to spare the bladder. Complete response rates after TURBT and chemotherapy range from 55%-60%. These findings suggest that the addition of chemotherapeutic agents (such as doxorubicin, *cis*-platin and mitomycin-C) to TURBT confers an advantage in terms of complete response rate.

Although chemotherapeutic agents are the treatment of choice for intravesicular therapy, other agents have been used, such as Bacille Calmette-Gue'rin (BCG). BCG is currently considered the intravesicular agent of choice for the treatment of superficial TCC of the bladder in the USA. Intravesicular therapy with BCG has been shown to be effective in reducing rates of disease progression, improving survival, and decreasing recurrence. The choice of intravesicular therapy with TURBT depends on the urologist's assessment of risk, toxicity and availability (*i.e.* national preference).

### **1.3.6.2 Treatment of Muscle Invasive Bladder Cancer**

Muscle invading tumours comprise 30% of all newly diagnosed bladder cancers. The most appropriate treatment for muscle-invading disease remains controversial.

### **1.3.6.2.1 Surgery**

Cystectomy is generally considered to be the 'gold' standard treatment for patients with muscle invasive tumours. Other possible primary surgical approaches are radical transurethral resection or partial cystectomy (Herr *et al.*, 1987). Cystectomy is the most effective local treatment for muscle invasive bladder cancer. It requires the removal of the whole bladder with bladder substitution and candidates for cystectomy must be operable and without evidence of lymphatic spread. Radical transurethral resection is appropriate for patients with small tumours, which can be completely resected. Partial cystectomy is indicated for solitary tumours situated in the mobile posterior part of the bladder, with normal surrounding urothelium allowing resectioning of the bladder.

Cystectomy remains the treatment associated with the highest local cure rate. However, debates and controversy relate to the indication of cystectomy. This is due to the perioperative mortality, loss of bladder function and possibly also incontinence and impotency. There is also the possibility that the operation was overtreatment, in that some patients may also have been cured by a more conservative approach such as partial cystectomy and radical transurethral resectioning, or indeed bladder preserving radiotherapy. In contrast, the cystectomy patients benefit from improved local control and removal of the possibility of disabling hematuria.

### **1.3.6.2.2 Radiation Therapy**

Radiotherapy is the second approach for treating muscle invasive bladder cancer. Radiotherapy ideally involves preservation of the functional organ without compromising survival. Definitive RT has been used for muscle invasive bladder cancer since the early 1900s and there is evidence that patients can achieve durable local control and maintain a functional bladder without compromising survival (Gospodarowicz *et al.*, 1989). Radiation therapy for bladder cancer is usually administered by the use of external beam radiotherapy using a linear accelerator. Less frequently, brachytherapy whereby a radiation source is implanted in the bladder wall, is used for small tumours,. With both techniques, the aim is to obtain a homogeneous dose of radiation within the target volume (tumour tissue), while minimising the radiation dose received by surrounding normal tissue. The total radiation dose used

for bladder cancer patients varies from 50 to 55 Gy, with 2.5 Gy to 2.75 Gy per fraction (Davidson *et al.*, 1990). Most patients are treated with one fraction per day five times a week. Patient's responses are evaluated by cystoscopic examination and biopsy 3 to 6 months after completion of RT. Patients with residual tumour and no known metastatic disease are offered salvage surgery. The overall complete response rate to radiotherapy approximates 50%, with complete response being higher in T<sub>2</sub> and T<sub>3a</sub> disease than in T<sub>3b</sub> and T<sub>4</sub> disease. Factors leading to a favourable effect on local control with radiotherapy include, early stage, absence of carcinoma *in situ*, small tumour size, solitary tumours and tumour configuration. However, in bladder cancer treatment, there has been considerable resistance to organ preservation via primary radiotherapy. The primary reason for the lack of acceptance of bladder preservation is the possibility of disease progression, poorer survival rates, impaired bladder function and reduction in the quality of life.

#### **1.3.6.2.3 Chemotherapy**

Chemotherapeutic agents were first used against metastatic bladder cancer in the 1950s. Chemotherapy as a monotherapy achieves a clinical complete response in only 25% of patients (Angulo *et al.*, 1996). This is more frequently reported in low stages and small papillary tumours. A wide range of two, three and four drug combination regimens were developed in an attempt to increase response rates and duration of survival (Raghavan *et al.*, 1990). For more than a decade the use of combination regimens appeared to improve response rates. The most effective combination regimens are MVAC and CMV, which combine methotrexate, vinblastin and *cis*-platin (with or without doxorubicin). A major benefit of using chemotherapeutic agents is that they can combine with other modalities. Chemotherapy in combination with cystectomy achieves better local control and clinical complete response as assessed on rebiopsy. While in conjunction with RT for bladder cancer preservation, many cytotoxic agents, in particular *cis*-platin, 5-fluorouracil and mitomycin-C, are capable of sensitising tumour tissue to radiation and increase cell killing in a synergistic fashion.

Multimodality therapy is emerging as the viable alternative treatment option for transitional cell carcinoma of the bladder. Refining the treatment choice by maximising quality of life without compromising survival is the ultimate goal.

### **1.3.6.3 Radiotherapy Versus Surgery**

Cystectomy and radiotherapy are the two main treatment options for transitional cell carcinoma of the bladder (TCC). Having established the extent of disease and the degree of invasion, the next decision is the choice between cystectomy or radiotherapy alone or in combination with chemotherapy.

The primary reason for the lack of acceptance of bladder preservation is the paucity of well executed trials comparing radical radiotherapy with radical cystectomy. Bladder preservation is not accepted as the standard treatment for TCC for a number of factors, including the possibility of disease progression, poor survival rate, impaired of bladder function and reduction in the quality of life. On the other hand, cystectomy is not appropriate for every patient and has the disadvantage of associated morbidity and mortality (Skinner *et al.*, 1988). The possibility that radiotherapy may be less effective than surgery has arisen in some trials. Radiotherapy appears to result in a poorer overall survival rate than surgery. However, the difference in overall survival may be attributed to a number of factors, including selection bias (younger fitter patients are considered more suitable candidates for surgery whilst older patients are considered for radiotherapy) and staging of the tumour. There is no evidence of trials comparing definitive radiation therapy with cystectomy alone. However, several studies have compared favourably the survival results following radical cystectomy (after preoperative radiation therapy) with separate cohorts of patients who had undergone radical radiation therapy (Salminen 1990). However, such studies exhibit inherent selection bias due to lack of randomisation, with both cohorts of patients rarely being comparable. Furthermore, in many reviews of radiotherapy, the use of early and aggressive salvage cystectomy has not been consistent (Turner *et al.*, 1999). Indirect evidence suggests that survival results are similar for both radical cystectomy and radical radiation therapy (despite the negative selection of radiation therapy patients).

Attempts to optimise radiation therapy are focusing on several areas. Modification to the mode and timescale of delivery of radiation therapy is being investigated, as is the use of radiosensitizing agents. Attempts have been made to determine prognostic factors capable of identifying patients likely to benefit from radiation therapy and work at the molecular level is underway to develop methods capable of predicting intrinsic radiosensitivity. It is this latter issue, together with the development of a method capable of predicting intrinsic chemosensitivity, that are the two aims of this project.

## **1.4 AIMS AND OBJECTIVES**

### **(A) Evaluation of the alkaline comet assay as a predictive test of bladder cancer cell radiosensitivity**

As described above, the standard treatment for muscle invasive transitional cell carcinoma of the bladder is cystectomy or radiotherapy. However, each of these modalities has only a 50% chance of success. This is most probably due to the uncertainty in selecting patients that should go for radical cystectomy or radical radiotherapy. The inadequacy of the precystectomy prognostic factors and their inability to distinguish reliably between patients who really benefit from cystectomy and those who will not is a probable reason for low survival in patients that underwent total cystectomy. Similarly, for radiotherapy, the lack of information regarding patients' response to radiotherapy and the inability to distinguish between radiosensitive and radioresistant patients is a possible reason for poor cure rates by this modality.

Consequently, if tumour radioresponse could be predicted in advance, it should be possible to significantly improve tumour control rates by selecting for immediate radiation therapy those patients whose tumours respond to radiation; additionally, those patients who would benefit from initial surgery whose tumours are unresponsive would be identified earlier, reducing the risk of metastatic spread and improving rates of survival.

The first aim of this study (Chapter 2) is to investigate the alkaline comet assay as a predictive assay of radiosensitivity in bladder cancer. Specific studies were conducted and they are as follows:

- Studies were conducted to evaluate use of the alkaline comet assay as a rapid predictive assay of bladder cancer cell radiosensitivity *in vitro*. These studies were conducted using six-bladder cancer cell lines of differing radiosensitivities. Radiogenic cell survival and viability of these cell lines were determined and their relationship to the induction and repair of DNA damage examined.
- Studies were conducted to determine the cellular factors responsible for the differing radiosensitivities. These entailed the alkaline comet assay assessment of various cell substrates.
- Finally, studies were undertaken to determine the alkaline comet assay response of epithelial cells isolated from human muscle invasive bladder tumour biopsies. The possibility of obtaining a range of radioresponses as determined by the alkaline comet assay would further emphasise the clinical potential of this assay.

### **(B) Evaluation of the alkaline comet assay as a predictive test of bladder cancer cell chemosensitivity**

In the continuing search for effective local control with improved survival in bladder cancer, the use of chemotherapeutic agents with local modalities could result in improved impact. However, in the treatment of some patients, local control is achieved without the use of cytotoxic chemotherapy and consequently, for this group of patients the inclusion of the latter would then be inappropriate. Consequently, in order to provide the most appropriate treatment, it is desirable to have a predictive test able to distinguish between chemosensitive and chemoresistant tumours. The two chemotherapeutic agents investigated, were *cis*-platin and mitomycin-C. These are

widely used in the treatment of bladder cancer with an overall response rate of 21% to 31% for *Cis-platin* (Fagg *et al.*, 1984), and 13% for mitomycin C (Carter *et al.*, 1979).

The second aim of this study (Chapter 3) is to investigate the alkaline comet assay as a predictive assay of chemosensitivity in bladder cancer. The specific study conducted was:

- Evaluation of the alkaline comet assay as a rapid predictive test of bladder cancer cell chemosensitivity *in vitro*. These studies were conducted using six-bladder cancer cell lines of differing chemosensitivities. Both cell survival and viability were determined and their relationship to the induction and repair of drug-induced DNA damage was examined.

## **CHAPTER TWO**

***MEASUREMENT OF RADIATION INDUCED  
DNA DAMAGE IN BLADDER CANCER CELLS:  
EVALUATION OF THE ALKALINE COMET  
ASSAY AS A PREDICTIVE TEST OF BLADDER  
CANCER CELL RADIOSENSITIVITY***

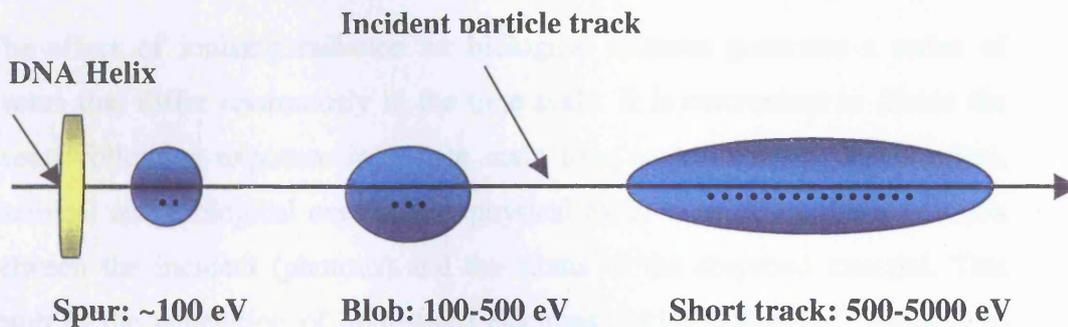
## 2.1 INTRODUCTION

Several attempts have been made to improve the outcome of radiation therapy for muscle invasive bladder cancer (reviewed by Colquhoun *et al.*, 2003). These improvements include the development and evaluation of assays that can predict tumour sensitivity on an individual basis (West *et al.*, 1990; Price *et al.*, 2000; Banasiak *et al.*, 1999; Whitaker *et al.*, 1995; Lynch *et al.*, 1991; Olive *et al.*, 1990 & 1995; McKelvey-Martin *et al.*, 1997). The ability to predict tumour radiosensitivity could improve the results of treatment centered on radiation therapy, allowing bladder preservation to remain a treatment option for muscle invasive bladder cancer. An evaluation of the alkaline comet assay as a highly sensitive method for determining radiosensitivity in bladder cancer is considered to be the ultimate objective of this first study. To evaluate the alkaline comet assay it is important to understand the basis of radiation biology. In the most general sense, radiation biology is the study of the effect of electromagnetic radiation on biological systems. Radiotherapy exploits a portion of the electromagnetic spectrum that is energetic enough to cause ionisation of atoms. This ultimately results in the breaking of chemical bonds that can lead to damage to important biomolecules. The most significant effect of ionising radiation in this context is cell killing, which is at the root of nearly all normal tissue and tumour responses noted in patients.

## 2.2 RADIATION AND BIOLOGICAL MATTER

One of the most important features of the interaction of ionising radiation with biological material is the random and discrete nature of energy deposition. Energy is deposited in discrete energetic packets referred to as “spurs” (a product of low energy deposition {100 eV}), “blobs” (a product of high energy deposition {100 to 500 eV}) and “short tracks” {500 to 5000 eV} (Figure 2.3) (Coleman *et al.*, 1996), each of which can generate approximately three to several dozen ionised atoms. The frequency of distribution and the density of the different types of energy deposition events along the track of the incident photon are measures of the radiation’s linear energy transfer or LET. Because the energy deposition events are discrete, it follows that although the average

energy deposited in a macroscopic volume of biologic material may be low, the distribution of this energy on a microscopic scale may be very large. Those biomolecules receiving a “direct hit” from a spur or blob receive a huge radiation dose that is a large deposition of energy in a small volume.



**Figure 2.1.** Energy deposition events along the track, each event can be classified according to the amount of energy deposited locally, which in turn determines how many ionised atoms will be produced.

Interaction of ionising radiation occurs at the atomic and nuclear level. Electromagnetic radiations, or photons, are particles that have wave-like qualities with zero mass, which transfer energy from one location to another by propagation of an electromagnetic wave at the speed of light. The process by which X-ray photons are absorbed depends on the energy of the photon and the chemical composition of the absorbing material. If the tissue absorbs an X-ray beam, a vast number of photons interact with a vast number of atoms, the net result is the production of a large number of fast electrons, which can ionise other atoms, break vital chemical bonds, and initiate changes that ultimately lead to biological damage. Although the differences between the various absorption processes (*e.g.* Compton versus photoelectric) are of practical importance, the biological consequences are minimal. Whether the absorption process is the photoelectric or the Compton process, much of the energy of the absorbed photon is converted to fast electrons.

For photon and particles, this energy deposition results in the ejection of the orbital electrons causing the target molecule to be converted first into an ion pair and then into a free radical. Further, the ejected electrons themselves can go on to produce additional ionisation. The cycle of ionisation, free radical production, and release of secondary electrons continues until all the energy of the incident photon or particle is expended.

The effect of ionising radiation on biological systems generates a series of events that differ enormously in the time scale. It is convenient to divide the events following exposure into three main time scales, namely the physical, chemical and biological events. The physical event consists of the interaction between the incident (photons) and the atoms of the absorbed material. This result in the generation of high-speed electrons, taking about  $10^{-18}$  seconds to traverse the DNA molecule and  $10^{-14}$  seconds to pass across mammalian cells, which in turn result in further ionisations. The chemical event ( $10^{-12}$  – 100 seconds) describes the period in which these damaged atoms and molecules react with other cellular components in a chemical reaction, resulting in broken molecular bonds. The biological event (hours - years) includes changes in the chemistry of the biomolecules and changes in the biological functions, leading to cell death or possible abnormal cell function.

### **2.3 EFFECTS OF RADIATION ON CRITICAL CELL TARGETS**

Radiation therapy is a physical medical modality whereby radiative energy is delivered to a target volume to kill cells. Identification of the target whose damage is primarily responsible for radiation induced cell killing has been the ultimate goal of several investigations. Current evidence indicates that the molecular target for radiation induced cell killing is DNA, the genetic material (Kaplan *et al.*, 1998). A surprising continuity exists between the physical events that occur in the first picoseconds (or less) after ionising radiation interacts with DNA and the ultimate consequences of that interaction on the tissue. Radiation kills cells by producing free radicals in the nucleus, which produce a variety of damages in the DNA, (as an example, each 1 Gy dose of radiation produces about 1000 initial single strand breaks and about 40 initial double strand

breaks). Some lesions are more important than others and radiation lethality correlates most significantly with the number of residual, unrepaired double-strand breaks after radiation (Radford *et al.*, 1985 & 1986).

To understand the effect of ionising radiation on DNA it is important to appreciate the structure of DNA. DNA is a large molecule that has a characteristic double-helix structure consisting of two strands, each made up of a sequence of nucleotides. A nucleotide is a subunit in which a purine or pyrimidine base is linked through a deoxyribose sugar moiety to a phosphate group. The “backbone” of the molecule consists of alternating sugar-phosphate groups (Figure 2.2). The connection of the phosphate groups to the sugar ring is through the 5' and 3' carbons of the deoxyribose moieties, which at the end of the DNA sequence define the 5' and 3' termini. There are four different bases, two are single-ring structures (pyrimidines) thymine and cytosine, and two are double-ring structures (purines) adenine and guanine (Figure 2.3). The order of these bases along the molecule is what specifies the genetic code. The two strands of the double helix are held together by hydrogen bonds, these bonds are between thymine and adenine (2-bonds), and between cytosine and guanine (3-bonds). The bases are paired in this way along the length of the DNA molecule.

All cellular molecules are potential targets for the localised energy deposition events that occur as spurs, blobs, or short tracks. Whether the ionisation of a particular biomolecule results in a measurable biological effect depends on a number of factors, including the probability/likelihood that a molecule is a target for the ionising particle/photon and how vital the molecule is. The effect of radiation on the molecular level can be classified into direct and indirect effects.

### **2.3.1 INDIRECT EFFECT OF RADIATION**

Water is the most abundant molecule in the cell, comprising 80% to 90% of a cell's mass. The highly reactive free radicals formed by radiolysis of water are capable of contributing to the DNA damage, by migrating to the DNA and damaging it. This mechanism is referred to as *indirect radiation action* (Figure 2.4).

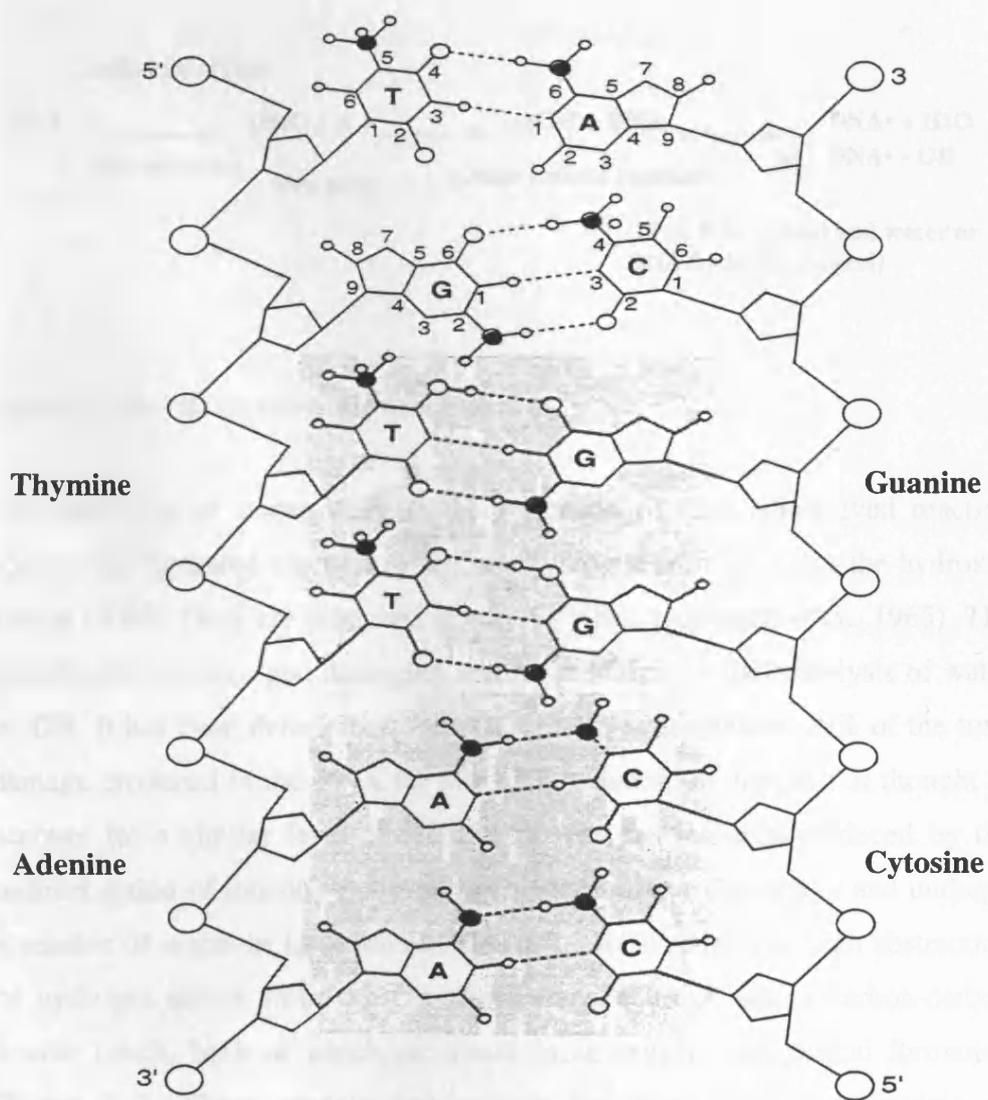


Figure 2.2. DNA structure.

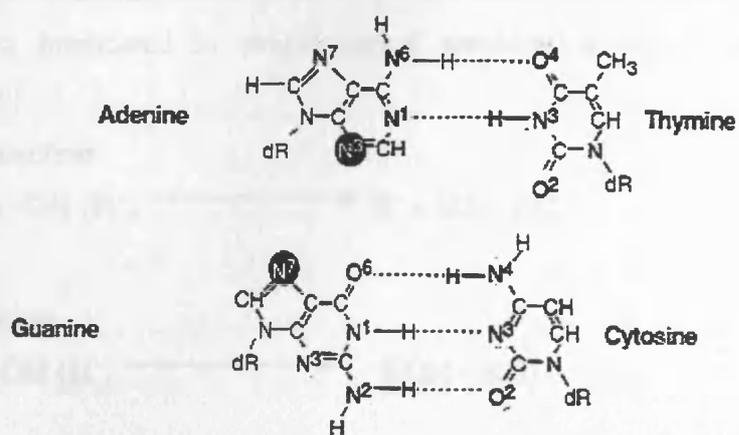
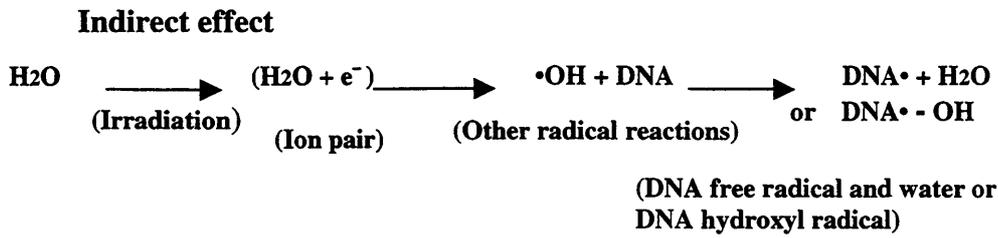


Figure 2.3. The structure of pyrimidines and purines.



**Figure 2.4.** The indirect action of ionizing radiation.

The radiolysis of water leads to the formation of three short-lived reactive species, the hydrated electron ( $\text{e}^- \text{aq}$ ), the hydrogen atom ( $\text{H}\cdot$ ) and the hydroxyl radical ( $\cdot\text{OH}$ ). They are produced in varying yields (Johansen *et al.*, 1965). The most highly reactive and damaging species produced by the radiolysis of water is  $\cdot\text{OH}$ . It has been determined that *indirect action* constitutes 70% of the total damage produced in the DNA for low LET radiation; in turn, this is thought to account for a similar level of cell killing. The free radicals produced by the indirect action of ionising radiation are highly reactive chemically and undergo a number of reactions to acquire an electron.  $\cdot\text{OH}$  is capable of both abstraction of hydrogen atoms from other molecules and addition across carbon-carbon double bonds, both of which can result in an organic free radical formation (Figure 2.5). These organic free radicals are short lived; they contain an unpaired electron and consequently react to satisfy their own valence needs. Interception of the organic radicals, by molecular oxygen can result in peroxy radical formation. Such action may avert restitution processes and is one of the factors implicated in radiobiological sensitisation (this is described further below).

**Abstraction**



**Addition**



**Figure 2.5.** Formation and reactions of organic free radicals.

With three reactive water species being present on radiolysis, it is clear that the radiation chemistry of aqueous systems can become quite complex, particularly in cases where the solute reacts with all three species. However, through the use of selective scavengers the system can be greatly simplified and it is possible to study each component in isolation.

Macromolecules that have been converted to free radicals can undergo a series of reactions in an attempt to rid themselves of unpaired electrons, many of which result in the breakage of nearby chemical bonds. In the case of DNA, these broken bonds may result in the loss of a base leading to a basic site or an entire nucleotide resulting in the frank scission of the sugar phosphate backbone, involving either one or both DNA strands (Figure 2.6). Strand breaks occur as two types, single strand breaks (SSB), and double strand breaks (DSB) (Powell *et al.*, 1990).

The best-known chemical modifier of indirect radiation action is oxygen. Under oxygenated conditions, the principle primary species of water radiolysis are  $\cdot\text{OH}$ , the hydroperoxide radical ( $\text{HOO}\cdot$ ) and its anion ( $\text{O}_2\cdot^-$ ), the latter also being known as superoxide.  $\text{O}_2\cdot^-$  is not very reactive and is not reported to produce DNA strand breaks but may contribute to the redox reactions. Oxygen acts as a radiosensitiser by forming peroxides in the DNA at induced carbon-centered radical sites, thereby “fixing” the radiation damage. In the absence of oxygen, DNA can be restored to its preirradiated condition by hydrogen donation from endogenous reducing species in the cell, such as the free radical scavenger glutathione (a thiol compound); this can be considered a type of fast chemical restitution or repair (Figure 2.7). These two processes, fixation and restitution, are considered to be in a dynamic equilibrium, such that changes in relative amounts of either the radiosensitiser (oxygen), or the protector, (e.g. glutathione), can tip the scale in favour of either fixation (more damage, more cell killing, greater radiosensitivity) or restitution (less damage, less cell killing, greater radioresistance) (Wouters *et al.*, 1997). Consistent with this free radical based interpretation of the oxygen effect in the finding that oxygen is required to be present during irradiation to produce an aerobic radioresponse (Gunderson *et al.*, 2000). The concentration of oxygen necessary to achieve essentially

maximum sensitisation is small; evidence for the high efficiency of oxygen as a radiosensitiser.

The overall effect of ionising radiation on DNA is to produce a variety of damage including single strand breaks, double strand breaks, base loss, modified bases, and DNA-DNA crosslinks. The extent of damage produced by the dose of radiation is directly proportional to the dose. The damage is produced by the direct action of the radiation on the DNA, because they contain unsaturated bonds.

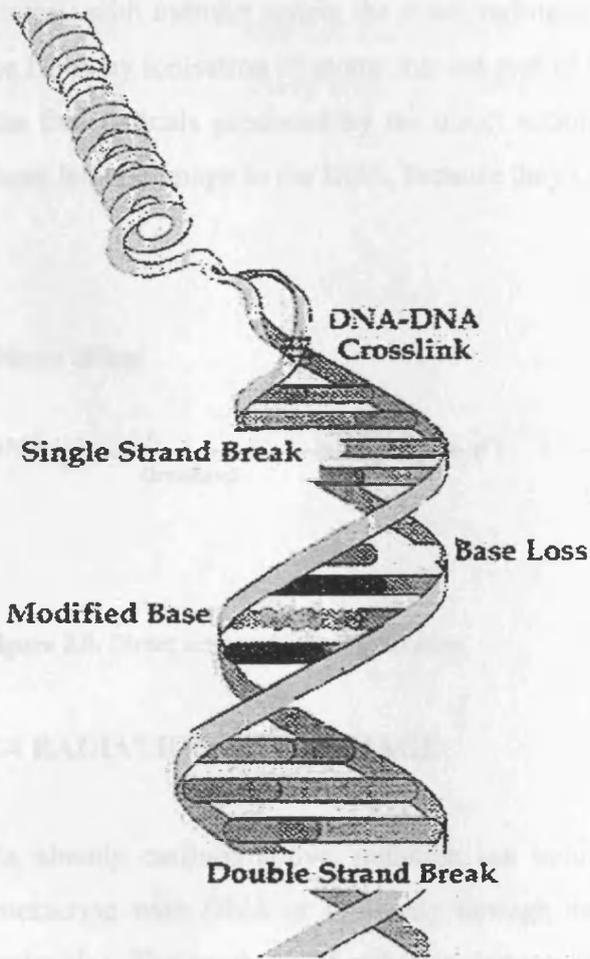


Figure 2.6. Types of DNA damage produced by ionising radiation.

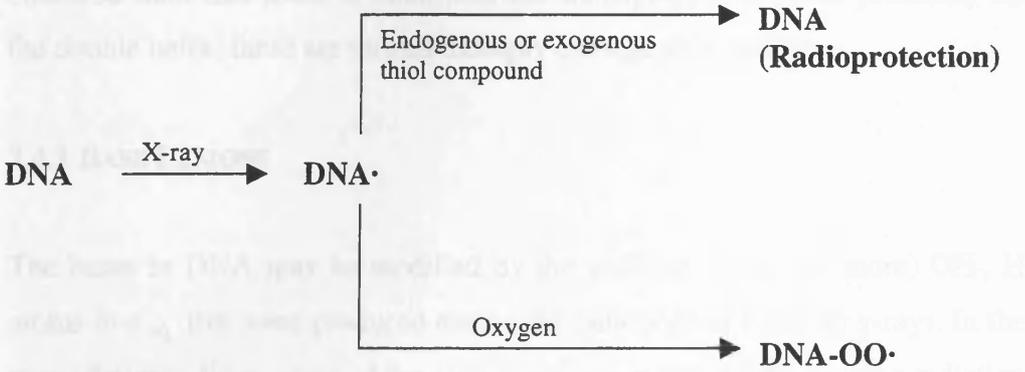


Figure 2.7. Mechanism of action for oxygen effect.

### 2.3.2 DIRECT EFFECT OF RADIATION

The second mode through which radiation can act is via *direct action*. In contrast with *indirect action*, the direct radiogenic mechanism causes damage to the DNA by ionisation of atoms that are part of the DNA molecule (Figure 2.8). The free radicals produced by the direct action of ionising radiation go on to cause lethal damage to the DNA, because they contain unpaired electrons.

#### Direct effect

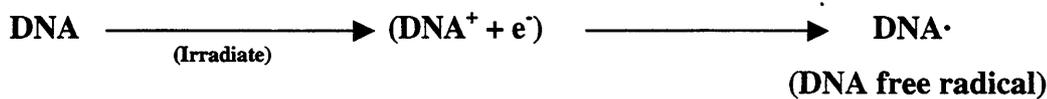


Figure 2.8. Direct action of ionising radiation.

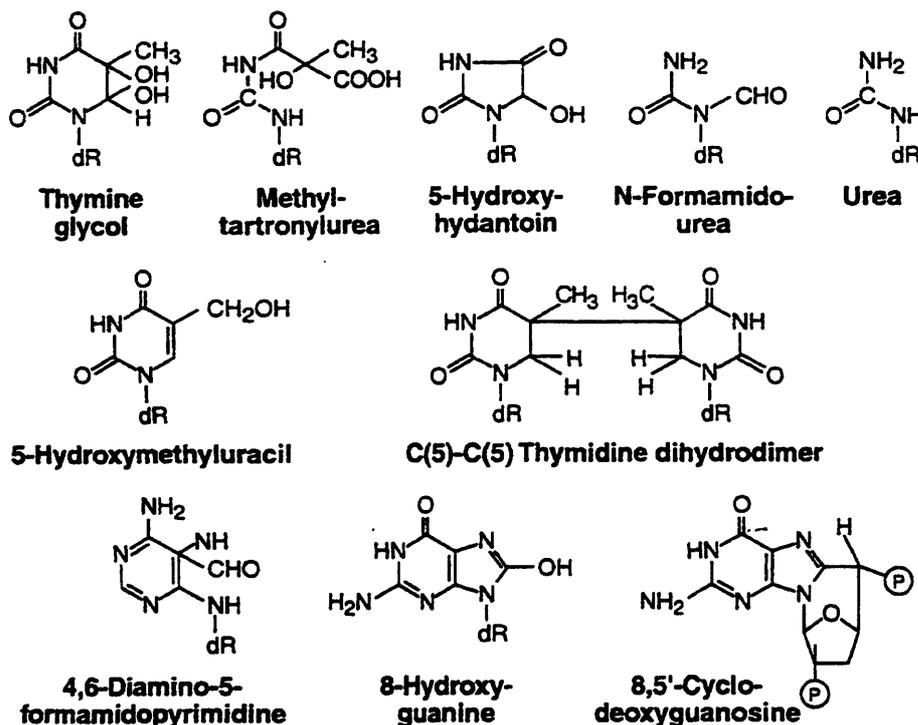
## 2.4 RADIATION DNA DAMAGE

As already outlined above, radiation can induce a range of lesions by direct interaction with DNA or indirectly through damage induced in nearby water molecules. The products of radiation damage include base damage, damage to sugar backbone, single strand breaks and double strand breaks (Leadon *et al.*, 1996). Furthermore, a feature of radiogenic DNA damage is that it can be clustered such that there is more than one damaged site in close proximity on the double helix; these are termed multiply damage sites (MDS).

### 2.4.1 BASE LESIONS

The bases in DNA may be modified by the addition of one (or more) OH·, H atoms or  $\text{e}^-_{\text{aq}}$  that were produced during the radiolysis of water by  $\gamma$ -rays. In the case of pyrimidines, some of the major products produced by ionising radiation are thymine glycol, 5-hydroxy-hydantion, and C(5)-C(5)-thymidine dihydrodimer. For purines, the biologically important derivatives induced by

ionising radiation are 4,6-diamino-5-formamidopyrimidine, 8,5'-cyclo-deoxyguanosine, and the mispairing lesion 8-hydroxyguanine (Figure 2.9) (Cadet *et al.*, 1985). Damage to the DNA bases by ionising radiation has little effect in terms of cell killing because they are repaired readily. However, if the damage was not repaired or misrepaired it may result in mutation.



**Figure 2.9** Examples of base damage induced by ionising radiation.

## 2.4.2 STRAND BREAKS

When cells are irradiated with X-rays, many SSBs occur. These can be observed and scored as a function of dose if the DNA is denatured and the supporting structure stripped away. In intact DNA, however, SSBs are of little biological consequence as far as cell killing is concerned because they are repaired readily using the opposite strand as a template. Single strand breaks are repaired by base excision repair (see Section 2.5.1). If both strands of the DNA are broken, and the breaks are well separated, repair again occurs readily, as the two breaks

are handled separately. Single strand breaks can also be secondary products from alkali labile sites.

By contrast, if the breaks in the two strands are opposite one another, or separated by only a few base pairs, this may lead to a DSB; that is, the DNA duplex physically separated into two pieces. A DSB is believed to be the most important lesion produced in chromosomes by radiation. The interaction of two DSBs can lead to chromosomal aberrations. These aberrations may result/ lead to mutation and ultimately carcinogenesis, or they may physically interfere with the processes of mitosis and cytokinesis and result in prompt cell death. In other cases, cell division can occur, but the loss or uneven distribution of genetic material between the cells progeny is ultimately lethal. Clearly, these aberrations are the result of unrepaired DNA damage that persists from the time of irradiation until the time of the next division (Steel *et al.*, 1983). Misjoined or unrepaired double strand breaks can produce deletion, translocation, and acentric or dicentric chromosomes, all of which are of serious consequence to the cell. There are many kinds of DSBs, varying in the distance between the breaks on the two DNA strands and the kinds of end groups formed. Their yield in irradiated cells is about 0.04 times that of single strand breaks, and they are induced linearly with dose, indicating that they are formed by single tracks of ionising radiation. Two processes repair DSBs, homologous recombination, requiring an undamaged DNA strand as a participant in the repair, and end-to-end rejoining *via* nonhomologous recombination (see Sections 2.5.4 and 2.5.5).

### **2.4.3 COMPLEX DAMAGE**

DNA damage that results from multiple radical attacks occurs if the events of high-energy deposition producing multiple ionisations and free radicals, physically overlap the DNA helix. This gives rise to a wide variety of complex lesions whereby multiple damages occur within close proximity on the DNA. These may include base damage as well as double and single strand breaks; the term multiply damaged sites describe these phenomena. Complex damage can cover up to 20 base pairs (Figure 2.10).



**Figure 2.10.** Illustration of a locally multiple damaged site (Hall 2000).

## 2.5 RADIATION DNA DAMAGE REPAIR

DNA is unique in so far as it is the only cellular macromolecule with its own repair system. Until quite recently, little was known about DNA repair processes in mammalian cells. The study of DNA repair in mammalian cells received a significant boost during the late 1960s with publications by Cleaver (Cleaver *et al.*, 1969 & 1968) that identified the molecular defect responsible for the human disease *xeroderma pigmentosum*. Cleaver showed that cells derived from such patients were sensitive to UV radiation and were defective in the nucleotide excision repair pathway. These cells were not especially sensitive to ionising radiation, but due to the defect in their repair ability they showed high levels of unrepaired damage. Each type of DNA damage induced by radiation is subjected to reversal by one of several DNA repair pathways. There are five major radiation DNA damage repair pathways, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombinational repair (HRR) and non-homologous end joining (NHEJ), each specialised for certain type/class of DNA damage. BER and NER are mainly responsible for removing inappropriate or damaged bases in DNA. MMR is involved in correcting mismatched bases during replication. HRR and NHEJ are required to repair DNA double strand breaks and are especially important in responding to ionising radiation (Braithwaite *et al.*, 1999). However with increasing levels of DNA damage the cell fails to achieve full repair and switches to cell cycle arrest and then maybe to apoptosis. Cell cycle arrest is sometimes permanent, but ordinarily reversible, allowing time for further DNA repair. Apoptosis is a natural process that occurs during embryonic development

or during tissue regression to eliminate cells without evoking an inflammatory response. Apoptosis is a permanent process and plays a critical role in the cell's response to ionising radiation.

### 2.5.1 BASE EXCISION REPAIR

The base excision repair pathway repairs base damage and single strand breaks (Figure 2.11). Enzymes called DNA *glycosylases* recognise specific types of damage bases and excise them without otherwise disturbing the DNA strand (Leadon *et al.*, 1996). DNA *glycosylases* are small proteins, which are <30 kDa in size, with no subunit structure and some require metal cofactors. Cells with lower activities of some DNA *glycosylases* appear to be more sensitive to irradiation. This is due to the fact that the damaged bases are not recognised and therefore cannot be excised. The action of the glycosylase itself results in the formation of another type of damage observed in irradiated DNA, an apurinic or apyrimidinic (AP) sites. The AP site is then recognised by another repair enzyme, an AP endonuclease, which nicks the DNA 5' to the lesion. The resulting strand break becomes the substrate for an exonuclease, which removes the a basic site, along with few additional bases. The small gap that results is "patched" by DNA polymerase, which uses the opposite, undamaged DNA strand as a template. Finally, DNA ligase seals the patch in place. Base excision repair is highly efficient, rapid and if one pathway enzyme fails, then another can take over. Important *glycosylases* for the removal of oxidised purines are *fpg* (in *E.coli*) and HOGG1 (in humans). These are *N-glycosylases* with an associated  $\beta,\delta$ -lyase activity, which catalyses the cleavage of both phosphodiester bonds 5' and 3' to the basic site in DNA, generating a single base gap limited by 3' and 5' phosphate ends. An important *glycosylase* for the removal of oxidised pyrimidines is endonuclease III (from *E.coli*). As well as an *N-glycosylase* specific for thymine glycol and other oxidised pyrimidines this is class I AP endonuclease. This acts by cutting the 3' phosphodiester bond of the AP site in a  $\beta$ -lyase activity. Defects in crucial proteins (*i.e.* DNA *glycosylases*) in the BER pathway can lead to an increase in radiosensitivity (Bernstein *et al.*, 2002).

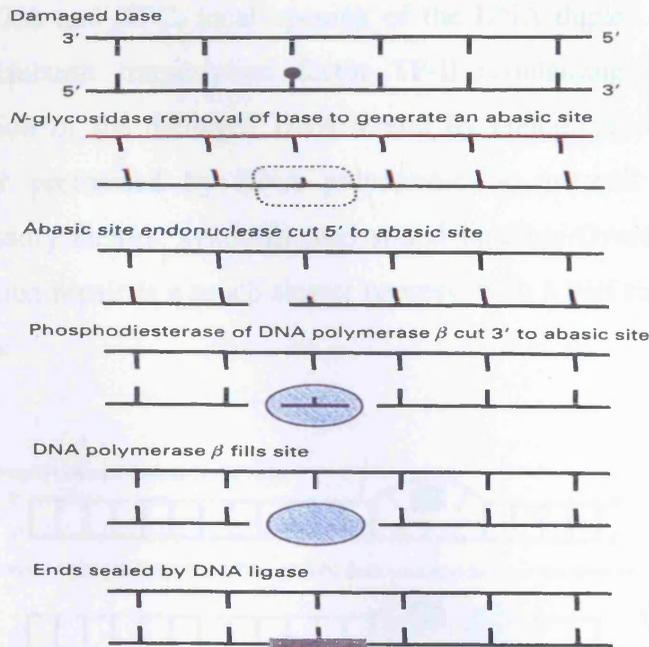
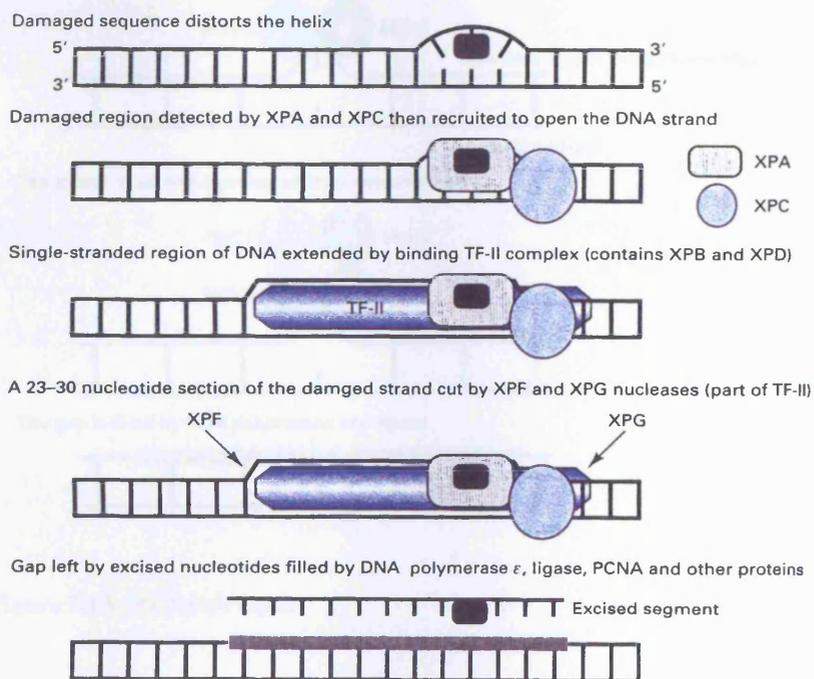


Figure 2.11. Base excision repair.

## 2.5.2 NUCLEOTIDE EXCISION REPAIR

Another major DNA repair pathway is nucleotide excision repair (Figure 2.12). The DNA glycosylases that begin the process of base excision repair do not recognise all known forms of base damage, particularly bulky lesions such as large DNA adducts induced by chemotherapeutic agents (Leadon *et al.*, 1996). In such cases, another group of enzymes, termed *structure specific endonucleases*, initiate the excision repair process. These repairs do not recognise the specific lesion, but they are thought instead to recognise more generalised structural distortions in DNA that necessarily accompany a bulky base lesion. The structure specific endonucleases incise the affected DNA strand on both sides of the lesion and release an oligonucleotide fragment made up of the damage site and several bases on either side of it. Because a longer segment of DNA, including both bases and the sugar phosphate backbone, is excised, this type of excision repair is referred to as nucleotide excision repair. Two subpathways of NER are global genomic repair (GGR) and transcription coupled repair (TCR). These pathways are initiated somewhat differently, with GGR acting on damages in non-transcribed regions of DNA and TCR acting on damages in actively transcribed DNA. The NER requires the action of more than 30 proteins in a stepwise manner that include damage recognition detected

by XPA and XPC, local opening of the DNA duplex around the lesion by a multisubunit transcription factor TF-II (containing XPB and XPD), dual incision of the damaged DNA strand by endonucleases XPG and XPF, gap repair performed by DNA polymerase  $\delta\epsilon$  as well as several replication accessory factors, synthesis, and strand ligation. Overall, however, nucleotide excision repair is a much slower process, with a half time of approximately 12 hours.



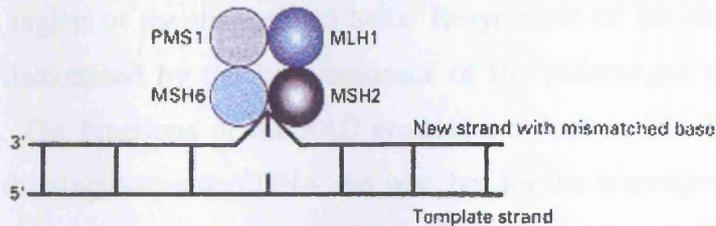
**Figure 2.12.** Nucleotide excision repair.

### 2.5.3 MISMATCH REPAIR

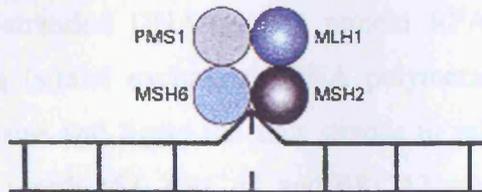
Mismatch repair is an important pathway for eliminating mutations from base mismatches arising from faulty replication or repair, or from chemical base modification. When a DNA polymerase misincorporates a base during replication, the incorrect base is subsequently recognised and removed by mismatch repair proteins, such as MSH6, MSH2, PMS1 and MLH1 (Figure 2.13). The resulting strand break becomes the substrate for an exonuclease, which removes the basic site, along with few additional bases. The small gap

that results is “patched” by DNA polymerase, which uses the opposite, undamaged DNA strand as a template. Finally, DNA ligase seals the patch in place. However, there is little evidence to suggest that mismatch repair plays a significant role in ionising radiation damage.

Mismatched bases recognised by MSH6 (or MSH3).MSH2 heterodimer  
 PMS1 and MLH1 then bind



The strand is cut and digested with an exonuclease



The gap is filled by DNA polymerase and ligase



**Figure 2.13.** Mismatch repair.

### 2.5.4 HOMOLOGOUS RECOMBINATIONAL REPAIR

DSBs can be repaired by two mechanisms, homologous recombination and non-homologous end joining. Homologous recombinational repair has been recognised as the most important pathway for repair of double strand breaks in yeast and to some extent in mammalian cells. In homologous recombinational repair (HRR), sequence information that is lost due to damage in one double strand DNA molecule is accurately replaced by physical exchange with a segment from a homologous intact DNA molecule (Figure 2.14). Genetic analysis has revealed the existence of a large number of genes that regulate double strand break repair (Ward *et al.*, 1994). Among the genes are breast cancer associated gene 1 (BRCA1), ATM, ATM-related (ATR), Werner

syndrome gene (WRN), Bloom syndrome gene (BLM), *Tip60* and p53. *RAD* proteins are also examined because of the major role they play in HRR. Homologous recombination requires extensive regions of base homology on the undamaged helix to provide a template for repair. 5'-3' exonucleases digest damaged strands to expose single-stranded regions either side of the break. A complex series of proteins (*RAD* proteins) promote the sensing of homologies between single-stranded damaged DNA and homologous base sequence in the same region of the undamaged helix. Resynthesis of the excised sequences is then determined by the base sequence of the undamaged strand of the sister helix. The functions of the *RAD* proteins are unclear but *RAD51* polymerises onto the single-stranded DNA and searches for the homologous sequence on the undamaged helix (Bezzubova *et al.*, 1997). The other *RAD* proteins plus the single-stranded DNA-binding protein RPA facilitate crossover between the strands (strand exchange). DNA polymerase, ligases and accessory proteins synthesise and ligate the four strands to reform two helices. *RAD51* will also interact with p53, BRCA1 and BRCA2 proteins, providing a mechanistic link with proteins known to be involved in DNA repair (King 2000). Cells deficient in any of these proteins are sensitive to ionising radiation (Leadon *et al.*, 1996).

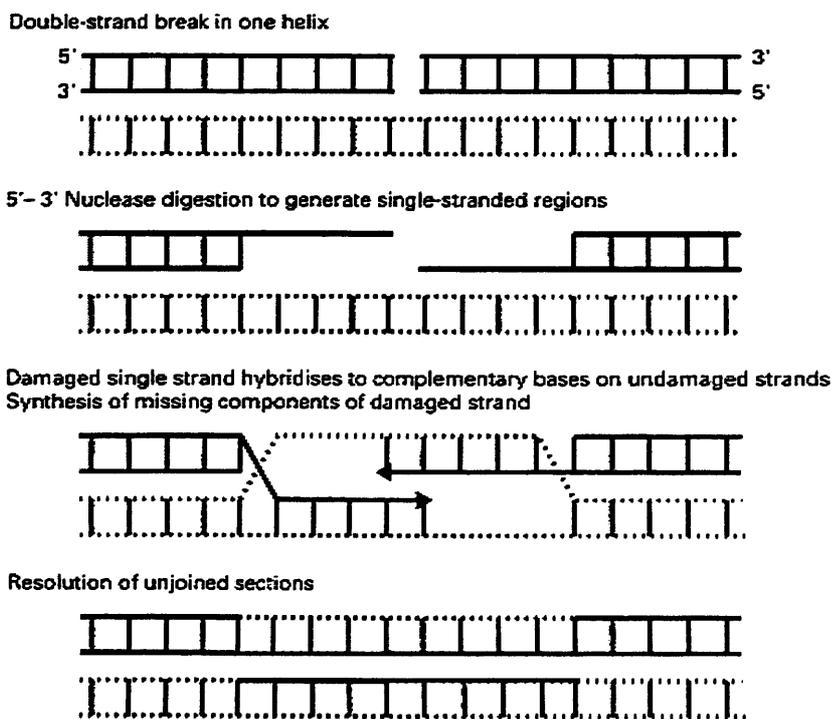


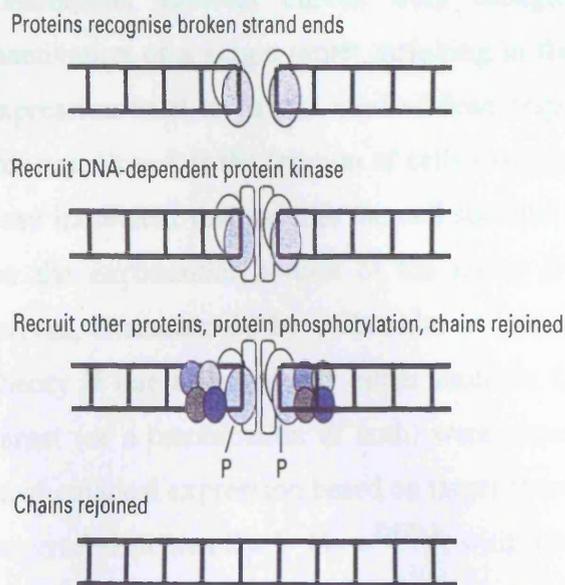
Figure 2.14. Homologous recombinational repair.

### 2.5.5 NON-HOMOLOGOUS END JOINING

Human cells appear to differ from yeast in that for the repair of DSBs the non-homologous end-joining pathway appears to predominate (Figure 2.15). Non-homologous end joining is regarded as largely inaccurate because it involves the end joining reactions with junctions containing deletions. Non-homologous end joining is governed, by a number of genes (Bernstein *et al.*, 2002). One essential gene involved in resistance to cell killing by ionising radiation and to the repair of double strand breaks is XRCC5, which encodes Ku80. This protein binds with high affinity to the ends of double strands in a complex that includes another protein, Ku70 (Jin *et al.*, 1997). Ku80 functions in normal cellular processes requiring double strand breaks rejoining. Ku80 exists in cells in a heterodimeric complex with Ku70, the product of the XRCC6 gene. The Ku80 and Ku70 heterodimer is required for the end-binding and repair functions. The Ku proteins carry out the important function of recognising the ends of double strand breaks and protect them from further degradation before the end-joining reactions that mediate double strand breaks repair occur. An associated protein in the complex with important function in DNA repair is DNA-PK, which is the product of the XRCC7 gene. The catalytic subunit of the DNA-PK becomes an active kinase, capable of further transmitting the damage signal, after it binds to the Ku80/70 complex, which has attached to DNA ends. The complex is able to align the ends of the double strand break and allow their ligation by DNA ligase, which, among one or more other factors and activities, completes NHEJ DNA repair.

### 2.6 RADIATION CELL SURVIVAL

Radiotherapy is the most common modality in treating cancer. Radiation therapy is most commonly given as a series of small daily dose fractions of approximately 2 Gy each, 5 days per week, over a period of 5 to 7 weeks to a total dose of 50 to 70 Gy. Although, the historical development of this “conventional” radiotherapy schedule was empirically based, certain early radiobiological experiments suggested this approach. One of these experimental approaches was the study of radiation cell survival curves.



**Figure 2.15.** Non-homologous end joining.

### 2.6.1 CELL SURVIVAL CURVES

Survival curve theory proposes to describe radiation cell survival curves taking into consideration the physics of energy deposition. Experiments with macromolecules and prokaryotes established that dose-response relationships could be explained, in principle, by random and discrete nature of energy absorption, if one assumed that the response resulted from critical “targets” receiving random “hits” (Gunderson *et al.*, 2000). With an increasing number of shouldered survival and dose-response curves being described for cells irradiated both *in vitro* and *in vivo*, various equations were developed to fit these data. Target theory pioneers studied a number of different endpoints in the context of target theory, including enzyme inactivation in cell free systems (Hutchinson *et al.*, 1961), cellular lethality, chromosomal damage, and radiation induced cell cycle perturbations in microorganisms (Gunderson *et al.*, 2000). Survival curves, in which the log of the “survival” of a certain biologic activity was plotted as a function of radiation dose, were found to be either exponential or shouldered, the latter usually noted for the survival of more complex organisms (Hutchinson *et al.*, 1961).

Exponential survival curves were thought to result from the single-hit inactivation of a single target, resulting in the loss of activity. A mathematical expression used to fit this kind of dose response relationship was  $S=e^{-D/D_0}$ . In this equation, S is the fraction of cells that survive a given dose D, and  $D_0$  is the dose increment that reduces the cell survival to 37% (1/e) of some initial value on the exponential portion of the curve (Figure 2.16 (A)). The shouldered curves, characterised by a shoulder at low doses, were consistent with target theory if one assumed that either multiple targets or multiple hits in a single target (or a combination of both) were necessary for radiation inactivation. A mathematical expression based on target theory that provided a fairly good fit to survival data was  $S= 1- (1- e^{-D/D_0})^n$ , with n being the back extrapolation of the exponential portion of the survival curve to zero dose (Figure 2.16 (B)). Implicit in this multitarget model was that damage had to accumulate before the overall effect was registered. It soon became apparent that some features of this model were inadequate (Alper *et al.*, 1980). The most obvious problem was that the single hit, multitarget equation predicted that survival curves should have an initial slope of zero; that is, for small doses per fraction, the probability of cell killing would approach zero. This was not observed in practice in mammalian cell survival curves or in clinical trials curves. A different interpretation of cell survival was formulated in the late 1960s and early 1970s, which was known as the “linear-quadratic” or “alpha-beta” equation. The survival curves for this interpretation were plotted with the dose on a linear scale and the surviving fraction on a logarithmic scale and the equation is  $S= e^{-(\alpha D + \beta D^2)}$  (Figure 2.17). This equation was shown to fit many survival data well, particularly in the low dose region of the curve (1- 4 Gy), and it also provided the negative initial slope. In this equation, S is the fractional cell survival following a dose D,  $\alpha$  is the rate of cell killing by a single hit process, and  $\beta$  is the rate of cell killing by a two hit mechanism. Qualitatively, the shape of the survival curve can be described in relatively simple terms, at low dose regions the curve starts out straight on a log linear scale and at higher doses the curve bend, and this bending extends over a range of few grays. The theoretical derivation of the linear-quadratic equation is based on two sorts of observations. Based on microdosimetric considerations, Kellerer and Rossi in 1972 proposed that radiation-induced lethal lesions resulted from the interaction of two sublesions.

According to this interpretation, the  $\alpha D$  term is the probability that these two sublesions would be produced by a single event, whereas  $\beta D^2$  is the probability that two sublesions would be produced by two separate events. The same assumption was made on double strand breaks, in that DNA double strand breaks are considered lethal lesions and such lesions could be produced by either a single energy deposition involving both strands in DNA or by two separate events, each involving a single strand.

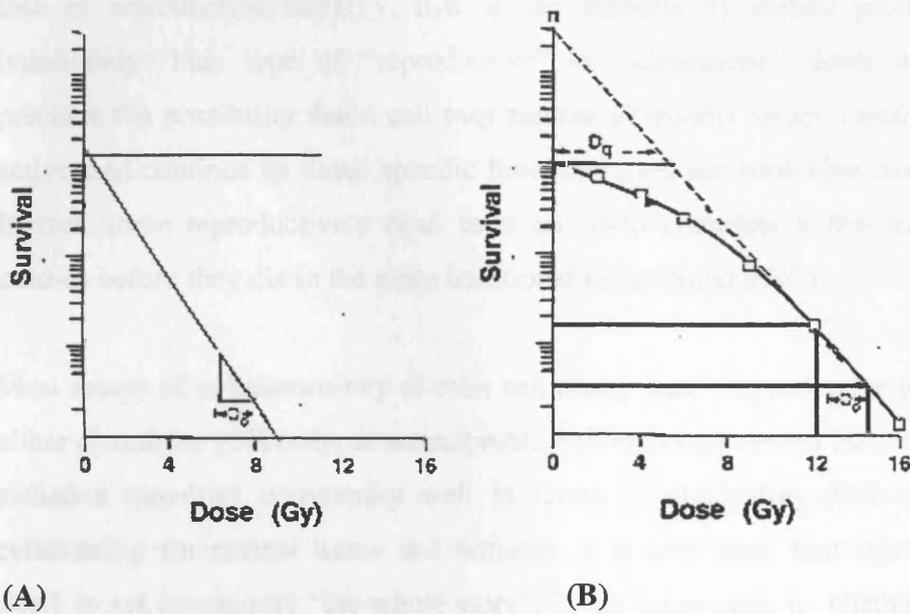


Figure 2.16. The two most common types of target theory, (A) single target hit; (B) multi target hit.

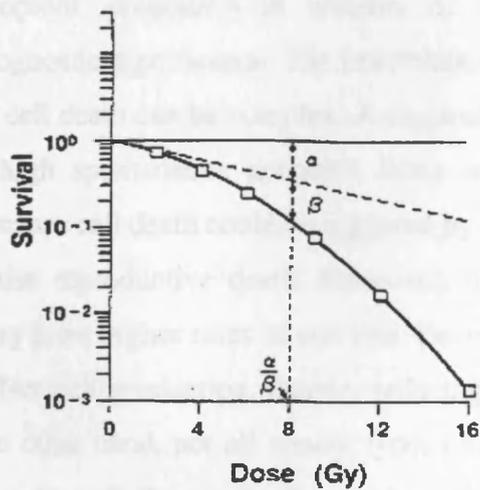


Figure 2.17. The linear quadratic model.

## 2.6.2 CLONOGENIC SURVIVAL

As described above, a survival curve is a curve that describes the relationship between radiation dose and the proportion of cells that survive. The traditional definition of death as a permanent, irreversible cessation of vital functions is not the same as that which constitutes “death” to the radiation specialists. For proliferating cells, including those maintained *in vitro*, the stem cell of normal tissue and tumour clonogens, cell death in the radiobiological sense refers to a loss of reproductive integrity, that is, an inability to sustain proliferation indefinitely. This type of “reproductive” or “clonogenic” death does not preclude the possibility that a cell may remain physically intact, metabolically active, and continue its tissue specific functions for some time after irradiation. In fact, some reproductively dead cells can even complete a few additional mitoses before they die in the more traditional sense (Steel 2002).

Most assays of radiosensitivity of cells and tissue assess reproductive integrity, either directly or indirectly, as an endpoint. Although such assays had served the radiation oncology community well in terms of elucidating dose response relationship for normal tissue and tumours, it is now clear that reproductive death is not necessarily “the whole story”. What is not clear is whether and to what extent apoptosis contributes to our traditional measures of radiosensitivity based on reproductive integrity, and whether pre-treatment assessment of apoptotic propensity in tumours or dose limiting normal tissue has any prognostic significance. The interrelationship between these different pathways of cell death can be complex. A suggestion has been made that the tumour with a high spontaneous apoptotic index may be inherently more radiosensitive because cell death could be triggered by lower doses than are usually required to cause reproductive death. Moreover, tumours that readily undergo apoptosis may have higher rates of cell loss, the net effect of which would be partially to offset cell production, thereby reducing the number of tumour clonogens. On the other hand, not all tumour types undergo apoptosis, and even among those that do, radiotherapy itself could have the undesirable side effect of selecting for subpopulations of cells that are apoptosis “resistant”. Nevertheless, with all these proposals, the conventional radiobiological view remains the same; the

loss of reproductive integrity is the critical response to radiation (Steel *et al.*, 2002).

### 2.6.2.1 Clonogenic Assay

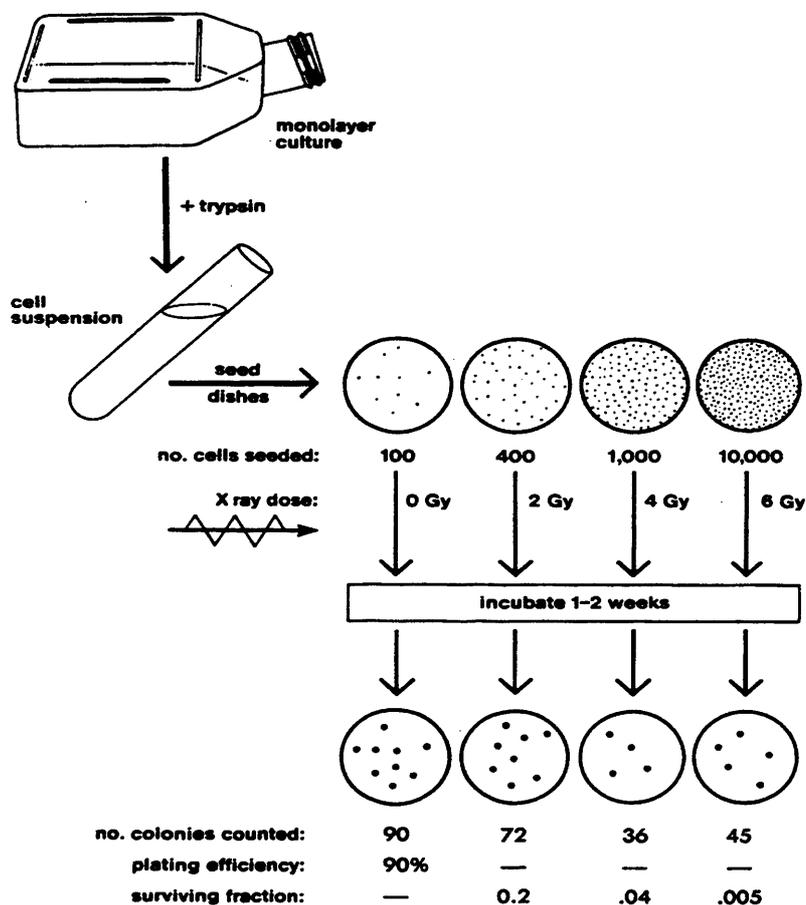
Clonogenic assay has formed the basis of the studies of cellular response to radiation, by allowing quantitation of radiation response to a single cell (Chadwick *et al.*, 1973). The basic idea of the clonogenic assay is to assess the ability of the cell to grow into a large colony, which can be seen with the naked eye, as proof that the cell had retained its reproductive integrity after radiation (Hall 2000). In the mid 1950s the cell culture techniques were sufficiently refined to allow radiation dose response experiments to be conducted. The reproductive integrity of a single HeLa cell was measured by its ability to form macroscopic colonies of at least 50 cells (corresponding to approximately 6 successful cell divisions) on petri dishes (Puck *et al.*, 1956). A single cell suspension is prepared and divided into several aliquots to perform the clonogenic assay. One aliquot is kept as an unirradiated control whilst the others are irradiated with increasing doses. The suspensions are then plated out in culture under the same circumstances, except that since it is anticipated that radiation has killed some cells, progressively larger numbers of the irradiated cells needs to be plated. After a suitable period of incubation, the grown colonies are scored (Figure 2.18). Surviving fraction was calculated as the ratio colonies formed to cells plated taking into account plating efficiency (which accounts for the number of colonies scored as percentage of the number of viable unirradiated control cells plated).

This process is repeated for each individual dose, and a survival curve is plotted traditionally as the logarithm of the surviving fraction against dose (of radiation, cytotoxic drug or others). For low doses of X-rays curves were characterised by a shoulder at low doses, and a roughly exponential region at higher doses.

To bridge the gap between the radiation responses of cells grown in culture and in animals, Hewitt and Wilson (Hewitt *et al.*, 1959) developed a method to assay single cell survival *in vivo*. To measure the survival curve *in vivo* they

established two groups of experimental tumours (often implanted in mice); one to be irradiated and the other kept as a control. Then some time after irradiation a single cell suspension for both groups is prepared and these are plated out under the same circumstances (except that the irradiated cells need a larger number of cells seeded). Plating efficiency, and surviving fraction are calculated and the survival curve is plotted. The difference here is that the cells are irradiated under *in vivo* conditions.

Overall, clonogenic assay has formed a central theme in the understanding of tumour radiobiology, though these assays are not without artefacts.



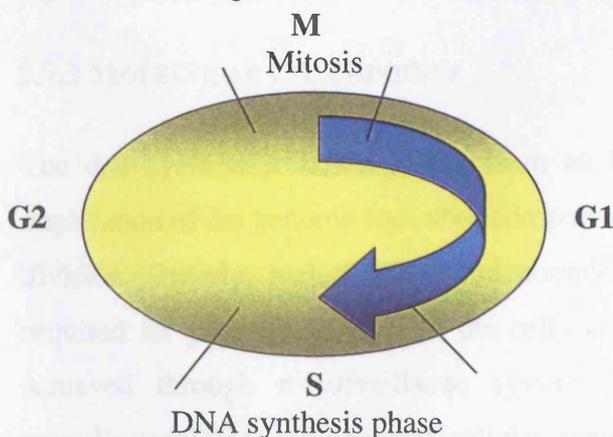
**Figure 2.18.** The cell culture technique used to generate a cell survival curve. Cells from a stock culture are prepared into a single cell suspension by trypsinization, and the cell concentration is counted. Known number of cells are inoculated into petri dishes and irradiated. They then are allowed to grow until the surviving cells produce macroscopic colonies that can be counted readily. The number of cells per dish initially inoculated varies with the dose, so that the number of colonies surviving is in the range that can be counted conveniently (Hall 2000).

## 2.7 TUMOUR CELL RADIOSENSITIVITY

Why is radiation therapy successful in controlling one patient's tumour but not another's, even when both tumours seem to be identical? Why is radiation treatment generally successful in controlling certain types of cancer but not others? A simple answer is that, although tumours appear identical macroscopically, they may be different phenotypically and genotypically. Different tissues isolated from many types of human and experimental cancers, differ in antigenicity, metastatic potential, and sensitivity to radiotherapy. With regard to the latter both intrinsic and extrinsic factors contribute to the difference in sensitivities. Intrinsic factors may include, inherent radiosensitivity, cell cycle kinetics, gene expression, biochemical repair processes, and the way in which the tissue is structurally and functionally arranged. Extrinsic factors, on the other hand tend to be related to physiological conditions, such as the degree of vascularity, availability of oxygen, pH, and the degree of contact between normal and malignant tissue. Some of these intrinsic and extrinsic factors are discussed in detail in the following section.

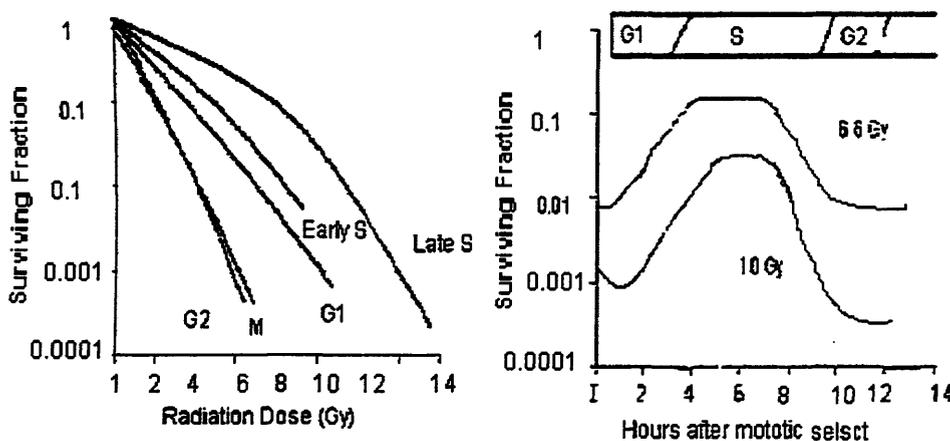
### 2.7.1 CELL CYCLE

It has been proposed that the perturbation of the cell cycle (Figure 2.19) is a basic feature of cellular response to ionising radiation. Such effects can modify the radioresponsiveness of tissue either directly or indirectly, depending on the fraction of cycling cells present in the tissue of interest, their proliferation rates and the kinetic organisation of the tissue or tumour as a whole.



**Figure 2.19.** The stages of the cell cycle for actively growing mammalian cells.

Advances in techniques for the study of cell cycle kinetics paved a way for the analysis of radiation responses of cells as a function of cycle position. In an experiment by Terasima and Tolmach, (Terasima *et al.*, 1963) using HeLa cells, it was noted that cells were most radioresistant in the late S phase. Cells in G<sub>1</sub> were resistant at the beginning of the phase, but they became sensitive towards the end of the phase (length of the G<sub>1</sub> phase determines whether the cells will be resistant or intermediately sensitive at this phase). In G<sub>2</sub> phase cells were increasingly sensitive as they moved toward the highly sensitive M phase (Figure 2.20). The survival curves of these studies confirms that the most sensitive cells to radiation are those in the M and late G<sub>2</sub> phase, in which survival curves are steep and are largely shoulderless, and the most resistant cells to radiation are those in the late S phase. These studies indicate that the radiosensitivity of cells varies substantially with the phase of the cell cycle at which the radiation is delivered.



**Figure 2.20.** Variation of radiosensitivity through the cell cycle (Steel 2002).

### 2.7.2 MOLECULAR CHECKPOINTS

The cell cycle is a series of events in an invariant order that leads to the duplication of the genome and other components of the cells and eventually cell division. Orderly, high fidelity, and complete duplication of the genome is required for progression through the cell cycle (Weinert *et al.*, 1998). This is achieved through a surveillance system that detects DNA damage and unreplicated DNA and activates cellular checkpoints that block the activity of

the cell cycle “engine” (Weinert *et al.*, 1997). Because ionising radiation acts by producing severe damage to DNA, cell cycle checkpoints are essential for cell survival during radiation and provide a target for strategies aimed at sensitising cells to radiation therapy (Abraham 2001). Cells that lose the checkpoint gene functions move directly into mitosis with damaged chromosomes and are therefore at a higher risk of dying; hence they show great sensitivity to radiation. It has been known for many years that mammalian cells exposed to a small dose of radiation tend to experience a block or a delay in a certain phase of the cell cycle. There are two kinds of delay, mitotic delay and division delay. The mitotic delay is defined, as a delay in the entry of the cells into mitosis, and is a consequence of transient “upstream” blocks or delays in the movement of cells from one cell cycle phase to the next (Shiloh 2001). The division delay, is a delay in the time of appearance of new cells at the completion of mitosis, and is caused by the combined effects of mitotic delay and any further lengthening of the mitosis process itself. Division delay increases with dose and is, on average, about 1 to 2 hours per Gray (Terasima *et al.*, 1963), depending on the cell line. The cell cycle blocks and delays primarily responsible for mitotic delay and division delay are, respectively, a block in the G2-to-M phase transition and a block in the G1-to-S phase transition (Bartek *et al.*, 2001). The duration of the G2 delay, like the overall division delay, varies with cell type, but for a given cell type is both dose and cell cycle age dependent. In general, the length of the G2 delay increases linearly with dose. For a given dose, the G2 delay is longest for cells irradiated in S or early G2 phase, and is shortest for cells irradiated in G1 phase (Terasima *et al.*, 1963). The role for DNA damage and its repair in the cause of division delay is demonstrated by the finding that certain cell types that either did not exhibit the normal cell cycle delays associated with radiation exposure (Painter *et al.*, 1980) or, conversely, were treated with chemicals (such as caffeine) that ameliorated the radiation-induced delays (Tolmach *et al.*, 1977), tended to contain higher amounts of residual DNA damage, and showed increased radiosensitivity. One of the most important cell cycle checkpoints is the Cdk2, which prevent DNA synthesis.

### 2.7.3 OXYGEN LEVEL

Until relatively recently, oxygen had been termed a dose modifying agent; that is, the ratio of doses to achieve a given survival level under hypoxic and aerated conditions was constant, regardless of the survival level chosen for a given dose of radiation. This dose ratio to produce the same biologic endpoint is the OER (oxygen enhancement ratio). Recent evidence indicates that oxygen may not be strictly dose modifying. Several studies have shown that the OER for sparsely ionising radiation is lower at lower doses (1- 4 Gy) than at higher doses. The OER appear to be smaller at a high level of survival, and larger at higher doses and lower level of survival. This finding has been observed for exponentially growing cells in culture when the OER was calculated from high-resolution survival determinations in the shoulder region of the survival curve. The OER typically has a value of between 2.5 and 3.0 for a large single dose of radiation, 1.5 to 2.0 for an intermediate dose of radiation, and 1.0 for a small dose of radiation (Gunderson *et al.*, 2000). Lower OERs for dose per fraction in the range commonly used in radiotherapy have also been inferred indirectly from clinical and experimental tumour data, in addition to some laboratory findings for cells in culture (Palcic *et al.*, 1984). Investigators have suggested that the lower OERs result from an apparent age response for the oxygen effect, not unlike the age response for inherent radiosensitivity and cell cycle delay (Hall 2000). Although the exact mechanism of the oxygen effect is unknown, a simplistic model can be used to illustrate the current understanding of this phenomenon. The radical competition model holds that oxygen acts as a radiosensitiser by forming peroxides in important biomolecules damaged by radiation exposure, thereby “fixing” the radiation damage. In the presence of oxygen the cells appear to be more sensitive to radiation, as the damage is fixed and unrepaired. In the absence of oxygen, DNA can be restored to its preirradiated condition and appears to be more resistant to radiation therapy (see Section 2.3.1).

#### **2.7.4 DAMAGE REPAIR AND RADIOTHERAPY**

From a phenomenological point of view, radiation damage in cells can be classified into three major categories: lethal damage (LD), which is irreversible and irreparable and leads to cell death. Sublethal damage (SLD), which under normal circumstances can be repaired, and only becomes lethal when it interacts with additional sublethal damage, that is, when the total amount of damage has accumulated to sufficient level to cause cell lethality; and potentially lethal damage (PLD), which is by definition, a spectrum of radiation damage that may or may not result in cell lethality depending on the cells' postirradiation conditions. Potentially lethal damage repair (PLDR) happens when the cells are maintained in overcrowded conditions, when incubated after irradiation at reduced temperatures, incubated in the presence of certain metabolic inhibitors, or in balanced salt solutions (Philips *et al.*, 1966). Investigators have also shown interest in the role that potential lethal damage repair (PLDR) could play in dose fractionation responses. In some cases, the ability of tumour cells to repair potentially lethal damage has been correlated with the radioresponsiveness of their tumour of origin, with tumours whose cells were proficient at potentially lethal damage repair (PLDR) tending to be relatively radioresistant, and vice versa (Weichselbaum *et al.*, 1980). This would be detrimental to the therapeutic ratio. On the other hand, potentially lethal damage repair (PLDR) by non-proliferation normal tissues would favourably modify the therapeutic ratio. When considering repair phenomena in intact tissues, one should remember that both the magnitude of the repair and the rate of the repair could influence how the tissue behaves during a course of radiation therapy.

#### **2.8 TUMOUR RADIOCURABILITY**

Tumour control is achieved only when all clonogenic cells are killed or are otherwise rendered unable to sustain tumour growth indefinitely. To estimate the likelihood of cure, it is necessary to know or at least to appreciate, approximately how many clonogenic cells the tumour contains, how radiosensitive these cells are, and what is the relationship between the number of clonogenic cells remaining after treatment and the probability of recurrence. The last is perhaps the easiest to ascertain, given our knowledge of both the

random and discrete nature of radiation damage and the general shape of the dose response curves for mammalian cells and tissues. For a given number of surviving cells per tumour, the probability of local control can be derived from Poisson statistics using the equation  $P = e^{-n}$ , where  $P$  is the tumour control probability and  $n$  is the average number of surviving clonogenic tumour cells. For example, when, for a large number of tumours, about two clonogenic cells remain per tumour at the end of the radiation therapy, the tumour control rate will be about 10 %, that is, that 9 of 10 tumours of the same size and relative radiosensitivity will recur. Should treatment reduce clonogenic cell numbers to an average of 0.1 per tumour, the tumour control probability would increase to 90%. The tumour control probability for a given fraction of surviving cells is not particularly helpful if the total number of cells at risk is unknown, and this is when an understanding of logarithmic relationships and exponential cell killing is useful. Based in the resolution of existing tools and technology of cancer detection, assume that a 1 cm<sup>3</sup> (1 g) tumour mass can be identified reliably. A tumour of this size has been estimated to contain approximately 10<sup>9</sup> cells (Norton *et al.*, 1977). A further assumption, that all such cells are clonogenic (rarely, if ever the case), suggests that at least 9 logs of cell killing would be necessary before any appreciable tumour control would be achieved, and 10 logs of cell killing would be required for a high degree of tumour control. After the first log or 2 of cell killing, however, some tumours respond by shrinking, and this is known as partial response. After 2 to 3 logs of cell killing, the tumour may shrink to a size less than the current limits of clinical detection, which is a complete response. Although partial and complete responses are valid clinical endpoints, a complete response does not necessarily mean tumour cure. At least 6 more logs of cell killing would still be required before any significant probability of cure would be expected. This explains why radiation therapy is not halted if the tumour disappears during the course of treatment. Finally, although the goal of curative radiation therapy is to reduce tumour cell survival by at least 9 logs, even for the smallest tumour likely to be encountered, it is much less clear how many logs of cell killing a particular normal tissue can tolerate before it loses its structural and functional integrity. This would depend on how the tissue is organised structurally, functionally and proliferatively,

which constituent cells are the most and least radiosensitive, and which cells are the most important to the integrity of the tissue.

## **2.9 PREDICTIVE ASSAYS**

In the regular day-to-day practice of radiotherapy, treatment schedules are planned for the average patient with a given type of malignancy at a given site. Although a lot of work has been rendered on this topic, little is done to adapt a treatment schedule to the individual case. The ultimate objective of predictive assay is to select a treatment protocol that is the optimal for each individual patient, and provide a better possibility of cure than the conventional therapy. In particular, such assays may one day be used to choose patients suitable for new experimental protocols. Patient groups vary in their response to ionising radiation, some demonstrate more sensitivity to radiotherapy than others, and a small fraction suffers unacceptable late effects. An extensive variety of assays have been developed to attempt to find ways and means to prospectively recognise radiosensitive individuals, equally to reduce patients suffering and to achieve the ultimate aim of discovering the best treatment option. A number of researchers have addressed the possibility of predicting the acceptance of normal tissue to ionising radiation, and determining the radiosensitivity of some cells taken from biopsies or blood samples. Some of these assays include, the clonogenic assay ( $SF_2$ ), microtetrazoline assay (MTT), pulse field gel electrophoresis, telomere length, and single-cell gel electrophoresis (comet assay). These assays and their possibilities are discussed in detail in the following section. Finally, it remains an appealing assumption that, in the future, a rapid and inexpensive test will be available to potentially recognise patients whose tumour are radiosensitive or who expect to suffer intolerable late effects if given a standard course of radiation therapy.

### **2.9.1 CLONOGENIC ASSAY ( $SF_2$ )**

The clonogenic cell survival assay has been considered to be the gold standard for judging the cellular response to ionising radiation. Formation of a macroscopic colony from a single cell suspension is the ultimate proof of

reproductive integrity. A single cell suspension is prepared, cells are then seeded in appropriate numbers into a dish. The dishes can then be irradiated and are then incubated for 1 to 2 weeks. Each single cell divides many times and forms a colony that is easily visible with the naked eye. Plating efficiency (PE) is determined as the number of colonies scored as a percentage of the number of viable control cells plated, and the surviving fraction (SF) is defined as the ratio of colonies formed to cells plated (with correction for plating efficiency).

West and colleagues (West *et al.*, 1993) developed a superior way of performing the clonogenic assay, by plating the cells into a soft agar. They investigated prospectively the intrinsic radiosensitivity of 50 patients, with stage I, II, and III carcinoma of the cervix, who were to receive radical radiotherapy. They found that patients with SF<sub>2</sub> greater than 0.55 had a significantly low probability of local control than those with an SF<sub>2</sub> of less than 0.55 (West *et al.*, 1991, 1993 & 1995). Conversely, stage alone was a poor prognostic factor for local control than radiosensitivity. These findings indicate that the intrinsic radiosensitivity may be a useful predictor of local recurrence after radical radiotherapy.

Nevertheless, two main problems associated with this assay are (1) the timescale in obtaining the results is not appropriate for treatment planning (typically it takes 4-5 weeks) and (2) the assay usefulness is limited as only a proportion of the sampled biopsies yield colonies. In fact, a study of primary tumours only ~8% of bladder tumour biopsies gave rise to a colony formation (Ho *et al.*, personal communication), and only ~70% of cervical tumour biopsies gave rise to colony formation (West *et al.*, 1991).

Consequently, new assays were needed that had clinical relevance but which do not rely on the reproductive integrity as an endpoint. Use of such assays require leap of faith, namely, that the endpoints assessed would have to be related to the killing of clonogenic cells, though not of necessity in a direct way. Because nonclonogenic assays do not directly measure cell survival as an endpoint, data derived from them and plotted as a function of radiation dose are properly called dose response curves rather than cell survival curves, although such data are

often analysed and interpreted in much the same way as cell survival curve data. Because of the difficulties of performing clonogenic assays on fresh tumour cells, several attempts have been made to develop nonclonogenic assays that depend on the growth, yielding a good result in a short time.

### 2.9.2 MTT ASSAY

Historically, among the first nonclonogenic assays were the MTT colorimetric assays, in which the radiation response is measured by a colorimetric reaction. These assays can be performed on established cell lines or tumour biopsies. The cells are plated, treated with radiation or drugs, and allowed to grow for several days. The cells are then stained in one way or another, with quantitation of cell growth based on the supposition that only viable tumour cells are stained. The density of the stain in a given sample measured by spectrophotometer correlates with the number of surviving cells.

One of the earliest colorimetric assays was the microtetrazolium (MTT) assay. The MTT assay was developed to estimate cell survival, and quantifies viable cells through their ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a soluble yellow tetrazolium salt to a blue formazan precipitate. This process requires active mitochondrial function because it utilises the activity of the mitochondrial enzyme, succinate dehydrogenase. This assay, therefore, detects living but not dead cells. The result generated depends on the degree of activation of cells and can be read using a multiwell scanning spectrophotometer with a high degree of precision. Radiation sensitivities of tumour cells determined via this assay have been reported to compare favourably with those determined by the clonogenic assay after the assay has been individually optimised for each cell line, although discrepancies have been reported using higher doses of ionising radiation (Price *et al.*, 1990). The semi-automated MTT assay has been used extensively for measuring drug cytotoxicity *in vitro* and has also been used successfully for radiosensitivity studies (Banasiak *et al.*, 1999). The MTT assay overcomes the need for cell lines to form colonies and is also less labour intensive.

Banasiak demonstrated that the MTT assay could be used as a suitable assay for estimating the radiosensitivity in a panel of bladder cancer cell lines (Banasiak *et al.*, 1999). In this study, clonogenic and MTT assays gave similar results for some cell lines. Importantly, the MTT assay was used to predict survival for a group of patients treated with radiation therapy (Banasiak *et al.*, 1999). However, because the absorbance is non-linear with high cell numbers, it is important that the controls do not reach confluence. However, this assay is relatively insensitive at low radiation doses and the need to optimise the assay conditions for every cell line limits its usefulness in determining radiosensitivity particularly in a clinical setting.

### **2.9.3 PULSED-FIELD GEL ELECTROPHORESIS**

Since it is widely known that DNA strand breaks, particularly DSBs, are the significant lesions produced by ionising radiation, substantial effort has been made into producing new methods by which they can be measured. One of these methods is pulsed field gel electrophoresis, in which DNA separation can be used, as a measure of strand breaks produced by radiation. DNA fragments carry a net negative charge and when incorporated into an agarose gel they migrate under an electrical field at a speed that is inversely related to their size. In order to detect the movement of the DNA, they are either rendered radioactive in the live cell prior to lysis or stained with a specific fluorescent dye after electrophoresis. With the careful choice of parameters, constant pulsed-field gel electrophoresis can illustrate sensitivity to modest doses of radiation (Whitaker *et al.*, 1995). However, pulsing and alternating the electric field improves the separation of the fragments. This technique overcomes the problems of irregular movement of large DNA molecules in an electric field, so that their separation can be translated into a measure of strand breakage produced by a small radiation dose. Finally, DNA of known molecular weight is used to calibrate the movement of irradiated DNA in the gels. The trouble associated with this method is that, it requires many cells to start with, and a high dose of radiation is required to see a sizeable distance of migration.

#### **2.9.4 TELOMERE LENGTH**

Telomeres cap and protect the terminal ends of chromosomes; the name telomere literally means, “end part”. Mammalian telomeres consist of long arrays of TTAGGG repeats that range in total length anywhere from 1.5 to 150 kilobases. Each time a normal cell divides, telomeric DNA is lost from the lagging strand, leading to progressive shortening (Blackburn 1992). Telomere length has been described as a molecular or generational clock, because it shortens with age in somatic tissue cells. Stem cells in renewing tissues, and cancer cells in particular, avoid this problem of aging by activating the enzyme telomerase. Telomerase is a reverse transcriptase that includes the complementary sequence to the TTAGGG repeats and so continually rebuilds the chromosome ends to offset the degradation that occurs with each division (Sitte *et al.*, 1998). Virtually all human cancer cell lines and approximately 90% of primary human cancer cells exhibit telomerase activity.

A recent study of breast cancer patient’s lymphocytes, pointed to the possibility that telomeres may play a role in cellular response to ionising radiation. The study demonstrated a seven-fold reduction in the telomere length of a radiosensitive cell in comparison with a radioresistant cell. A significant inverse correlation between telomere length and chromosomal radiosensitivity in lymphocytes from breast cancer patients seems to support this hypothesis. Therefore, it was reasoned that telomere length might be used as a marker for chromosomal radiosensitivity (McIlrath *et al.*, 2001).

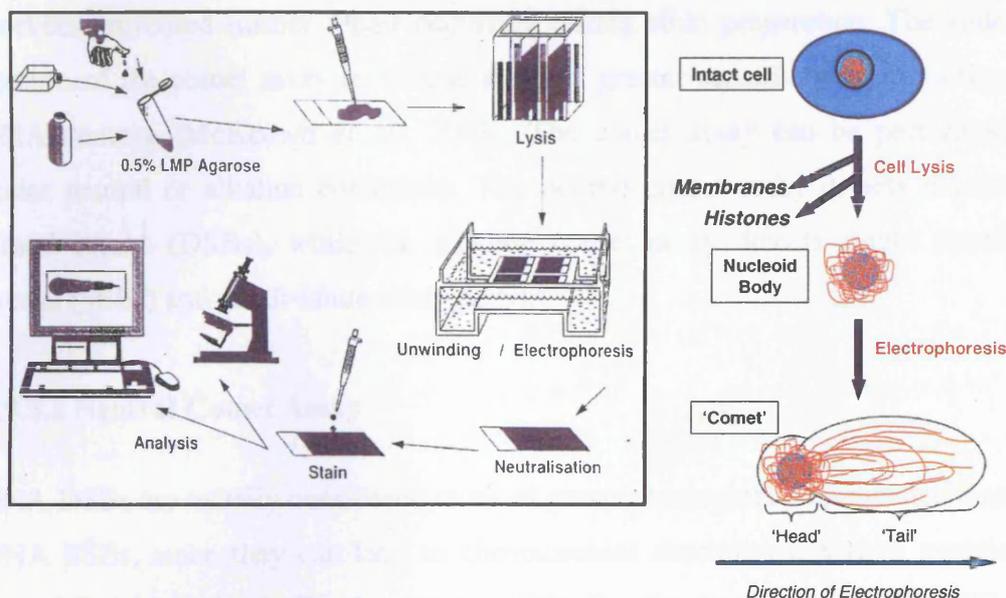
#### **2.9.5 COMET ASSAY**

Over the past 20 years, methods have been developed to measure DNA strand breaks produced in individual cells. Rydberg and Johanson (1978) described a method of single cell analysis based on lysis in alkali solution following irradiation. Their observation was that treating the cells with 2 M NaCl and anionic detergents (to remove cytoplasm, membranes and most nuclear proteins) produced ‘nucleoids’. These nucleoids are composed of supercoiled DNA arranged in loops attached to the nuclear matrix. This discovery led to the

development of the halo assay (RotiRoti and Wright 1987). The halo assay showed that strand breaks in the DNA cause relaxation of the supercoiled DNA, and allow development of a 'halo' of relaxed/broken DNA loops attached to the nuclear matrix protein of individual cells. Ostling and Johanson then presented a novel idea of measuring DNA damage in individual cells based on migration of DNA in an electric field. Cells embedded in agarose are lysed to produce nucleoids, and briefly subjected to electrophoresis. The electrophoresis acts by 'pulling' negatively charged damaged DNA away from the nucleoid towards the anode. The relaxation of the supercoiled DNA due to strand breaks, allows the migration of the damaged DNA from the nucleoid 'head' to form a 'tail' of a comet like image, viewed by fluorescence microscopy after staining with a suitable dye (Figure 2.21).

The microgel method first described by Ostling and Johanson (1984, 1987) involved five general steps. (1) Single cells were exposed to ionising radiation and then embedded in agarose on a microscopic slide. (2) Slides were placed in neutral high salt detergent solution to remove proteins, and if strand breaks were present, DNA supercoiling would relax into the space originally occupied by the whole cell. (3) DNA was exposed to low-voltage electrophoresis for a short period, which acted to pull relaxed coils and broken DNA ends towards the anode. (4) DNA was stained with fluorescent DNA binding dye. (5) DNA from individual cells was viewed using a fluorescence microscope. Olive in 1989 introduced the name 'comet assay' as an alternative name for single cell gel electrophoresis (Olive *et al.*, 1989). The comet assay has become a relatively simple, rapid and sensitive method for measuring DNA strand breaks at the level of single cells. The original method has undergone considerable changes to improve sensitivity and reliability. In particular the method has been progressively improved in terms of sensitivity and reproducibility for detecting single strand breaks; low gelling temperature agarose is routinely employed, more protein lysis was accomplished, and an alkaline lysis step was included before or during electrophoresis (Olive *et al.*, 1990). The timing and voltage used for electrophoresis are largely empirical; the important concern is that all of the DNA from an individual cell should remain within the field of view of the fluorescence microscope. Any DNA damage that ultimately gives rise to strand

breaks can be detected directly using the comet assay, with the percentage of DNA in the tail being proportional to the number of strand breaks (Olive *et al.*, 1990).



**Figure 2.21.** The comet assay (courtesy of McKelevy-Martin).

Ultimately, although the general steps for single cell gel electrophoresis remains the same, most laboratories developed their own variation in lysis, electrophoresis or staining. A more difficult problem is that it has not been possible to standardise the most important parameter commonly used to measure DNA damage in this assay. Tail length and tail moment are some of several parameters measured in this assay. However, tail moment is a popular and sensitive endpoint for measuring DNA damage. It was firstly introduced by Olive and originally it was defined as the average distance migrated by the DNA multiplied by the fraction of DNA in the comet tail (Olive *at al.*, 1990). The comet assay was first employed to measure radiation-induced DNA damage using the 'ependorf method', this involve irradiating cells in eppendorfs and then slides are prepared by mixing the cells with an equal volume of low melting point agarose. This is layered on top of a layer of normal melting point

agarose. Finally, one major approach in improving the sensitivity and reliability of the comet assay was introduced by Price *et al.*, (2000). This involves embedding the cells in agarose on slides prior to irradiation at 0°C. After irradiation the slides are then placed in lysis solution and then subjected to electrophoresis. By using this method, termed the 'slide method' Price and co-workers prevented further repair occurring during slide preparation. The slide version of the comet assay technique allowed greater sensitivity for detecting DNA damage (McKeown *et al.*, 2003). The comet assay can be performed under neutral or alkaline conditions. The neutral comet assay detects double strand breaks (DSBs), while the alkaline comet assay detects single strand breaks (SSBs) and alkali-labile sites (ALS).

#### **2.9.5.1 Neutral Comet Assay**

DNA DSBs are usually considered to be of greater biological consequence than DNA SSBs, since they can lead to chromosomal aberrations, loss of genetic material and cell death (Blocher *et al.*, 1982). For the detection of DNA DSBs by neutral comet assay, cells are embedded in agarose and lysed in neutral conditions. Ostling and Johanson developed this method in the early 1980s (Ostling *et al.*, 1984 & 1987). The original single cell electrophoresis method was performed under neutral conditions; only low radiation doses (0- 10 Gy) were employed. This neutral method was therefore understood to be sensitive to the loss of DNA supercoiling caused by single strand breaks. However, the neutral comet assay is able to detect double strand breaks, which occur 25 to 40 times less frequently than single strand breaks. For this it is essential to use much higher radiation doses and more extensive lysis. The limit of sensitivity for detecting double strand breaks is an X-ray dose of 5 Gy, which is equivalent to about 200 double strand breaks per cell. The neutral comet assay shows a linear dose-response relationship for double strand breaks over the range of 5-300 Gy (Olive *et al.*, 1995). Depending on the lysis conditions, the shape of the initial dose-response curve (tail moment as a function of dose) for double strand breaks from 0 to 2 Gy can exhibit an initial high slope. This is indicative of the loss of DNA supercoiling by single strand breaks (Olive *et al.*, 1998), and should not be confused with damage due to double strand breaks.

Using the neutral comet assay (NCA) to measure DSBs, McKeown, McKelvey-Martin & colleagues report reasonable correlations between DSB manifestation and survival at 10 Gy (SF 10) for a range of bladder tumour cell lines, but a lack of correlation with survival at 2 Gy (SF 2) (Price *et al.*, 2000). However, neutral comet assay requires high radiation doses to identify differences in DNA double strand breaks (typically up to 60 Gy); doses which have no relevance to clinical practice and which may yield false impressions of radiosensitivity at low clinically relevant doses (2 Gy).

#### **2.9.6.2 Alkaline Comet Assay**

The alkaline comet assay is a highly sensitive method for detecting radiation induced single strand breaks and alkali-labile sites, and can readily detect levels of DNA damage induced by radiation at low clinically relevant doses ranging from 1 to 4 Gy (Olive *et al.*, 1998). The alkaline version of the comet assay was discovered in an attempt to improve the sensitivity and the reproducibility of the original comet assay method. These improvements were accomplished by adding an alkaline lysis step before or during electrophoresis (Olive *et al.*, 1990). The alkali causes denaturation of the duplex DNA, and allows the individual strands to separate and migrate independently. The alkaline comet assay (ACA) is traditionally and widely conducted using the eppendorf method. However, ACA has been performed using the slide method, which increases the sensitivity for detecting DNA damage (McKelvey-Martin *et al.*, 1998). The ACA shows a linear dose response relationship for single strand breaks in a range of doses from 0 to 10 Gy (McKelvey-Martin *et al.*, 1998). In a previous study of just three bladder cancer cell lines, an inverse correlation was obtained between clonogenic survival and mean tail moment for comet formation, suggesting that ACA could potentially be used to predict the radioresponse of the single cell lines (McKelvey-Martin *et al.*, 1998).

### **2.10 RATIONALE FOR THIS STUDY**

The poor survival rate for patients with muscle invasive transitional cell carcinoma of the bladder (TCC), following radical radiotherapy or radical

cystectomy has led to attempts to improve the outcome of the treatment plan and prognosis. Various attempts have been made to optimise radiation therapy and these have focused on many areas including, the mode and timescale of delivering radiation therapy, the use of radiosensitizing agents, and predicting radiosensitivity (particularly, intrinsic radiosensitivity).

If the intrinsic radiosensitivity of invasive transitional cell carcinoma of the bladder (TCC) could be predicted in advance, it may be possible to radically improve tumour control by selecting for immediate radiation therapy those patients whose tumours are radiation sensitive; moreover, those patients whose tumours are radiation resistant, who would benefit from initial surgery, would be identified earlier reducing the risk of metastatic spread.

The present study evaluates the alkaline comet assay as a measure of bladder cancer cell radiosensitivity *in vitro* using a panel of six bladder cancer cell lines and demonstrates that the extent of DNA damage formation and repair, as determined by the assay, reflects bladder cancer cell radiosensitivity. Furthermore, it is demonstrated that a feature manifest at the level of the nucleoid body plays a significant role in determining the ACA response of bladder cancer cell lines to ionising radiation. Finally, results are presented of a preliminary study to assess the differing radiation response of epithelial cells isolated from invasive human bladder cancer biopsies as determined by the ACA.

## **2.11 MATERIALS AND METHODS**

### **2.11.1 CELL LINE AND CULTURE CONDITIONS**

Six bladder cell lines derived from high grade TCC, namely RT112, UM-UC-3 and HT1376 (purchased from the American Tissue Culture Collection) and J82, T24, RT4 (provided by Professor Kilian Mellon, University Division of Urology, Leicester General Hospital), were used in the study. All culture media were obtained from Life Technology and Sigma Chemical Company.

The cells were maintained as monolayer by subcultivation. The RT112 and HT1376 cells were cultured in Eagles minimal essential medium (EMEM) supplemented with 10% foetal calf serum (FCS), 1% non essential amino acid and 1% penicillin-streptomycin. For the J82 cell line 1% sodium pyruvate and 1% glutamax was also added. The UM-UC-3 cell line was subcultivated in EMEM supplemented with 10% foetal calf serum, 1% penicillin-streptomycin and 1% sodium pyruvate. The T24 cell line was subcultivated in McCoy's medium supplemented with 10% foetal calf serum and 1% penicillin-streptomycin. RT4 cell line was subcultivated in RPMI medium supplemented with 10% foetal calf serum, 1% penicillin-streptomycin and 1% sodium pyruvate. Cells were harvested at 70-80% confluence by washing with prewarmed 37°C phosphate buffered saline (PBS) and then exposing the cultures to trypsin-EDTA (0.1% trypsin, 0.4% EDTA in PBS) at 37°C. Culture medium was then added to the cells before centrifugation at 1500 rpm for 5 minutes. The pellet was resuspended and incubated in culture medium (37°C, 30 minute) at  $1 \times 10^5$  cells/ml (for comet assay, clonogenic assay and MTT assay) before use. All cells were tested for viability following this procedure using trypan blue exclusion and was consistently found to be >99%.

With the cell cultures established exponential growth was observed and the doubling time for each cell line was calculated, by allowing the cells to grow over a period of five days (144 hours). The doubling time was calculated using the following equations:

$$\text{Numbers of doublings} = \frac{\left( \frac{\text{Number of cells after certain incubation}}{\text{Starting number of cells}} \right)}{\text{Log } 2}$$

$$\text{Doubling time} = \frac{\text{Time of growth}}{\text{Numbers of doublings}}$$

The doubling time for J82 was 17.4 hours, for T24 21 hours, for RT112 24 hours, for UM-UC-3 18.2 hours, for HT1376 30.7 hours and for RT4 36 hours.

### 2.11.2 CLONOGENIC ASSAY

Cells from exponential growing culture are prepared into suspension by the use of trypsin as described in section 2.11.1. The number of cells per unit volume of this suspension was counted using a hemocytometer and seeded in appropriate numbers in 60 x 15 mm petri dishes with 10 ml of corresponding culture medium. The number of cells seeded per dish was adjusted based on the plating efficiency of each cell line, so that a countable number of colonies would result. The plating efficiency (PE) for each cell line was calculated and the results are as follows: J82 12%, T24 56.25%, RT112 47%, UM-UC-3 66%, HT1376 15% and for RT4 25%. For J82 and HT1376, unirradiated control cultures were seeded at 500 cells/dish, whilst for X-ray doses of 2, 4 and 6 Gy the numbers of cells plated were increased to 600, 800 and 1000 cells/dish, respectively. For RT112, unirradiated control cultures were seeded at 200 cells/dish, whilst for X-ray doses of 2, 4 and 6 Gy the numbers of cells plated were increased to 400, 500 and 800 cells/dish, respectively. For T24 and UM-UC-3, unirradiated control cultures were seeded at 100 cells/dish, whilst for X-ray doses of 2, 4 and 6 Gy the numbers of cells plated were increased to 300, 500 and 800 cells/dish, respectively. Finally, for RT4, unirradiated control cultures were seeded at 300 cells/dish, whilst for the X-ray dose of 2, 4 and 6 Gy the numbers of cells plated were increased to 500, 800 and 1000 cells/dish, respectively. Following 4 hours

incubation at 37°C, cells were irradiated in the dishes with 2, 4, and 6 Gy; unirradiated cultures were processed in parallel. Dishes were subsequently incubated at 37°C in humidified 95% air, 5% CO<sub>2</sub> for 2-3 weeks. Cells were then fixed with 3:1 ethanol:acetic acid and stained with crystal violet. Colonies were counted for the control and dose groups, and each experiment was performed on at least two separate occasions in triplicate. Plating efficiency (PE) was determined as the number of colonies scored as a percentage of the number of viable unirradiated control cells plated. The surviving fraction (SF) was defined as the ratio of colonies formed to cells plated (with correction for plating efficiency (PE)), and calculated using the formula:  $SF = \text{colonies counted} / (\text{cells seeded} \times (\text{PE} / 100))$ .

### **2.11.3 MICROTETRAZOLIUM ASSAY (MTT)**

Cells from exponential growing culture were harvested into a single cell suspension by the use of trypsin as described in section 2.11.1. The number of cells per unit volume of this suspension was counted and an appropriate number suspended in a falcon tube with 5 ml of corresponding culture medium. Cells were then irradiated at 2, 4 and 6 Gy; unirradiated cultures were processed in parallel. Cells were then dispersed (200 µl/well) in a 96-well microtiter plate; eight replicate wells were used for each determination. Preliminary experiments that were conducted to determine the optimal plating number. For UM-UC-3 and J82 the optimal plating number was  $3 \times 10^4$  cells/well, for RT112  $4 \times 10^4$  cells/well, for T24  $3.5 \times 10^4$  cells/well, for HT1376  $6 \times 10^3$  cells/well and finally for RT4 the optimal plating number was  $5 \times 10^4$  cells/well. Plates were maintained at 37°C in a humidified 95% air, 5% CO<sub>2</sub> for 24 hours. To perform the MTT assay, 12 mM of MTT solution was prepared by dissolving 5 mg of the MTT in 1 ml of sterile phosphate buffered saline; and the resultant solution was filtered through a 0.22µm filter to remove the formazan crystals. The culture medium was then removed from each well carefully to ensure that the monolayer of cells is not disturbed. MTT solution is then added, 20 µl to each well, and the plates are incubated at 37°C for one hour. Following this incubation, cells were inspected using low power microscopy to confirm reduction of the tetrazolium and to assess confluency of the monolayer. The

remaining MTT solution was then removed and 100  $\mu$ l of DMSO was added to each well to dissolve the formazan crystals. Plates were shaken for 5 minutes on a plate shaker to ensure adequate solubilisation. Absorbance readings on each well were performed at 550 nm (single wavelength) using a Titertek Multiskan MCC plate reader. Control wells (containing no cells) for determination of background absorbance were processed in parallel. The viable fraction of the treated cells (as a % of the untreated cells) was then calculated, using the following equation:

$$\text{Viable fraction} = \frac{\text{Absorbance of the treated cells}}{\text{Absorbance of the untreated cells}}$$

This process is repeated for each individual dose, and a response curve is plotted as the logarithm of the viable fraction against the radiation dose. All experiments were performed on at least two separate occasions in triplicate.

#### 2.11.4 TELOMERE LENGTH

To investigate whether telomere length plays a role in cellular response to ionising radiation, telomere lengths in the six-bladder cancer cell lines (J82, T24, RT112, UM-UC-3, HT1376 and RT4) were determined. Cells were harvested and the count was adjusted to  $1 \times 10^6$  cells/ml of the corresponding culture medium. 1 ml aliquots of cells were centrifuged at 1500 rpm for 5 minutes and the cell pellets then suspended in low melting point agarose to produce 'plugs'. The prepared plugs were then treated with proteinase K for 24 hours at 50°C. After washing with TE buffer (4.5 mM tris, 0.1 mM EDTA), the DNA in the plugs was digested with 2  $\mu$ l of *Hinf* I (Boehring Mannheim) in 1 ml of the supplied react 1 buffer (50 mM tris pH 8 plus 10 mM MgCl<sub>2</sub>) and at 37°C for 16 hours. *Hinf* I, is a restriction enzyme that cuts the DNA, resulting in fragments of ~ 250 bp, but does not cut the repeated sequence of the telomere (TTAGGG). Plugs were then washed with TE buffer (4.5 mM Tris, 0.1 mM EDTA), this was then heated at 65°C (to melt the low melting point agarose) and placed into the well of a 1% agarose gel and analysed by electrophoresis.

The gel was also loaded with both low molecular weight (1 Kb) and a high molecular weight markers (8 – 40 Kb) and then placed in a horizontal electrophoresis tank, filled with 1 x TBE buffer (4.5 mM Tris, 4.5 mM boric acid, 0.1 mM EDTA, pH 8). The electrophoresis is then carried out for 16 hours at a current of 50 mA. Gels were placed in a depurinating solution (0.14 M of concentrated HCl) for 30 minutes and then placed in denaturation buffer (1.5 M NaCl, 0.5 M NaOH dissolved in two litres of water) for 30 minutes. Finally, the gel was placed in a neutralization buffer (1.5 M NaCl, 0.5 M Tris in two litres of water, pH 7.5) for 30 minutes.

Gels were then blotted to Hybond-*N*-membranes and hybridised with digoxin (DIG high prime DNA labelling and detection starter kit II; Roche) for 16 hours at 42°C. After the incubation was complete, the membrane was washed twice using a washing buffer provided by the kit. Finally, the membrane is exposed to a photographic film and this developed it. To analyse the telomere length in these cells, the film was scanned in a densitometer (GS 670, Biorad) and analysed using the Sigma plot (Jandel) and EXCEL (Microsoft) software. Average telomere fragment length per lane was calculated from the weighted mean of the optical density.

### **2.11.5 ALKALINE COMET ASSAY**

The alkaline version of the comet assay is a highly sensitive method for detecting mainly radiation-induced single strand breaks and alkali-labile sites, and can readily detect levels of DNA damage induced by low radiation doses. A modified version of the comet assay has been performed using the 'slide method', which increases the sensitivity for detecting DNA damage by minimising the opportunity of repair for the induced damage prior to cell lysis (Price *et al.*, 2000).

#### **2.11.5.1 Cell and Slide Preparation**

ACA was performed on the six-bladder cancer cell lines (J82, T24, RT112, UM-UC-3, HT1376 and RT4). The method was carried out as follows: Dakin

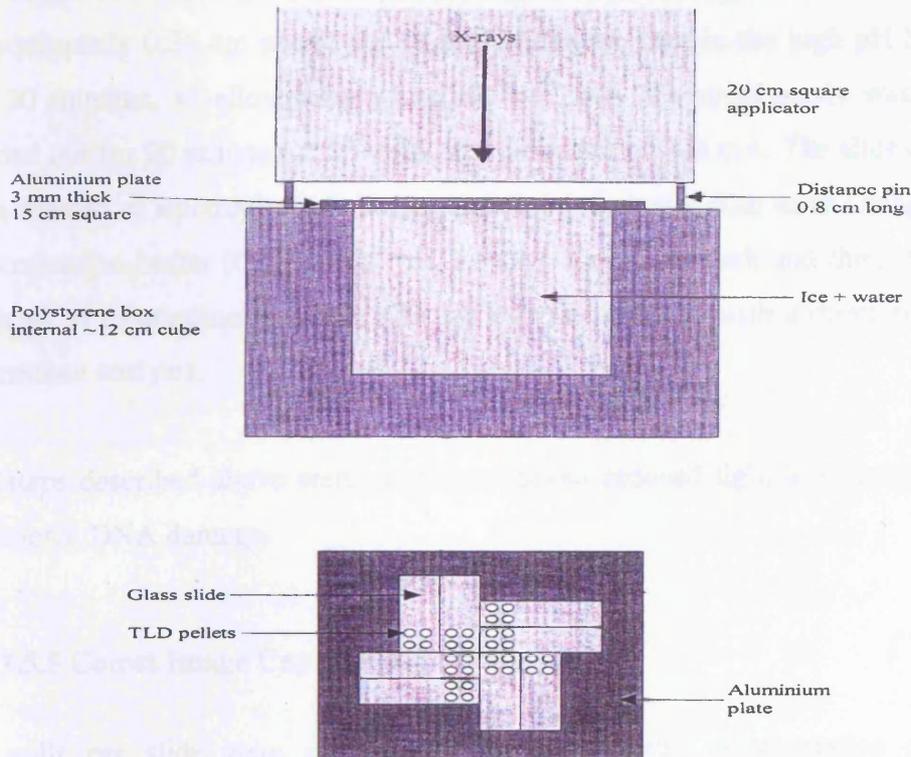
fully frosted slides were each covered with 100  $\mu$ l of 0.6% normal melting point agarose (dissolved in calcium and magnesium free phosphate buffered saline at 45°C), and the agarose allowed to solidify under a cover slip on ice. Cells were harvested, the viability was assessed using trypan blue, and the count adjusted to  $1 \times 10^5$  cells per one ml of culture medium. 1 ml aliquots of the cells were then centrifuged at 1500 rpm for 5 minutes; the pellets are then resuspended in 80  $\mu$ l of 0.6% low melting point agarose (dissolved in calcium and magnesium free phosphate buffered saline at 37°C). When the cover slip was removed from the solidified normal melting point agarose, 80  $\mu$ l of the low melting point agarose containing the cells were layered onto the normal melting agarose and allowed to solidify under a fresh cover slip, on ice. For the 'repair slides', equal volumes (40  $\mu$ l) of the cell suspension and 1.2% of low melting point agarose (dissolved in RPMI medium containing 20% foetal calf serum held at 37°C) were mixed, layered onto the normal melting point agarose and allowed to solidify under a fresh cover slip on ice.

#### **2.11.5.2 X-ray Irradiation**

The slides containing cells were irradiated on ice, using a Pantak DXT 300 X-ray machine at the Radiotherapy Unit, Leicester Royal Infirmary, operated at 300 kVp with a filter of 0.8 mm Sn + 0.25 mm Cu + 1.50 mm Al, giving a beam with an HVL of 3.5 mm Cu. Up to 12 slides can be irradiated at once, these are placed flat, in a prescribed fashion, on an aluminium sheet in a good thermal contact with the ice, 0.8 cm from the end of a 50 cm FSD, 20 cm square, normal therapy applicator. The dose rate (0.98 Gy/ minute) and uniformity of field ( $\pm 10\%$ ) have been determined for this configuration using thermo luminescent dosimeters (TLDs) (Figure 2.21 see below). Duplicate slides were irradiated with a dose of 0, 2, 4, and 6 Gy to generate an immediate damage dose response. Additional duplicate slides irradiated with 2.5 Gy were used to monitor repair at 15 and 30 minutes.

The dose rate was calculated using thermo luminescent dosimeters. Harshaw-sintered pellets made from natural lithium fluoride, measuring 4 mm in diameter by 1 mm in height were used. Each pellet was numbered and four were placed

on each glass slide corresponding to the area covered by the gel. The slides were then irradiated on ice and all pellets were analysed using a TLD reader. The light output from each pellet was measured and the results of each group of four pellets were then averaged. The light intensity measures were then converted to dose, via a previously determined calibration curve.



**Figure 2.22.** Dose measurement using thermo luminescent dosimeters.

### 2.11.5.3 Lysis

For studies of immediate damage, after exposure of the slides to irradiation, they were immediately placed in a cold (4°C) lysis buffer (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris, pH 10 and 1% triton X-100 was added fresh,) for a minimum of one hour. For repair studies, after irradiation the 'repair slides' were incubated in RPMI containing 20% foetal calf serum at 37°C for 15 and 30 minutes, then placed in cold lysis buffer for a minimum of one hour. The unirradiated control slides were placed in the lysis buffer immediately after slide preparation.

#### **2.11.5.4 Electrophoresis and Neutralisation**

After lysis, the slides were drained of lysis buffer and placed in a horizontal gel electrophoresis tank, surrounded by ice and filled with fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na<sub>2</sub>EDTA, pH 13) to a level of approximately 0.25 cm above the slides. Slides are kept in the high pH buffer for 20 minutes, to allow unwinding of the DNA. Electrophoresis was then carried out for 20 minutes at 25 volts and a current of 300 mA. The slides were then drained of electrophoresis buffer and flooded slowly with three changes of neutralisation buffer (0.4 M Tris, pH 7.5) for 5 minutes each and then stained with 40 µl of ethidium bromide (20 µg/ ml) and covered with a cover slip for immediate analysis.

All steps described above were conducted under reduced light level to prevent additional DNA damage.

#### **2.11.5.5 Comet Image Capture and Analysis**

50 cells per slide were analysed to obtain a result representative of the population of cells (Price *et al.*, 2000). The analysis was made using Komet software (Version 4, Kinetic Imaging Ltd.) and an epi-fluorescence microscope (Olympus BH2), equipped with an excitation filter 515 – 535 nm, 100 W mercury lamp and at a magnification X 200. Several parameters of each cell were calculated by the software package and Olive tail moment was selected as the parameter that best reflected DNA damage. The Olive tail moment is defined as the fraction of tail DNA multiplied by the distance between the profile centres of gravity for DNA in the head and DNA in the tail (Olive *et al.*, 1990).

#### **2.11.6 HUMAN BLADDER SAMPLES**

Samples of human bladder cancer tissue were acquired from patients diagnosed with invasive transitional cell carcinoma of the bladder; muscle invasion was

confirmed by subsequent histological analysis. In this study four samples were analysed from patients, with full approval of Leicester General Hospital and the authorised Ethical Committee. Samples were procured by transurethral resection (diathermy loop) and were transported to the laboratory in chilled saline (5 - 10 ml) within hours of excision. Only exophytic areas of tumour were sampled, avoiding the bladder wall. Upon receiving the sample, the tumour material was finely chopped into pieces and placed in 20 ml of collagenase (1 mg/ml) in a shaking water bath at 37°C for 20 minutes. The sample is then filtered through a nylon mesh (120 gauge filter) and the filtrate then washed three times with PBS. The resulting single cells were counted and viability assessed by trypan blue exclusion; typically this was >80%, samples with lower viability were discarded.

Due to the likelihood of infiltrating cells contaminating the biopsy material, cells expressing human epithelial antigen (HEA) were enriched via biomagnetic techniques using HEA microbeads (Miltenyi Biotech) according to the supplier's instructions. HEA is broadly expressed on cells of epithelial origin including the associated tumour cells (Moldenhauer *et al.*, 1987). For separation, the cells are magnetically labelled with HEA and separated on a column that is placed in a magnetic field. The labelling is accomplished by incubating the cells with the HEA microbeads (100 µl/ 10<sup>7</sup> cells/ml) plus 300 µl of mini MACs buffer (PBS pH 7.2, supplemented with 0.5% bovine serum albumin and 2 mM EDTA) plus 100 µl of blocking reagent, for 30 minutes, on ice. A series of three washes is then applied to the column using (500 µl) of mini MACs buffer. Cells were then placed onto the column, the magnetically labelled HEA<sup>+</sup> cells are retained whilst the unlabelled HEA<sup>-</sup> cells flow through. After removal from the magnetic field, the HEA<sup>+</sup> cells are eluted as the positively selected fraction.

The viability of the positively selected HEA<sup>+</sup> cells excised from the tumour biopsy were assessed using trypan blue; typically this was >75%, and the count adjusted to 1 x 10<sup>5</sup> cells per one ml of PBS. Slides were prepared as described in Section 2.11.5.1, irradiated as described in section 2.11.5.2 and ACA analysis was undertaken as described in Sections 2.11.5.3 – 2.11.5.5. To account for

experiment-to-experiment variation, additional cell slides were prepared using an established lymphoblastoid cell-line Raji (provided by Dr. McKelvey-Martin, University of Ulster) and these were irradiated and analysed in parallel for data normalisation. The Raji cell-line was maintained in suspension by subcultivation in RPMI medium supplemented with 10 % foetal calf serum (FCS).

#### **2.11.7 PREPARATION OF NUCLEI**

Cell nuclei were prepared from six-bladder cancer cell lines studied. The nuclei were prepared from  $1 \times 10^7$  cells using the initial cell lysis stage of a Qiagen DNA preparative kit (Blood and Cell Culture DNA Midi Kit; Qiagen), according to the manufacturers instructions. The cell lysis stage was accomplished by incubating the cells,  $1 \times 10^7$  cells in 2 ml of corresponding culture medium, with 2 ml of C1 buffer and 6 ml of distilled water, on ice for 10 minutes. The resulting solution is then centrifuged at 10,000 rpm for 15 minutes at 4°C. The pellet is then resuspended in 1 ml of C1 buffer plus 3 ml of distilled water and centrifuged at 10000 rpm for 15 minutes at 4°C. The prepared nuclei were then washed three times with calcium and magnesium free PBS. Slides were prepared as described in Section 2.11.5.1, irradiated as described in Section 2.11.5.2 and ACA analysis was undertaken as described in Sections 2.11.5.3 – 2.11.5.5.

#### **2.11.8 PREPARATION OF NUCLEOID BODIES**

Nucleoid bodies were prepared from the six-bladder cancer cell lines studied. The nucleoid bodies were prepared by one-hour lysis of the unirradiated cell slides. Slides were prepared as described in Section 2.11.5.1, lysed for one hour as described in Section 2.11.5.3. Following lysis the slides were washed three times with calcium and magnesium free PBS. Slides were then irradiated as described in Section 2.11.5.2 and ACA analysis was undertaken as described in Sections 2.11.5.3 – 2.11.5.5.

### **2.11.9 STATISTICS**

Data was analysed using one-way analysis of variance (ANOVA) using Minitab software (Version 13, Cleocom Ltd).

## 2.12 RESULTS

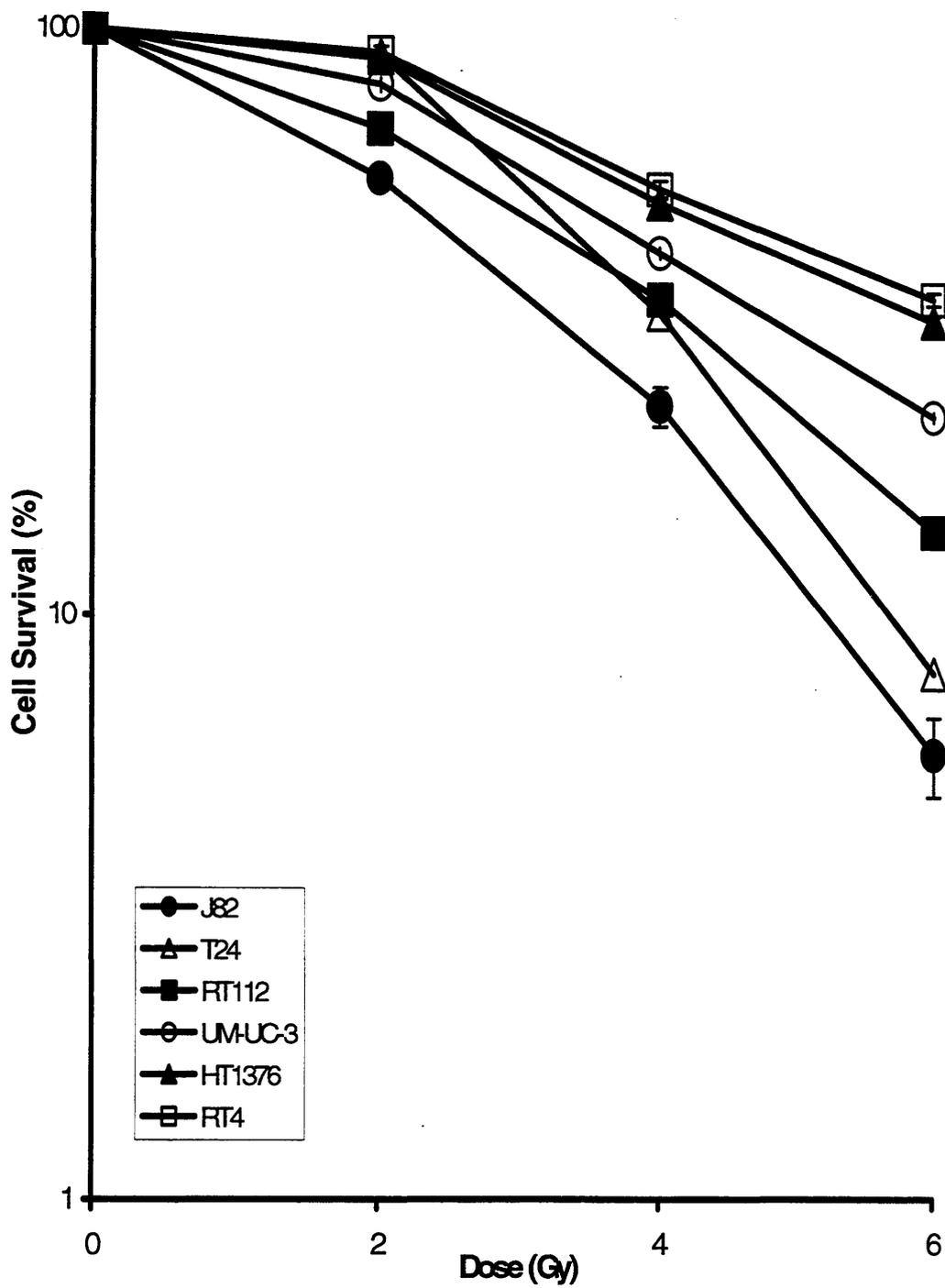
Radiation survival curves have been determined for the six bladder cancer cell lines investigated over a dose range of 0 - 6 Gy, using the clonogenic assay. Results are shown in Figure 2.23. These cell lines comprise a range of radiogenic sensitivities, with J82 being the most sensitive to radiation-induced cell killing, and HT1376 and RT4 being the most resistant to radiation induced-cell killing. Notably, one cell line, T24, demonstrates a radioresistant response at low dose (2 Gy) but a radiosensitive response at higher doses. The derived surviving fractions at 2 Gy (SF2) for each cell line are given in Table 2.1.

Cell line	SF2
J82	0.56 ± 0.062
RT112	0.68 ± 0.04
UM-UC-3	0.81 ± 0.051
HT1376	0.90 ± 0.052
T24	0.91 ± 0.054
RT4	0.92 ± 0.08

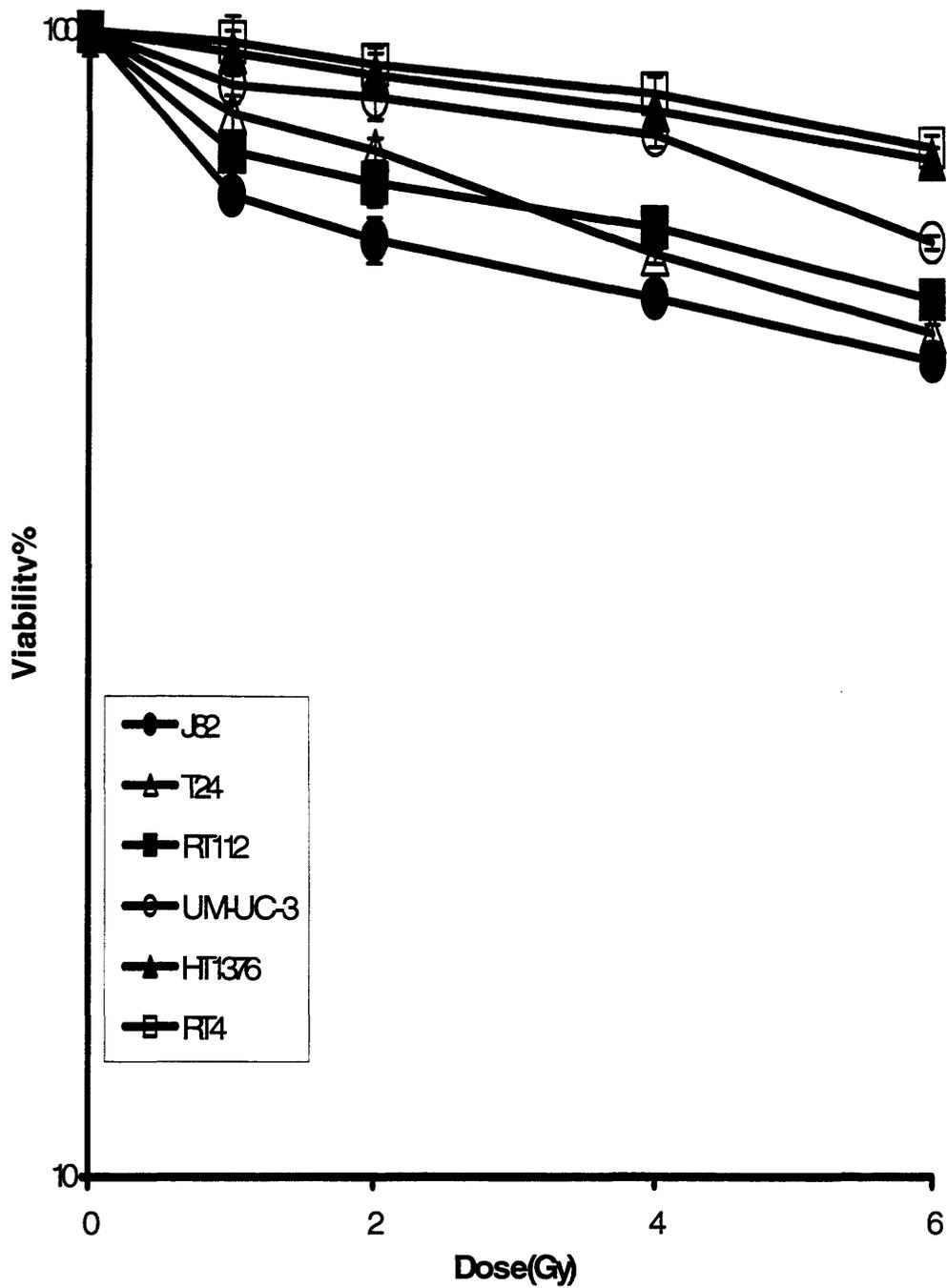
**Table 2.1.** Fraction of cells surviving exposure to 2 Gy of X-ray irradiation.

The radiation-induced loss of cell viability has been measured for the six-bladder cancer cell lines, over a dose range of 0 - 6 Gy using the MTT assay. The results are shown in Figure 2.24. As for the clonogenic response, cell lines exhibited a range of radiogenic responses, with J82 being the cell line most 'sensitive' to radiation-induced loss of cell viability, and HT1376 and RT4 being the most 'resistant'. Furthermore, the T24 cell line shows a relatively moderate/intermediate radioresponse at low irradiation dose (1 and 2 Gy) but exhibited a relatively greater loss of cell viability at higher radiation doses (4 and 6 Gy).

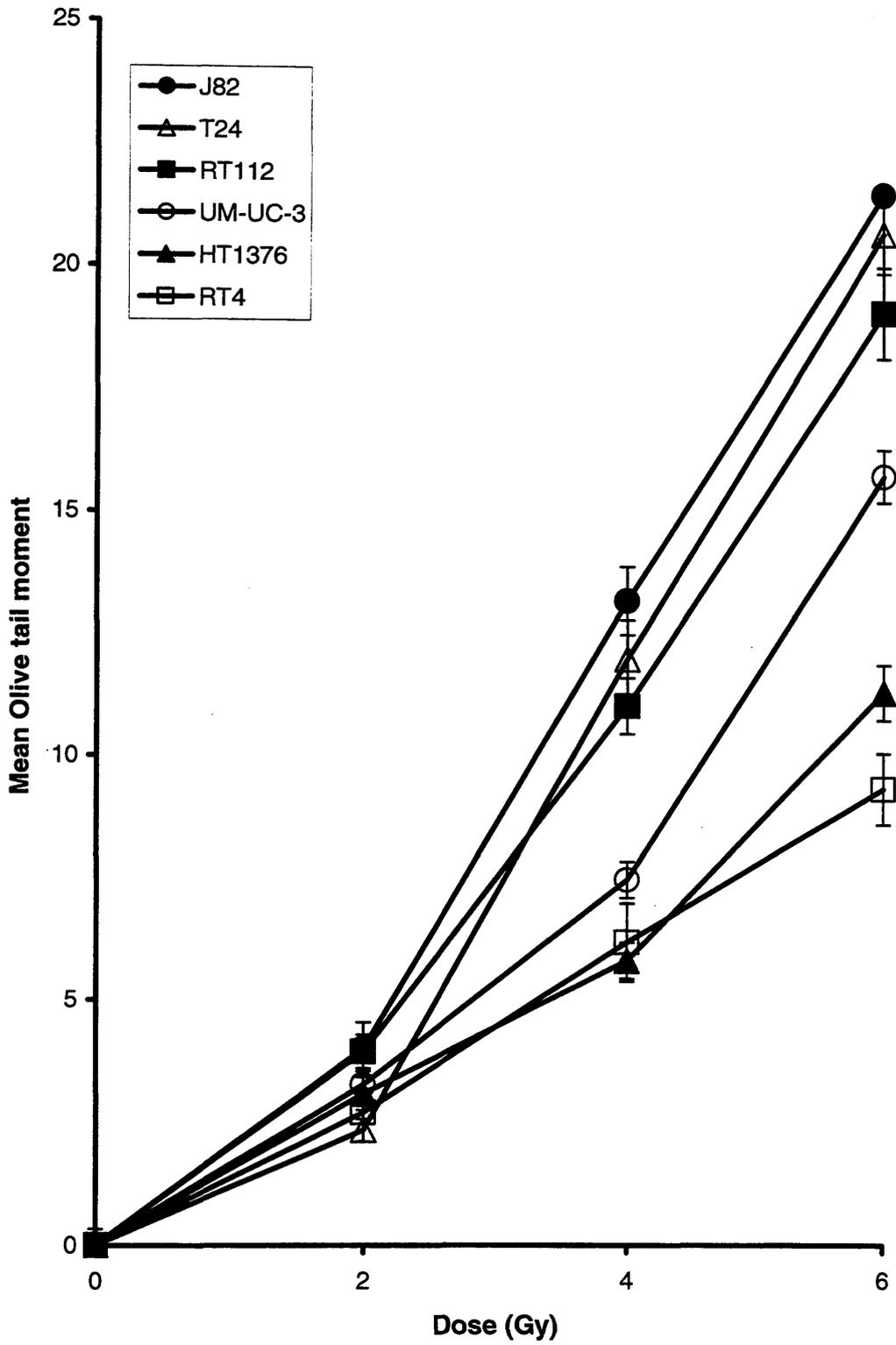
The initial measures of comet formation (as assessed by mean Olive tail moment) for the six-bladder cancer cell lines, over a dose range of 0- 6 Gy were assessed using ACA. The results are shown in Figure 2.25. The mean Olive



**Figure 2.23.** The radiation survival curve responses for the six bladder cancer cell lines investigated, over a dose range of 0- 6 Gy, as determined by the clonogenic assay. Survival was determined as the number of colonies formed following X-ray exposure. Each data point is the mean of three independent experiments  $\pm$  SE.



**Figure 2.24.** The radiation response for the six-bladder cancer cell lines investigated, over a dose range of 0- 6 Gy, as determined by the MTT assay. Viability was determined, as the number of viable cells remains following X-ray exposure. Each data point is the mean of three independent experiments  $\pm$  SE.

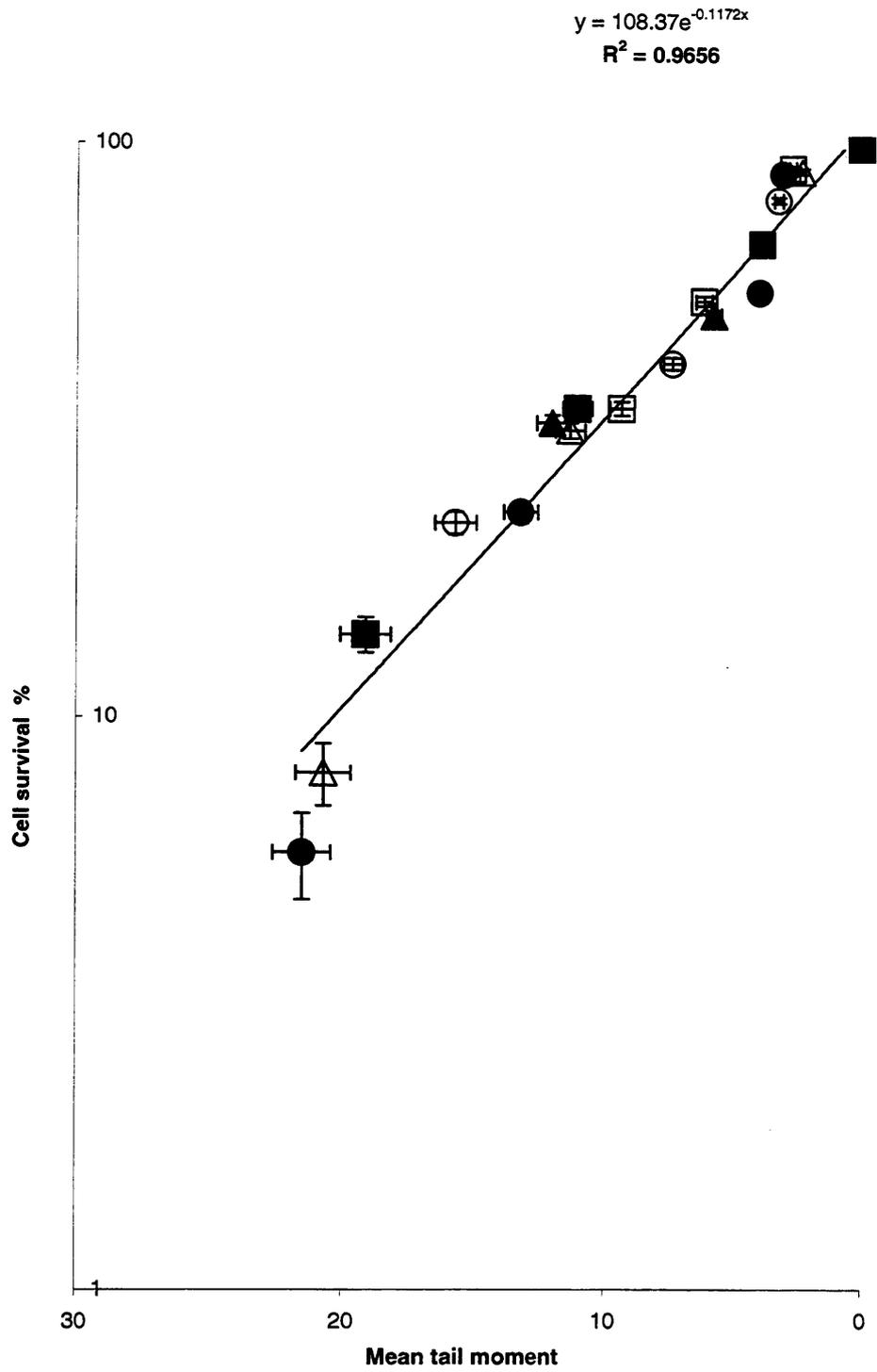


**Figure 2.25.** The level of initial comet formation for the six-bladder cancer cell lines, over a dose range of 0- 6 Gy, as determined by the alkaline comet assay. Induced DNA damage was measured as Olive tail moment, each data point is the mean of three independent experiments  $\pm$  SE.

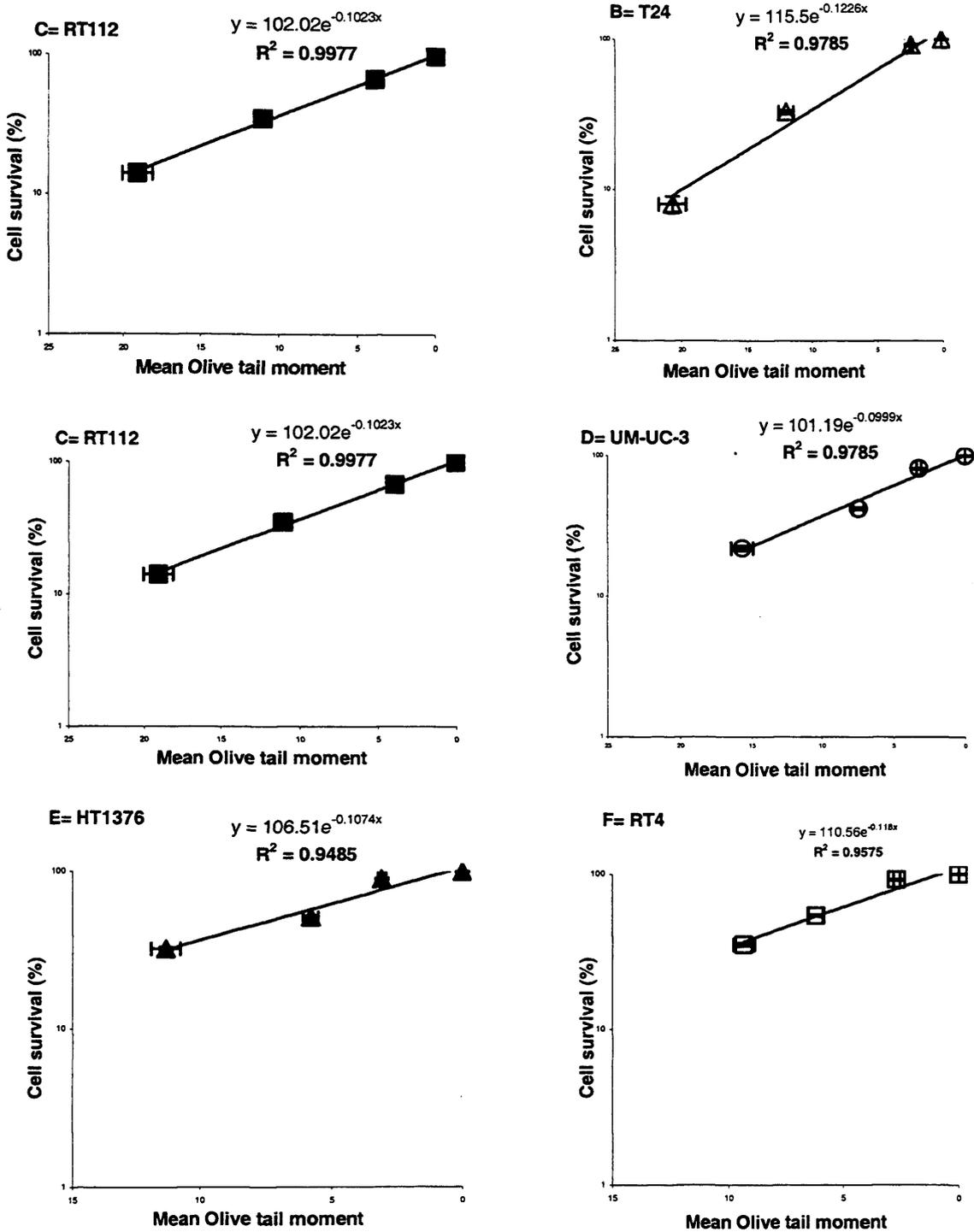
tail moment for each cell line was measured and a clear dose response curves were obtained, with the most radiosensitive cell line J82 displaying the highest values of comet formation, and the most radioresistant cell lines RT4 and HT1376 exhibiting the lowest. At 4 Gy and 6 Gy the rank order for initial comet formation matches the rank order of cell killing for all six cell lines J82> T24> RT112> UM-UC-3> HT1376> RT4. Furthermore, the T24 cell line exhibits a relatively low measure of mean Olive tail moment at low dose (2Gy) but relatively greater measures at higher doses (4 & 6 Gy). Using the alkaline comet assay as described, there was a significant difference after 2 Gy X-irradiation between the Olive tail moment measures for the more radiosensitive cell lines (J82 & RT112) and the radioresistant cell lines (RT4 & HT1376) ( $p < 0.05$ , Tukey One-way ANOVA).

The relationship between the measures of mean Olive tail moment for initial radiation-induced comet formation, as detected by ACA, and the measures of clonogenic cell survival, for all six-bladder cancer cell lines at 0, 2, 4 and 6 Gy is illustrated in Figure 2.26. A high degree of correlation exists between the two measures for all six-bladder cancer cell lines ( $R^2 = 0.9656$ ) indicating that the measure of damage (Olive tail moment) accurately reflects clonogenic survival for these six cell lines over the dose range studied. Similarly, for each individual cell line, the relationship between the measure of initial comet formation and the measure of clonogenic survival at 0, 2, 4 and 6 Gy is illustrated in Figure 2.27. A high degree of correlation exists between comet formation and survival for each individual cell line. The correlation coefficients ( $R^2$ ) for each cell line are given in Table 2.2. These vary between 0.9977-0.9575, with the values of the individual slopes varying by no more than ~15% of the collated value. Again, the high degree of correlation for each cell line reveals that one measure (initial comet formation) accurately reflects the other (survival).

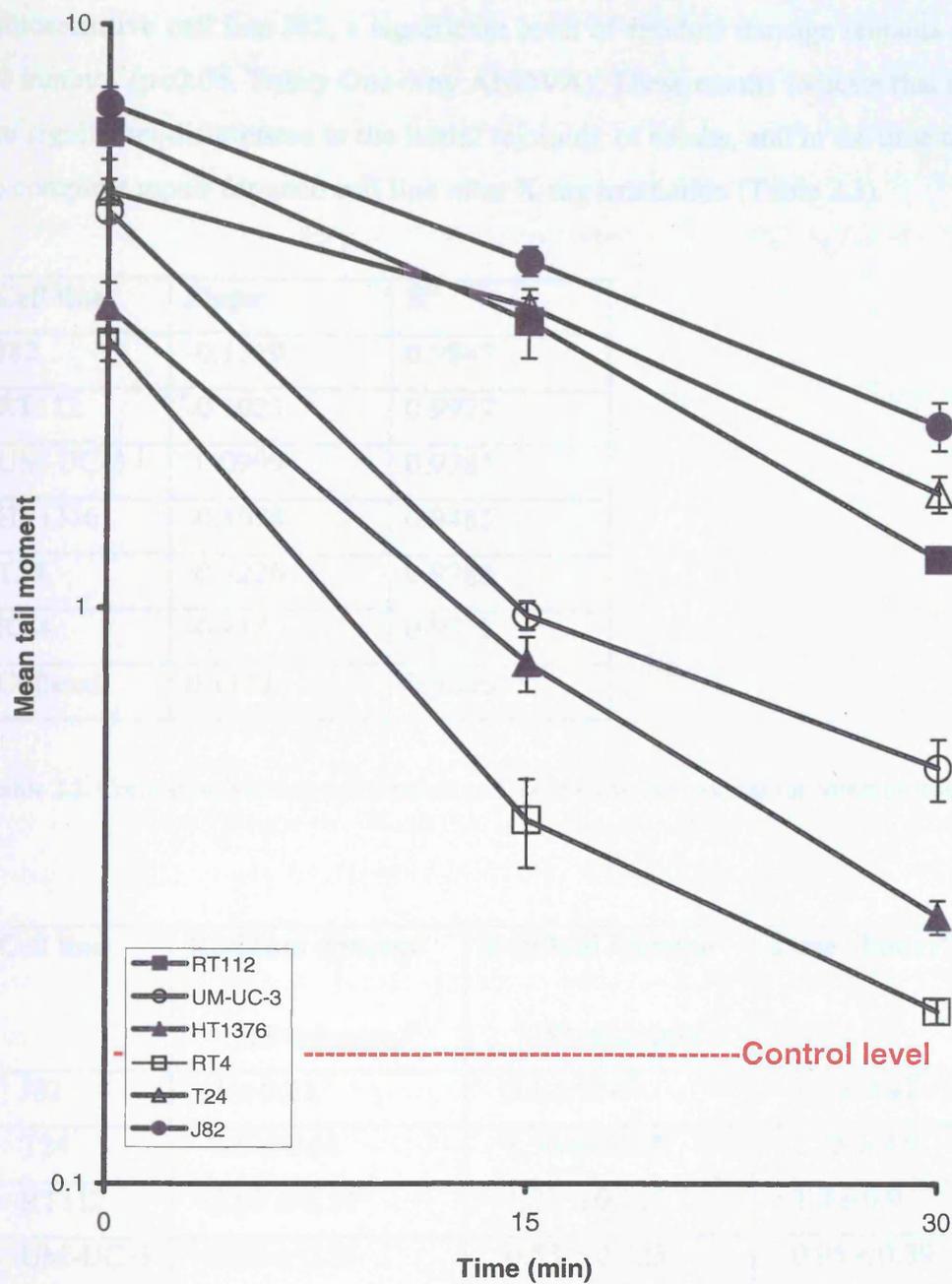
The repair of DNA radiation-induced single strand breaks and alkali labile site damage was determined using ACA, by incubating the cell-slides at 37°C for either 15 or 30 minutes after their being exposed to an X-ray dose of 2.5 Gy. The results are shown in Figure 2.28. For the most radioresistant cell lines, RT4



**Figure 2.26.** The relationship between the measures of mean Olive tail moment for initial comet formation, as detected by the alkaline comet assay, and the measures of clonogenic cell survival, for all six bladder cell lines at 0, 2, 4 and 6 Gy.



**Figure 2.27.** The relationship between the measures of mean Olive tail moment for initial comet formation, as detected by the alkaline comet assay, and the measures of clonogenic cell survival, for each cell lines at 0, 2, 4 and 6 Gy.



**Figure 2.28.** The extent of radiation-induced single strand break plus alkali labile site damage repair as determined by the alkaline comet assay, after irradiation with 2.5 Gy of X-ray. Each data point is the mean of three independent experiments  $\pm$  SE.

and HT1376, the majority of repair occurs by 15 minutes, while for the most radiosensitive cell line J82, a significant level of residual damage remains after 30 minutes ( $p < 0.05$ , Tukey One-way ANOVA). These results indicate that there are significant differences in the initial rejoining of breaks, and in the time taken to complete repair for each cell line after X-ray irradiation (Table 2.3).

Cell line	Slope	R <sup>2</sup>
J82	-0.1319	0.9847
RT112	-0.1023	0.9977
UM-UC-3	-0.0999	0.9785
HT1376	-0.1074	0.9485
T24	-0.1226	0.9785
RT4	-0.118	0.9575
Collated	0.1172	0.9656

**Table 2.2.** Correlation between cell survival and mean Olive tail moment for comet formation.

Cell line	Residual damage	Residual damage	Time (hour) <sup>b</sup>
	(15 minutes) <sup>a</sup>	(30 minutes) <sup>a</sup>	
J82	4 ± 0.21	2.1 ± 0.43	2.0 ± 0.47
T24	3.5 ± 0.11	1.54 ± 0.145	1.75 ± 0.5
RT112	2.69 ± 0.11	1.23 ± 0.116	1.0 ± 0.9
UM-UC-3	0.94 ± 0.56	0.53 ± 0.223	0.95 ± 0.39
HT1376	0.63 ± 0.277	0.33 ± 0.07	0.68 ± 0.71
RT4	0.338 ± 0.259	0.231 ± 0.65	0.58 ± 0.23

**Table 2.3.** (a) The residual DNA damage after 2.5 Gy irradiation, these are calculated by subtracting the control Olive tail moment from that remaining at 15 and 30 minutes. (b) The time (hours) taken to restore DNA damage to pre-irradiated control level. Where necessary this was estimated by extrapolation of the curve.

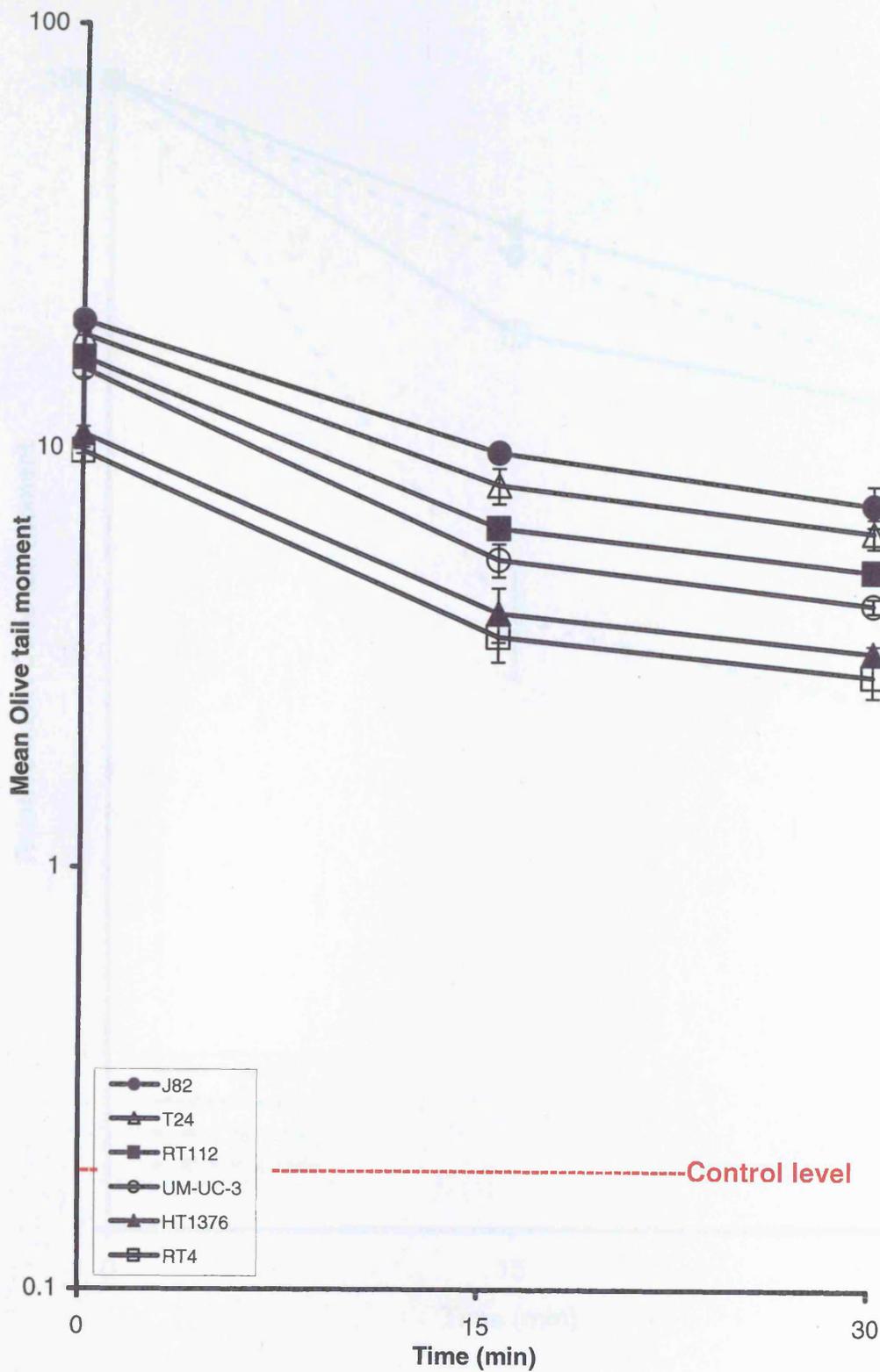
The extent of repair of radiation-induced single strand breaks and alkali labile sites damage was examined, after exposure to 6 Gy of X-ray. The results are

shown in Figure 2.29. The extent of repair after 6 Gy maintains the same rank order as noted after the 2.5 Gy, with the most radioresistant cell lines RT4 and HT1376 displaying the highest level of repair and the most radiosensitive cell lines J82 and RT112 exhibiting the lowest level of repair. It can be clearly seen that irradiation of the cells with a higher dose of radiation 6 Gy, induces a greater level of immediate damage, which lead to incomplete repair and a significant level of residual damage remaining after 30 minutes.

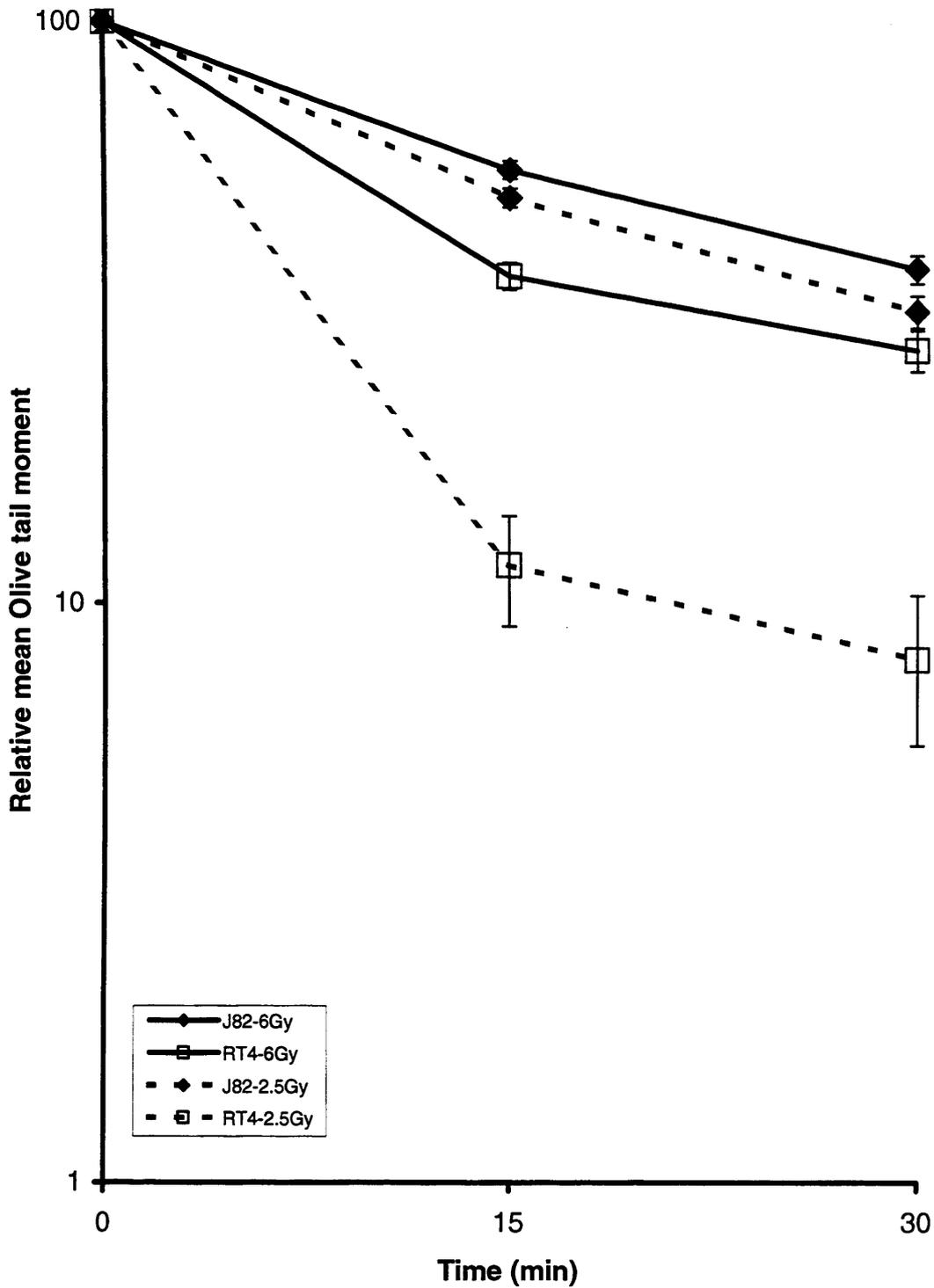
The repair profiles of the most radiosensitive (J82) and the most radioresistant (RT4) cell lines, after 2.5 and 6 Gy X-ray irradiation, are shown in Figure 2.30. The relative repair profile of the most radioresistant cell line RT4 at 6 Gy was similar in extent to the repair of the radiosensitive cell line J82 after 2.5 Gy X-ray irradiation.

The relationship between the measures of residual radiation-induced damage at 0 minutes and at the two repair incubation time points of 15 and 30 minutes after 2.5 Gy irradiation, as determined by the alkaline comet assay, and the determined SF2 values for the five cell lines (J82, RT112, UM-UC-3, HT1376 & RT4) are shown in Figures 2.31, 2.32 and 2.33. The level of residual damage after 0, 15 and 30 minutes repair correlates with the surviving fraction SF2 for these five cell lines with  $R^2$  values of 0.9659, 0.9776 and 0.9712 for 0, 15 and 30 minutes repair, respectively. However, the addition of data for the T24 cell line significantly worsens the correlation, yielding  $R^2$  values of 0.7897, 0.4461 and 0.486 for 0, 15 and 30 minutes repair, respectively; this data is illustrated in Figures 2.34, 2.35 and 2.36.

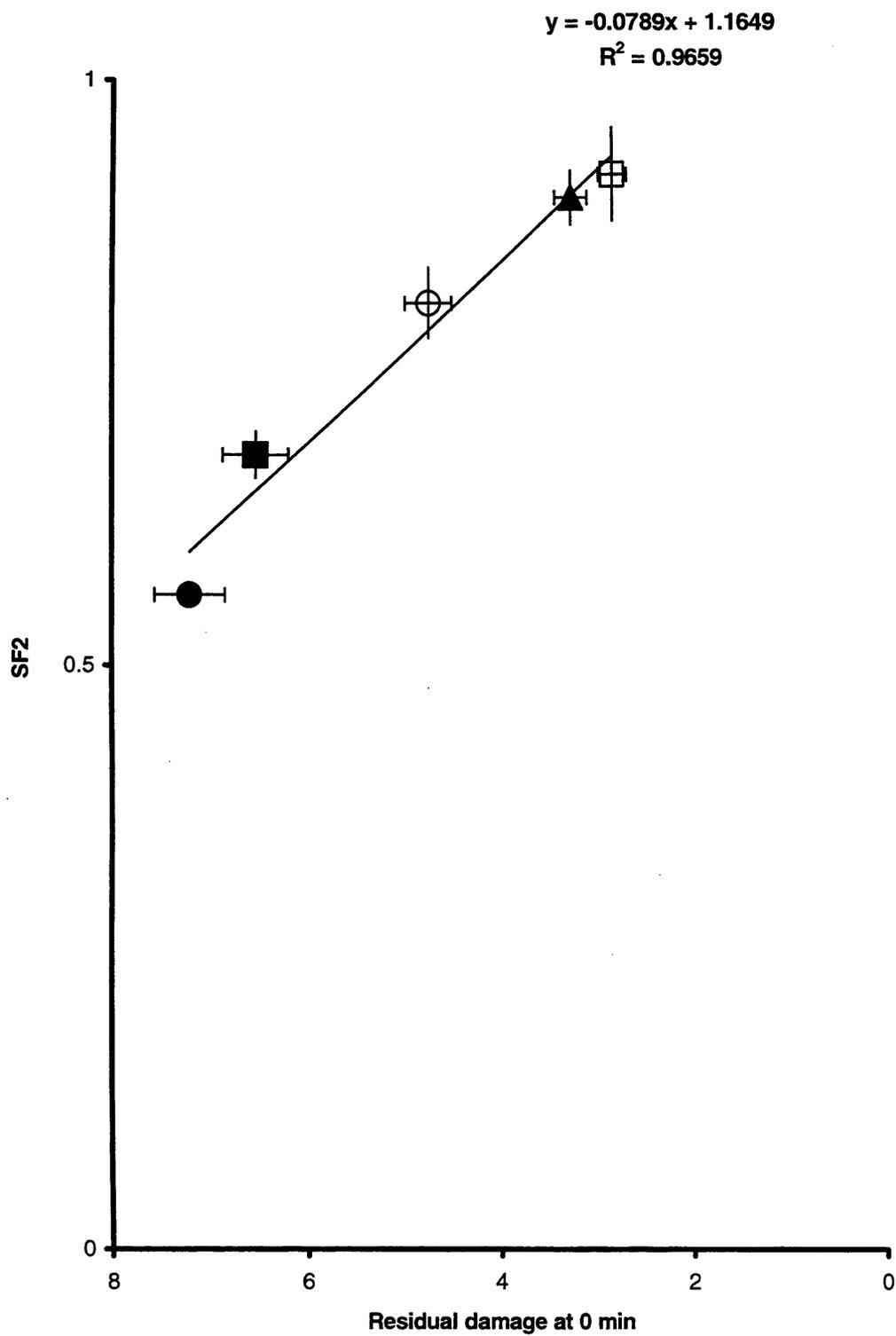
The initial level of radiation-induced comet formation in prepared nucleoid bodies versus isolated nuclei versus intact cells for the six-bladder cancer cell lines, over a dose range of 0-6 Gy was compared using ACA. These results are shown in Figure 2.37. The plots of the determined values of the mean Olive tail moment reveal clear dose response curves for each intact cell, isolated nuclei and prepared nucleoid body. Irradiation of the isolated nuclei and prepared nucleoid bodies result in a ~1.5 – 3-fold and a 5 – 10-fold increase, respectively, in immediate comet formation compared to the parent intact cells. At 2 Gy, 4



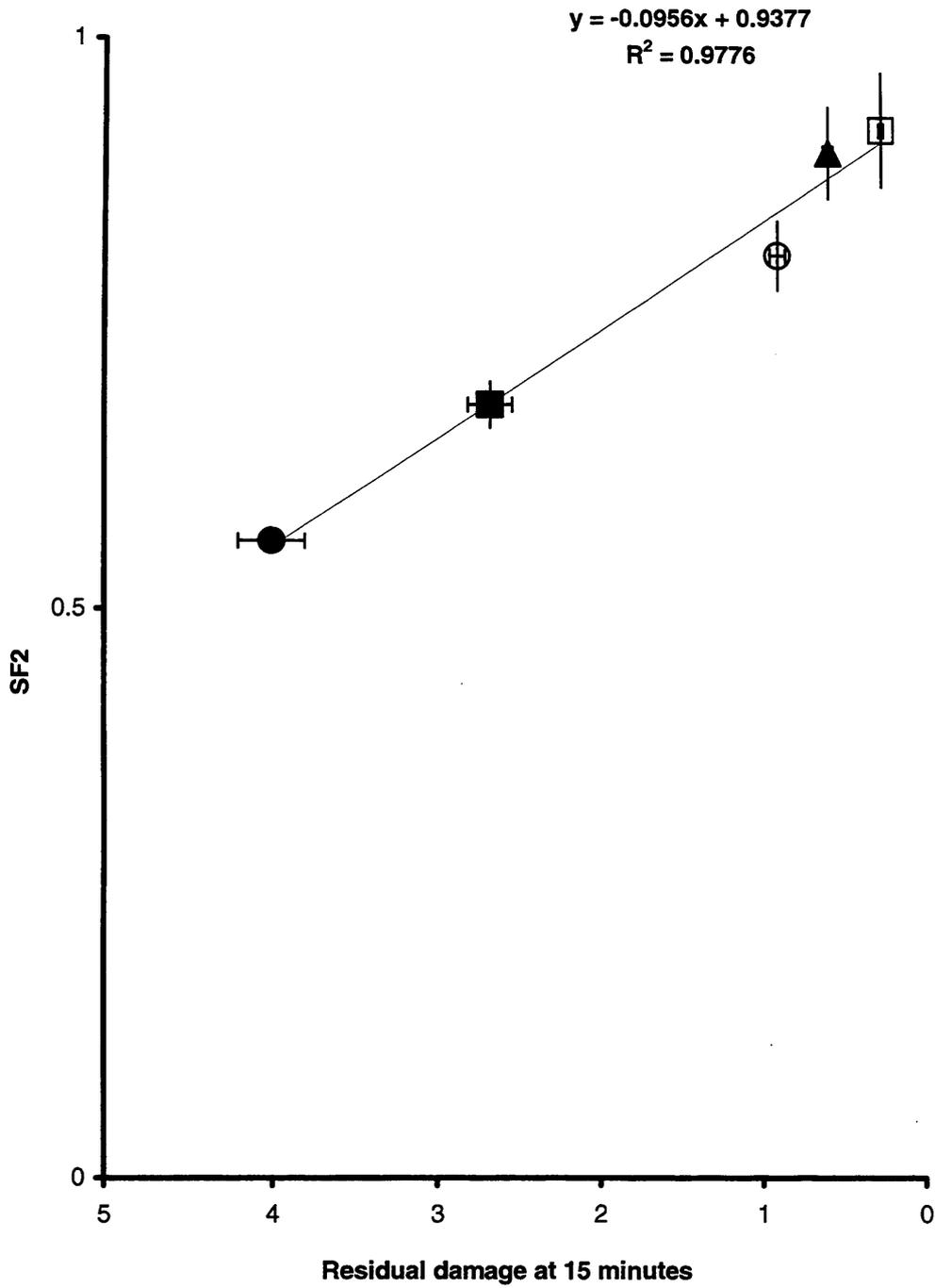
**Figure 2.29.** The extent of radiation-induced single strand break plus alkali labile site damage repair as determined by the alkaline comet assay, after irradiation with 6 Gy of X-ray. Each data point is the mean of three independent experiments  $\pm$  SE.



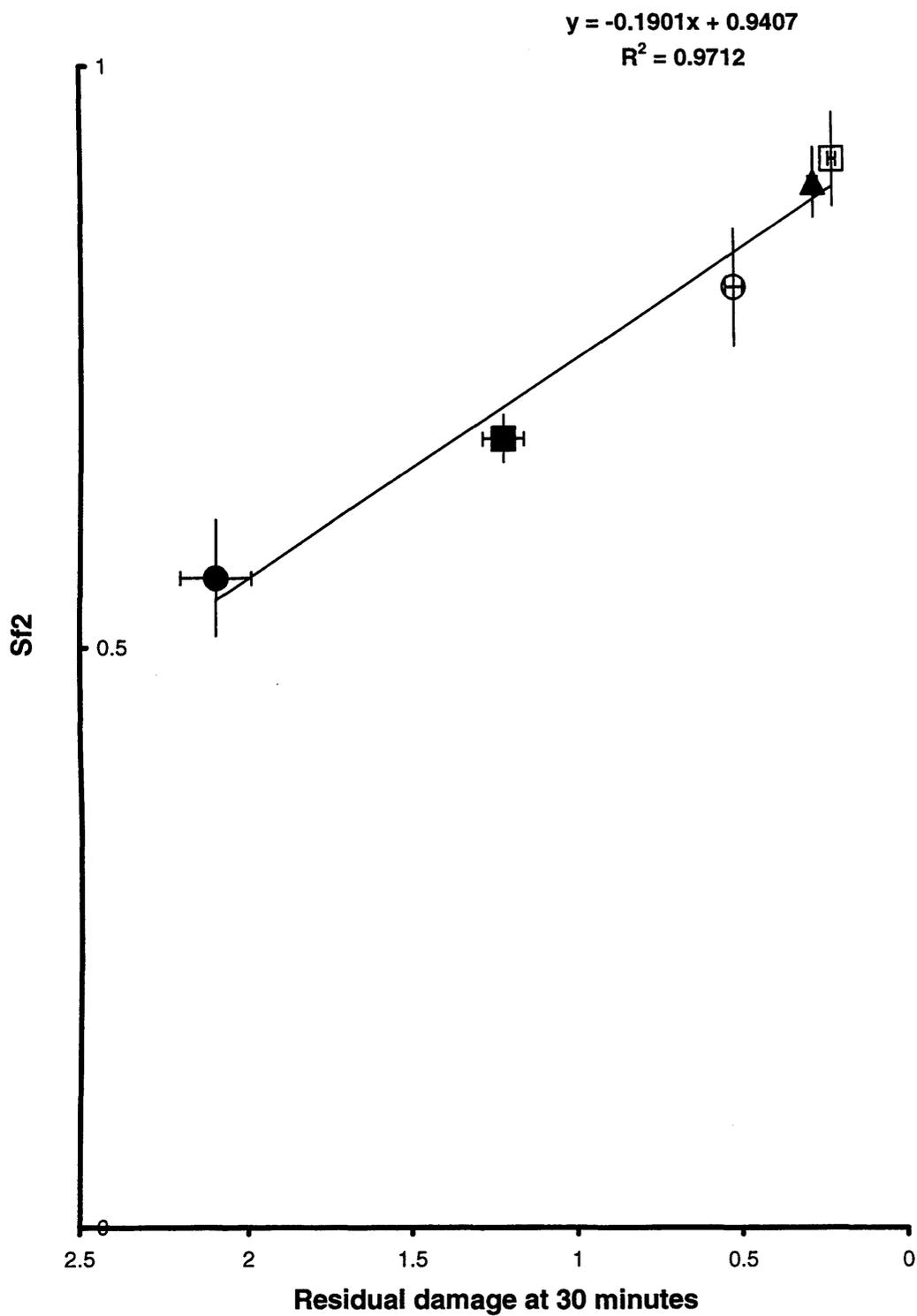
**Figure 2.30.** The extent of relative repair of radiation-induced single strand breaks plus alkali labile site damage formation as determined by the alkaline comet assay, for J82 and RT4, after irradiation with either 2.5 Gy or 6 Gy of X-rays. Each data point is the mean of three independent experiments  $\pm$  SE.



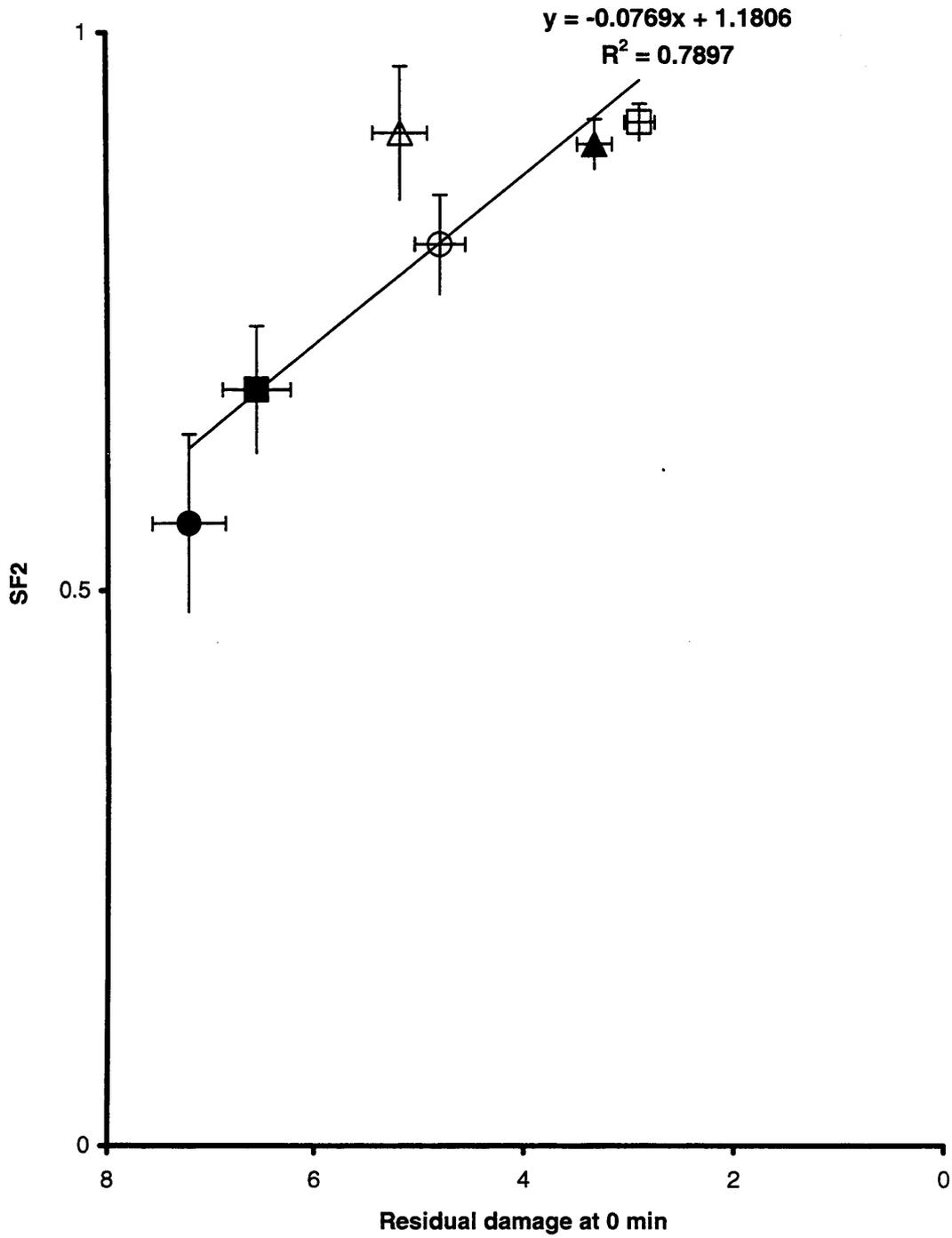
**Figure 2.31.** The correlation between the measures of residual radiation-induced single strand breaks plus alkali labile site damage at 0 minutes, as determined by the alkaline comet assay, and the determined SF2 values for the five cell lines J82, RT112, UM-UC-3, HT1376 and RT4.



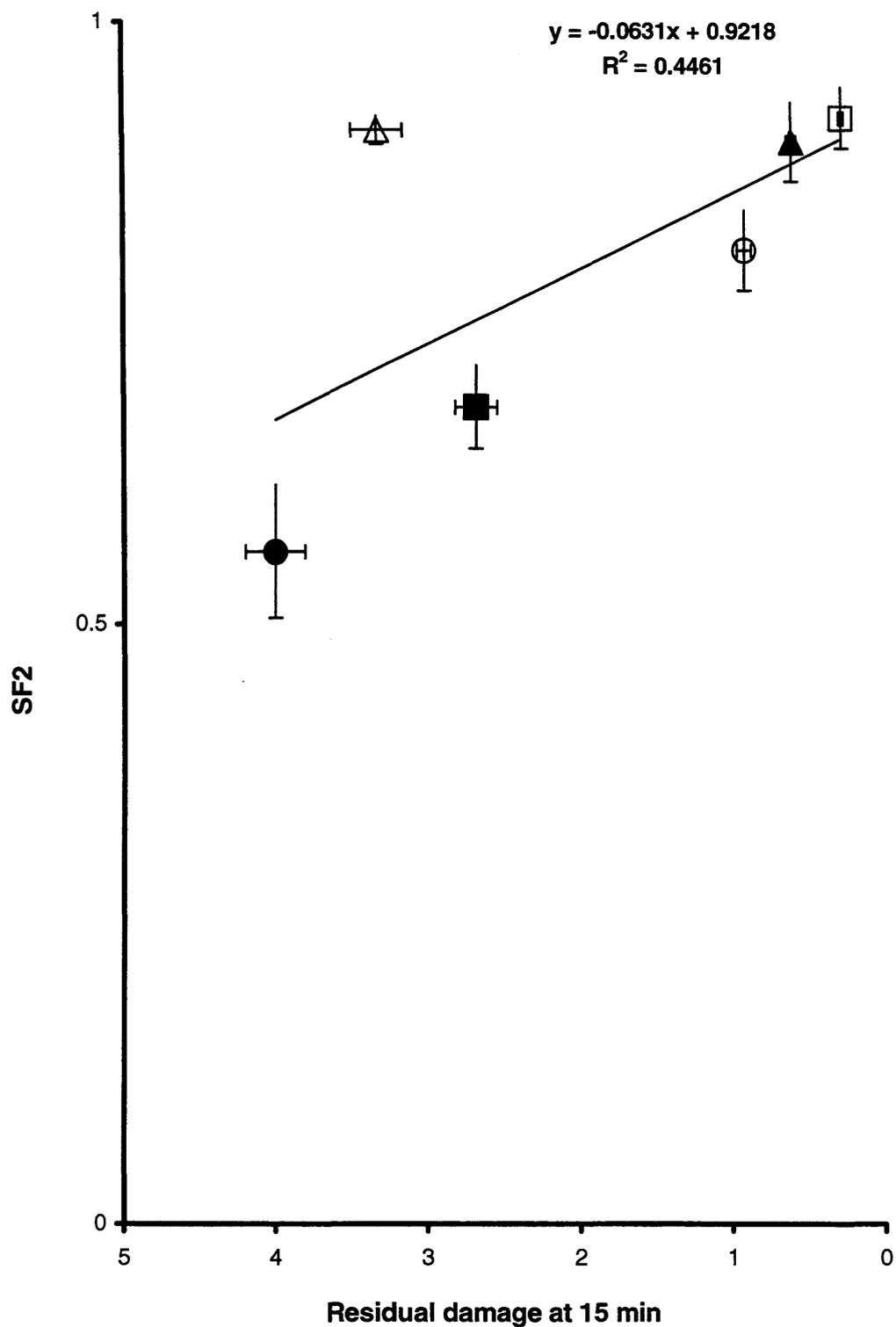
**Figure 2.32.** The correlation between the measures of residual radiation-induced single strand breaks plus alkali labile site damage at 15 minutes, as determined by the alkaline comet assay, and the determined SF2 values for the five cell lines J82, RT112, UM-UC-3, HT1376 and RT4.



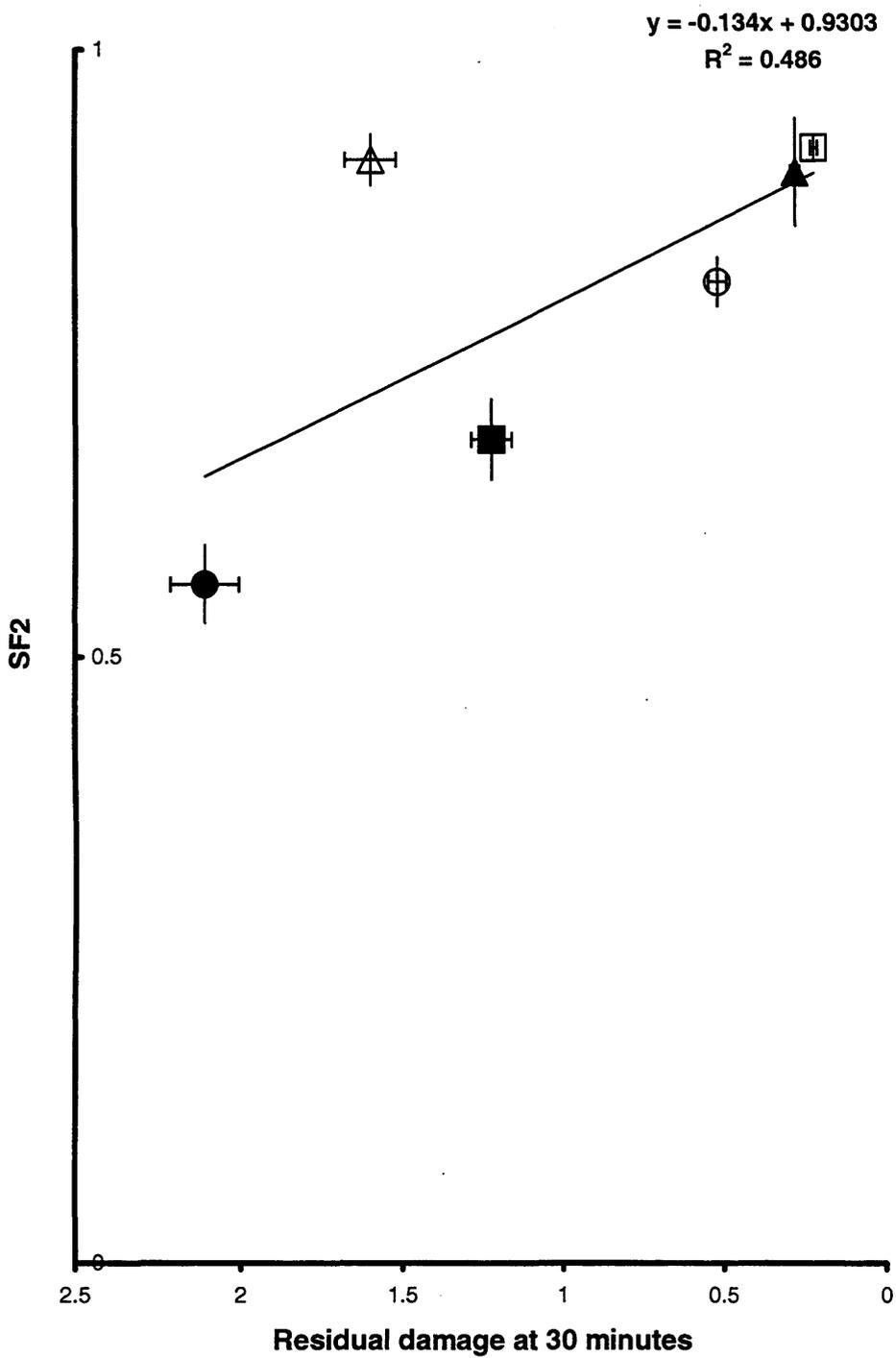
**Figure 2.33.** The correlation between the measures of residual radiation-induced single strand breaks plus alkali labile site damage at 30 minutes, as determined by the alkaline comet assay, and the determined SF2 values for the five cell lines J82, RT112, UM-UC-3, HT1376 and RT4.



**Figure 2.34.** The correlation between the measures of residual radiation-induced single strand breaks plus alkali labile site damage at 0 minutes, as determined by the alkaline comet assay, and the determined SF2 values for all six-bladder cancer cell lines J82, T24, RT112, UM-UC-3, HT1376 and RT4.



**Figure 2.35.** The correlation between the measures of residual radiation-induced single strand breaks plus alkali labile site damage at 15 minutes, as determined by the alkaline comet assay, and the determined SF2 values for all six-bladder cancer cell lines J82, T24, RT112, UM-UC-3, HT1376 and RT4.



**Figure 2.36.** The correlation between the measures of residual radiation-induced single strand breaks plus alkali labile site damage at 30 minutes, as determined by the alkaline comet assay, and the determined SF2 values for all six-bladder cancer cell lines J82, T24, RT112, UM-UC-3, HT1376 and RT4.

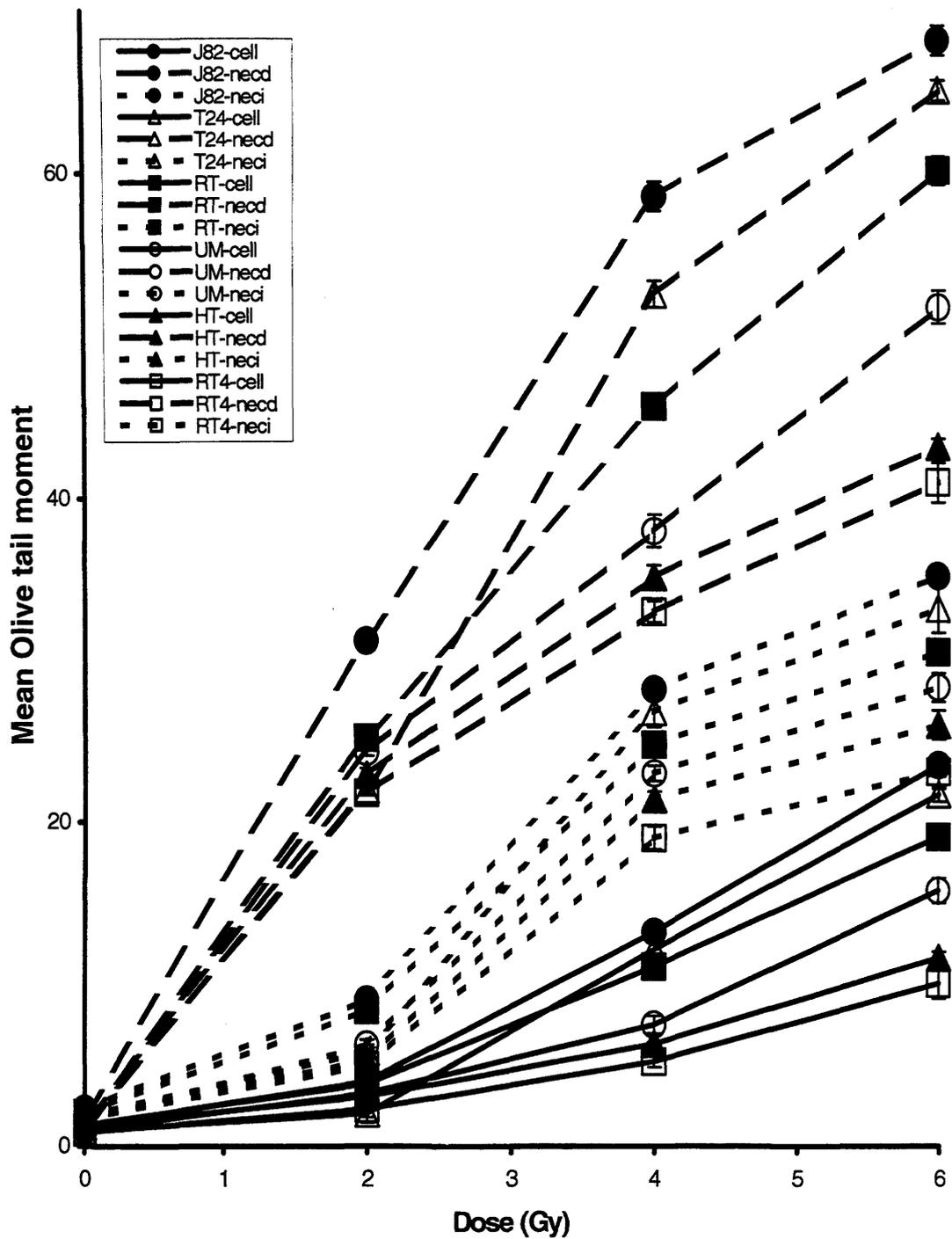
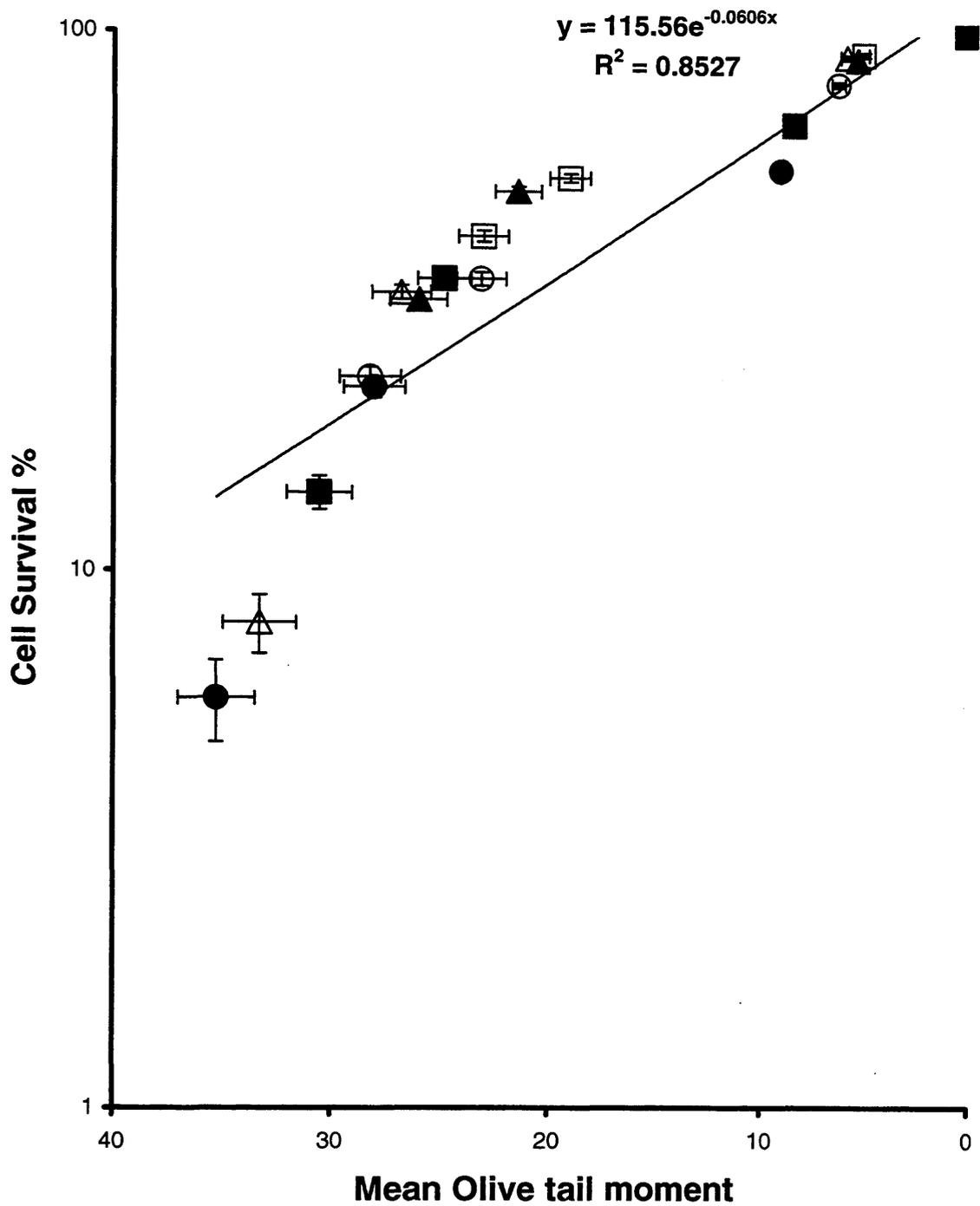


Figure 2.37. The level of initial radiation-induced comet formation in prepared nucleoid bodies versus isolated nuclei versus intact cells of the six-bladder cancer cell lines investigated, over a dose range of 0 – 6 Gy, as determined by the alkaline comet assay. Induced DNA damage was measured as Olive tail moment; each data point is the mean of three independent experiments  $\pm$  SE.

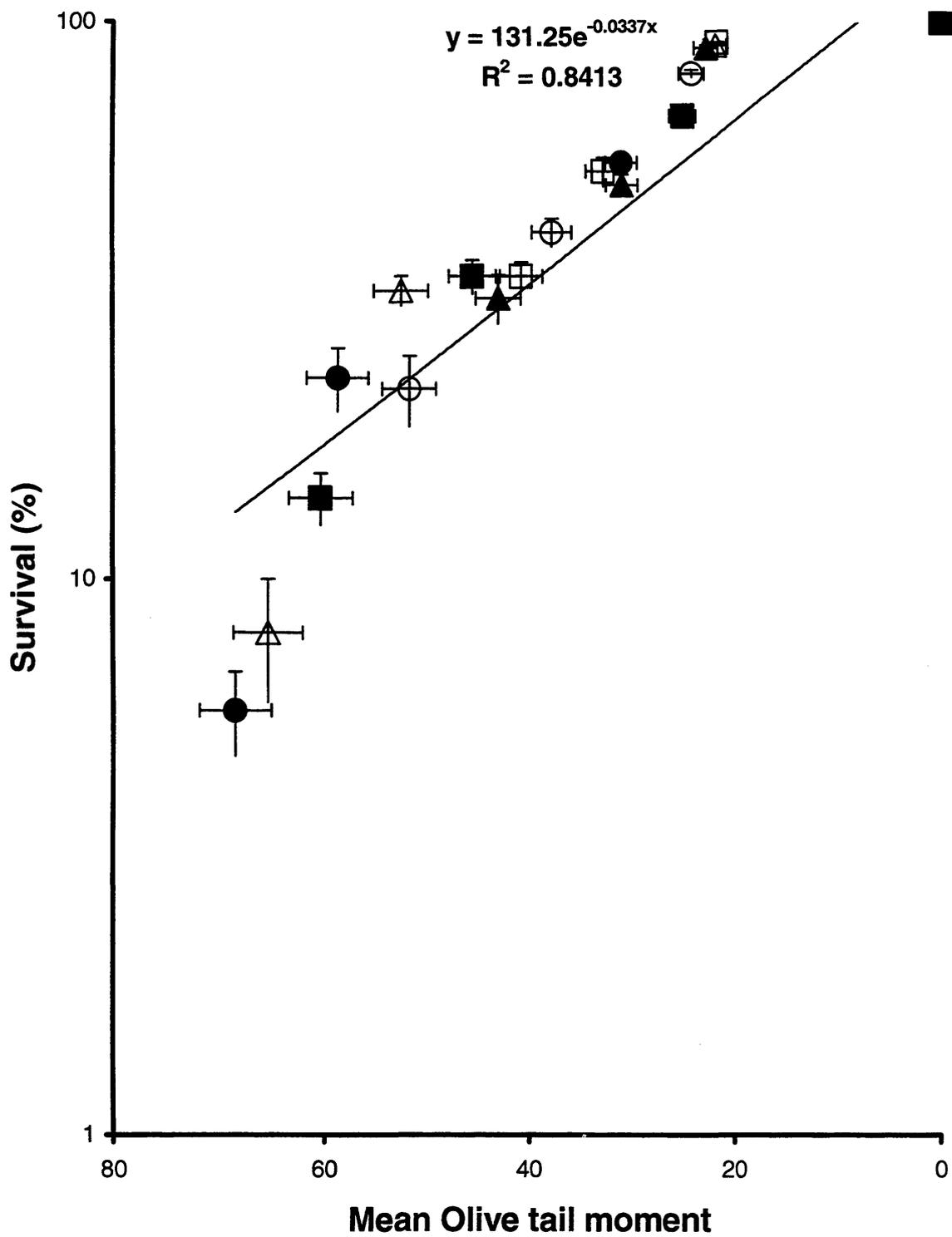
Gy and 6 Gy the level of immediate comet formation noted in the isolated nuclei and the prepared nucleoid bodies maintain the same rank order as noted in the intact parent cell, with those prepared from the most radiosensitive cell line J82 displaying the highest level of radiation-induced comet formation, and those prepared from the most radioresistant cell line RT4 exhibiting the lowest levels of irradiation-induced comet formation. The higher level of comet formation observed in the isolated nuclei and prepared nucleoid bodies, compared to the parent intact cells, permits the values of the mean Olive tail moment to better distinguish between the radiosensitive and radioresistant cell lines ( $p < 0.05$ , Tukey One-way ANOVA). Finally, for the T24 cell line, as noted for the intact cells, the irradiated nuclei and irradiated nucleoid bodies exhibit a relatively low level of immediate comet formation at low dose 2 Gy but a higher relative level of immediate comet formation at higher doses.

The relationship between the measures of mean Olive tail moment for initial radiation-induced comet formation in isolated nuclei and prepared nucleoid bodies, as detected by the alkaline comet assay, and the measures of clonogenic cell survival, for all six-bladder cancer cell lines at 0, 2, 4 and 6 Gy are illustrated in Figures 2.38 and 2.39. A notable degree of correlation exists between the two measures for all six-bladder cancer cell lines, with  $R^2$  values of 0.8527 for the isolated nuclei and of 0.8413 for the prepared nucleoid bodies. These values indicate that the measures of Olive tail moment for the nuclei and prepared nucleoids reflects clonogenic survival for these six cell lines over the dose range studied.

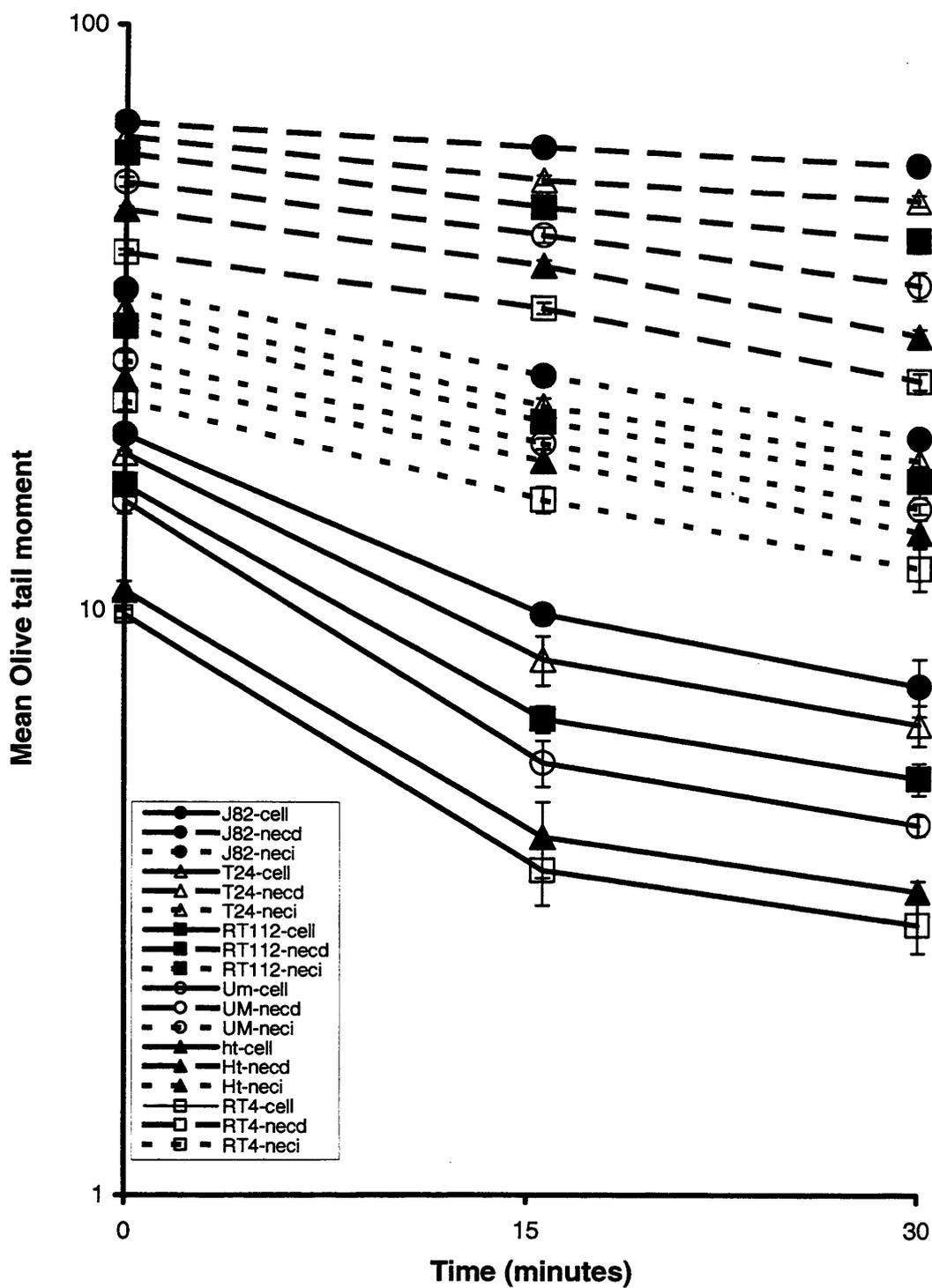
The apparent repair of DNA radiation-induced single strand breaks and alkali labile sites damage in prepared nucleoid bodies and isolated nuclei compared to damage repair in intact cells for the six-bladder cancer cell lines, was assessed using ACA, by incubating the respective cell slides at 37°C for either 15 or 30 minutes, after exposure to an X-ray irradiation dose of 6 Gy. The results are shown in Figure 2.40. At 6 Gy, for the intact cells, the most radioresistant cell lines, RT4 and HT1376, exhibited the highest level of repair and the most radiosensitive cell lines J82 and T24 demonstrated the lowest level of repair. For the isolated nuclei partial evidence of repair was observed, which maintained the same rank order as noted in the intact parent cell, with those nuclei prepared from the most radioresistant cell lines RT4 and HT1376



**Figure 2.38.** The relationship between the measures of mean Olive tail moment for initial radiation-induced comet formation in isolated nuclei, as detected by the alkaline comet assay and the measures of clonogenic cell survival, for all six-bladder cancer cell lines at 0, 2, 4 and 6 Gy.



**Figure 2.39.** The relationship between the measures of mean Olive tail moment for initial radiation-induced comet formation in prepared nucleoid bodies, as detected by the alkaline comet assay and the measures of clonogenic cell survival, for all six-bladder cancer cell lines at 0, 2, 4 and 6 Gy.



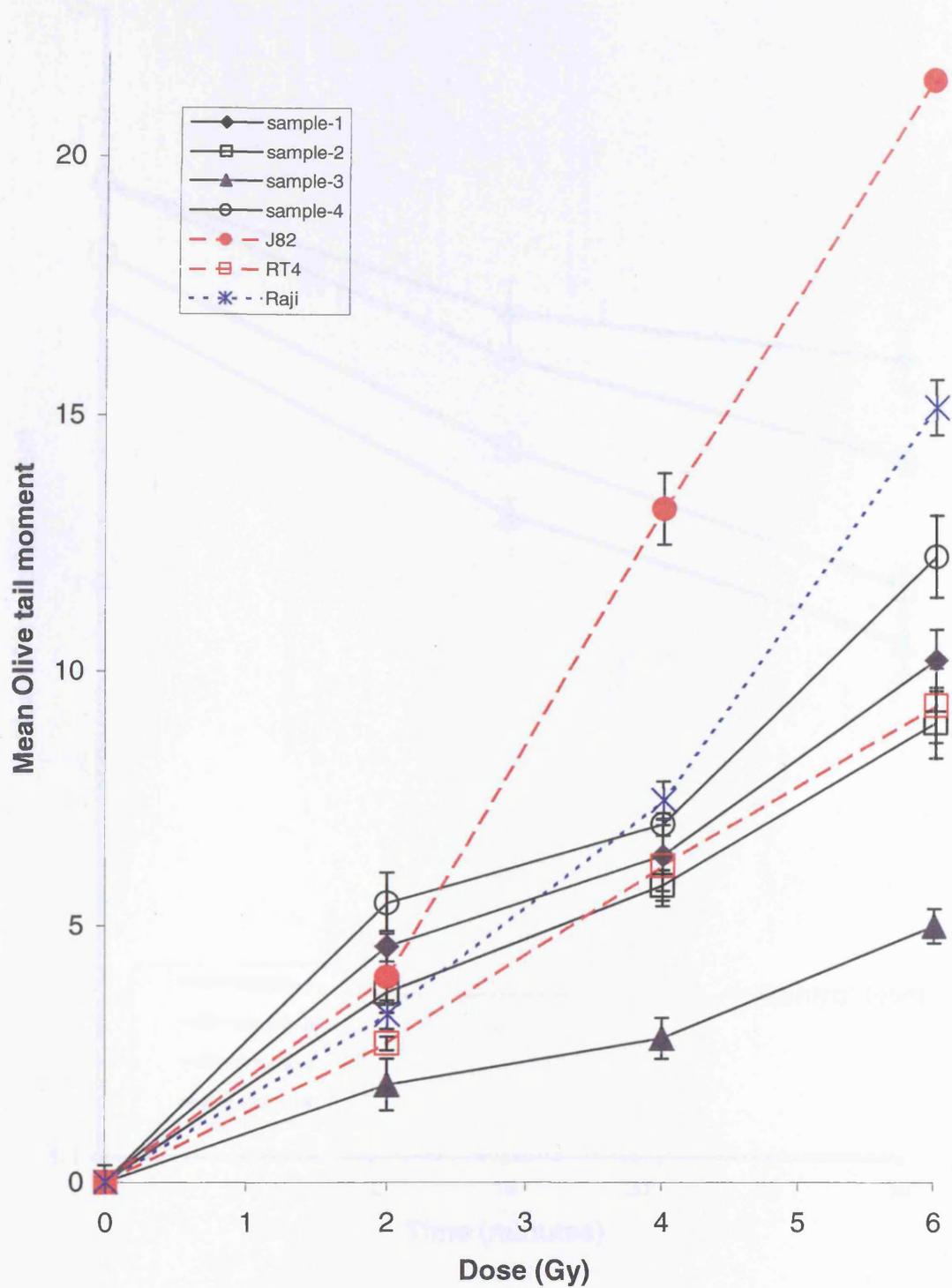
**Figure 2.40.** The extent of radiation-induced single strand breaks plus alkali labile sites damage repair in prepared nucleoid bodies versus isolated nuclei versus intact cells for the six-bladder cancer cell lines, as determined by the alkaline comet assay, after irradiation with 6 Gy of X-rays. Each data point represents the mean of two or three independent experiments  $\pm$  SE.

exhibiting the highest level of 'repair', and those nuclei prepared from the most radiosensitive cell lines J82 and T24 displaying the lowest level of 'repair'. For the prepared nucleoid bodies, a low decrease in mean Olive tail moment was observed after 15 and 30 minutes.

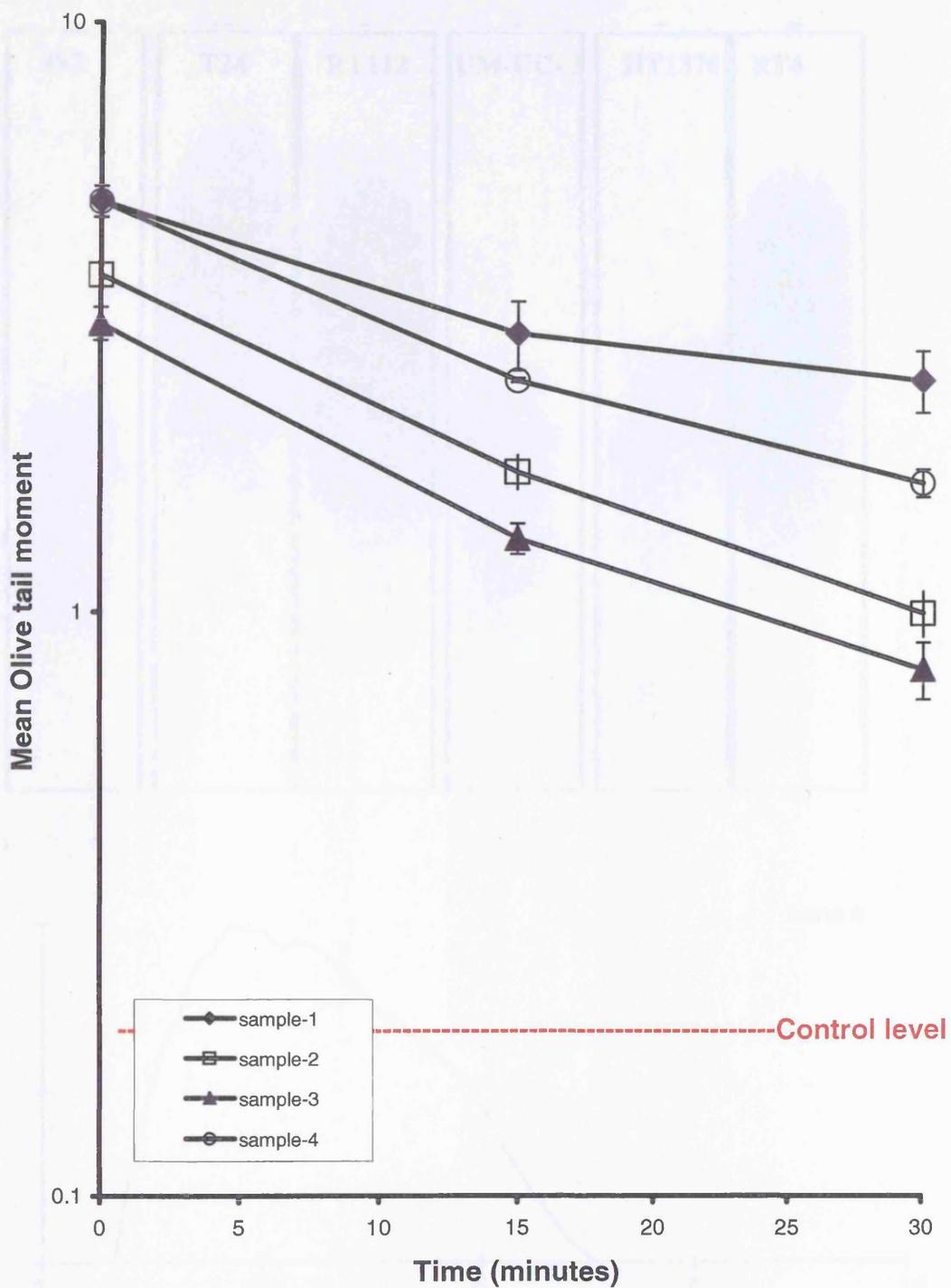
The initial level of radiation-induced comet formation in epithelial cells prepared from invasive bladder tumour biopsies, over a dose range of 0- 6 Gy, was examined using ACA. The results are shown in Figure 2.41. The plots of the mean Olive tail moment for the bladder cells reveal clear dose response curves, with a greater than 2-fold difference in immediate comet formation being noted between the most 'sensitive' and 'resistant' cells. Using the alkaline comet assay there was a significant difference after 2 Gy X-irradiation between the Olive tail moments measures for the most 'sensitive' cells (sample 4) and the most 'resistant' cells (sample 3) ( $p < 0.05$ , Tukey One-way ANOVA). A control Raji cell line was used to monitor experiment-to-experiment variations; the Raji cell line demonstrated an intermediate level of irradiation-induced immediate comet formation similar to that observed with the UM-UC-3 cells.

The repair of DNA radiation-induced single strand breaks and alkali labile site damage in epithelial cells prepared from invasive bladder tumour biopsies was examined using ACA, by incubating the cell-slides at 37°C for either 15 or 30 minutes, after exposure to an x-ray irradiation dose of 2.5 Gy. The results are shown in Figure 2.42. It was noted that the most 'sensitive' biopsies (samples 1 and 4) tended to display the lowest level of repair, while the most 'resistant' biopsy (sample 3) exhibited the highest level of repair. For the most 'resistant' biopsy the majority of repair occurs within 15 minutes, while for the more 'sensitive' biopsies a significant level of residual damage remains after 30 minutes. However, the results were not definite, the rank order of comet formation was observed to be higher in sample 4>1>2>3 and whilst the extent of repair was observed to be higher in sample 1>4>2>3.

The telomere lengths of the six-bladder cancer cell lines were assessed using the method outlined in Section 2.11.4. The results are shown in Figures 2.43 and 2.44.

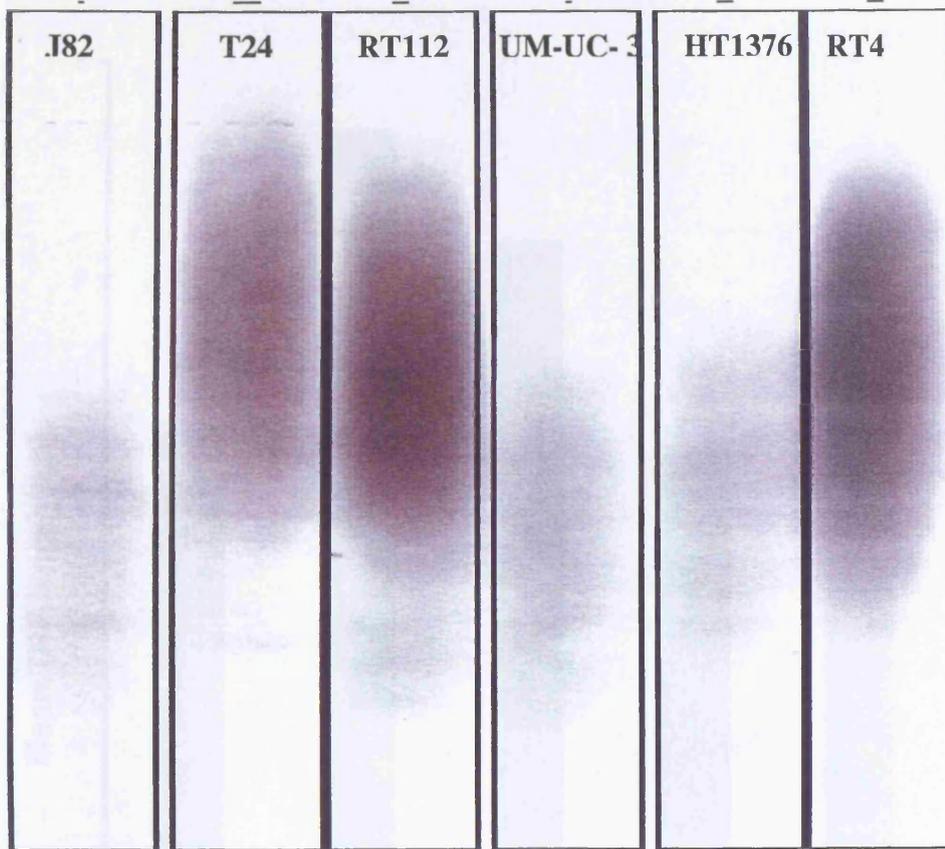


**Figure 2.41.** The initial level of radiation-induced DNA single strand breaks plus alkali labile sites damage formation in epithelial cells prepared from invasive bladder tumour biopsies, over a dose range of 0 – 6 Gy, as determined by the alkaline comet assay. Induced DNA damage was measured as Olive tail moment; each data point is the mean of duplicate irradiations  $\pm$  SE.

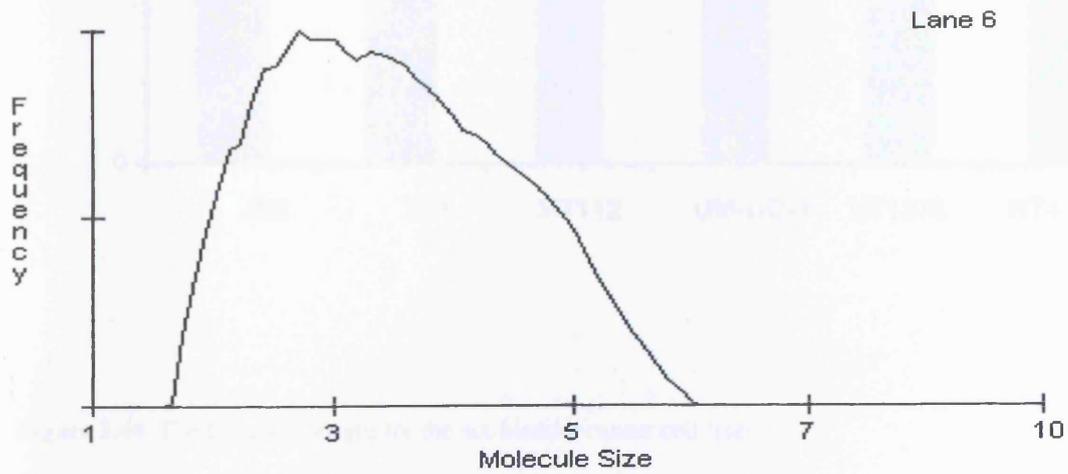


**Figure 2.42.** The extent of radiation-induced single strand breaks plus alkali labile sites damage repair in epithelial cells prepared from invasive bladder tumour biopsies, as determined by the alkaline comet assay, after irradiation with 2.5 Gy of X-rays; each data point is the mean of duplicate irradiations  $\pm$  SE.

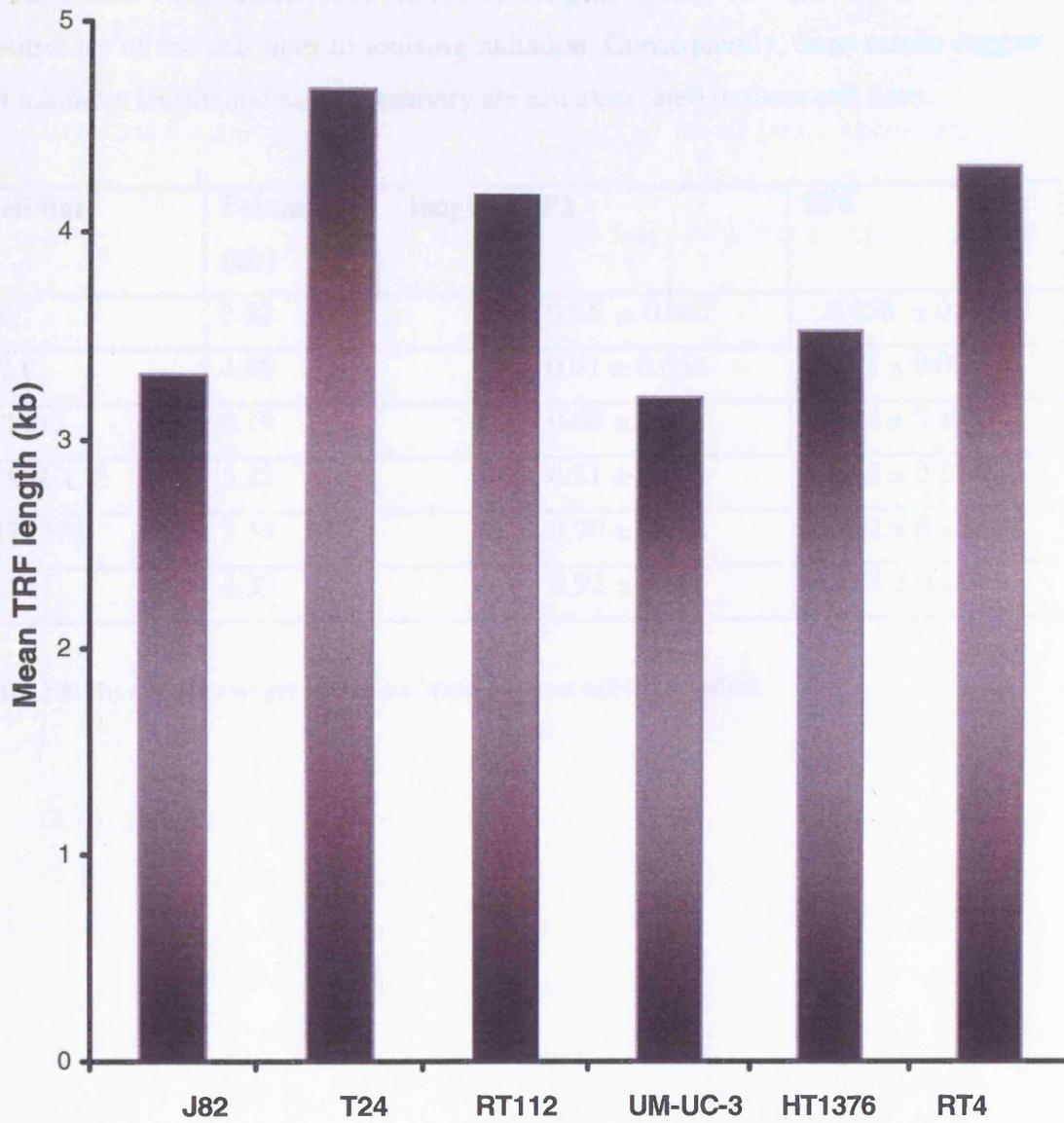
(A)



(B)



**Figure 2.43.** (A) The telomere length as presented on the agarose gel for the six-bladder cancer cell lines investigated. (B) The absorbance as for lane 6 (RT4).



**Figure 2.44.** The telomere length for the six-bladder cancer cell lines.

The derived values of telomere lengths for all six-bladder cancer cell lines are given in Table 2.4. The determined telomere lengths failed to correlate the relative sensitivities of the cell lines to ionising radiation. Consequently, these results suggest that telomere length and radiosensitivity are not associated in these cell lines.

Cell line	Telomere length (kb)	SF2	SF6
J82	3.32	0.56 ± 0.062	0.058 ± 0.07
T24	4.69	0.91 ± 0.054	0.08 ± 0.090
RT112	4.19	0.68 ± 0.04	0.14 ± 0.101
UM-UC-3	3.22	0.81 ± 0.051	0.22 ± 0.134
HT1376	3.54	0.90 ± 0.052	0.32 ± 0.150
RT4	4.33	0.92 ± 0.08	0.35 ± 0.154

**Table 2.4.** The telomere length for the six-bladder cancer cell lines studied.

## 2.13 DISCUSSION

The development of a rapid and valid predictive test for tumour cell radiosensitivity would make an important contribution towards enhancing the value of radiotherapy. If a predictive test could provide opportune information based on an individual's cell radiosensitivity, this would allow treatment to be planned specifically for each individual patient. In particular, the development and validation of a method for the prediction of bladder tumour cell radiosensitivity could significantly improve tumour cure rates and patients' survival as there is a choice of beneficial therapy dependent on the predictive outcome; those patients with predicted radiosensitive tumours could be considered candidates for immediate radical radiotherapy, whilst those patients with predicted radioresistant tumours could be possible candidates for immediate surgery. By improving the decision-making of which patients should be treated with radiotherapy and which should not, it is hoped to reduce the number of patients whose disease is not cured by radiotherapy, who are less likely to be offered secondary curative treatment.

Over the past decade, much work has been undertaken to develop assays capable of predicting tumour cell radiosensitivity, however, few have been found to be of value. Traditionally, the clonogenic assay has been considered to be the optimal assay for determining survival after radiation (Chadwick *et al.*, 1973). It relies on the ability of cells to form viable colonies derived from a single 'clone'. However, it is labour intensive, requires long incubation times to form colonies, is less reliable for cells with low cloning efficiencies and not all cells investigated undergo colony formation. Consequently, other faster and more automated assays have been investigated.

The MTT assay was also developed to estimate cell survival; it is a colorimetric assay, which quantifies viable cells. Radiation 'sensitivities' of tumour cells determined via this assay have been reported to compare favourably with those determined by the clonogenic assay after the assay has been individually optimised for each cell line. However, the clinical utility of this assay is limited by low sensitivity at low radiation doses and poor specificity.

DNA is the most important cellular target molecule for the lethal effects of ionising radiation; current evidence indicates that the extent of cell killing has been shown to relate to the level of DNA damage induced (Price *et al.*, 2000; Roos *et al.*, 2000; Bacova *et al.*, 2000). To assess DNA damage and repair in individual cells, Singh (Singh *et al.*, 1988) developed the microgel electrophoresis method (comet assay). This method is regarded as relatively simple, rapid and a highly sensitive method for assessing DNA damage formation and repair in cells. Furthermore, this assay is attractive as a clinical test as it is inexpensive, data is available on the same day or next day basis and the assay requires only low numbers of cells; typical of the numbers obtained from small clinical biopsies.

DNA double strand breaks are proposed to be the principal lesions responsible for the cell killing as an effect of ionising radiation (Ward *et al.*, 1988). Previously, using the neutral comet assay (NCA), the level of immediate radiation-induced double strand break formation has been shown to correlate with radiosensitivity for a panel of bladder cancer cell lines (Price *et al.*, 2000), with the highest damage level being noted in the most radiosensitive cell line and the least damage on the most radioresistant cell line. Unfortunately, the relative yield of radiogenic double strand breaks is low, and high doses of radiation were required to produce measurable levels; doses that are far greater than those used clinically. In contrast, the yield of radiation-induced single strand breaks is far greater than that of radiation-induced double strand breaks (Ward *et al.*, 1988), and can be more readily measured at low clinically relevant doses of radiation. Furthermore, the mechanisms, which are proposed to vary immediate radiation-induced double strand breaks, are also expected to vary the radiation-induced single strand breaks yield (Ward *et al.*, 1990). Consequently, the extent of immediate radiation-induced single strand break formation can be considered as a valid surrogate marker of initial radiogenic double strand break formation.

The alkaline comet assay (ACA) is a highly sensitive method for the assessment of SSBs and alkali labile sites (ALSs), and can readily detect levels of damage induced by clinically relevant doses of radiation (Singh, 1966; Singh *et al.*, 1994). In a previous study of just three bladder cancer cell lines, an inverse correlation was obtained between clonogenic survival and mean tail moment for comet formation,

suggesting that ACA could potentially be used to predict the radioresponse of the single cell lines (McKelevy-Martin *et al.*, 1998).

In the present study radiation survival curves for the six-bladder cancer cell lines have been obtained using clonogenic assay. The results obtained from the MTT assay maintain the same rank order as survival as determined by the clonogenic assay, with the most radiation sensitive cell line displaying the lowest level of viability after irradiation and the most radiation resistant cell line exhibiting the highest level of viability. However, the clonogenic assay was far more sensitive in determining survival than was the MTT assay at determining viability.

Discrepancies between the clonogenic assay and the MTT assay have been reported by others (Banasiak *et al.*, 1999) and have been attributed to the presence of dead cells that retain residual dehydrogenase activity. Furthermore, results are dependent on incubation time and formazan end product does degenerate. MTT results may also be affected by pH, culture age, length of incubation, and D-glucose concentration (Vistica *et al.*, 1991). The MTT assay was found to be relatively insensitive in the cell lines investigated in this study especially at low radiation doses. This might be due to differences in assay end-points, and therefore is likely to be an unsuitable short-term measure of survival.

The alkaline comet assay in this study was demonstrated to be capable of predicting radiation cell survival for a panel of six-bladder cancer cell lines. The initial level of radiation-induced comet formation had a strong inverse correlation with clonogenic survival for all six-bladder cell lines, with the most radiation sensitive cell line displaying the greatest measures of comet formation and the most radioresistant cell line displaying the least. Furthermore, for one cell line, T24, the notable change of radiation resistant to radiation sensitive phenotype with increasing dose was mirrored in the alkaline comet assay response.

For the determined measures of radiation-induced DNA single strand break plus alkali labile site damage repair, which includes measures of residual damage at various repair time points, there was good correlation with survival (SF2) for five of the six bladder cancer cell lines investigated, with the extent of repair being greater in the

radioresistant cell lines. However, the excellent correlation of radiation clonogenic survival with the extent of immediate radiation-induced comet formation for all six-bladder cancer cell lines studied (including the anomalous behaviour of the T24 cell line) as compared to the correlation of survival with the extent of damage repair (which was only apparent for five of the six cell lines studied) signifies that it is immediate comet formation that best predicts survival for the six cell lines. Virtually identical findings have been reported in two other recent studies (Dunne *et al.*, 2003; McKeown *et al.*, 2003).

For each individual cell line, the measure of mean Olive tail moment increases with dose due to the dose-dependency of radiation-induced DNA damage formation. However, a conclusion that it is the differing levels of immediate radiogenic DNA damage noted between cells which is responsible for their differing radiosensitivities, conflicts with previous reports that fail to detect such damage (SSB or DSB) differences/or correlations, using comet or other assays (Evans *et al.*, 1993; Olive *et al.*, 1991; Woudstra *et al.*, 1996). This may reflect the fact that studies which fail to correlate initial damage or repair with cell survival tend to have compared cell lines of different origins (which may have different DNA conformations and therefore may exhibit considerable differences in radiogenic DNA damage assays), e.g. (Olive *et al.*, 1994; Olive *et al.*, 1991; Woudstra *et al.*, 1996). However, studies using cell lines of the same origin at least show correlations between survival and measures of repair/or residual damage, e.g. (Eastham *et al.*, 1999; Marples *et al.*, 1998).

The extent of repair was examined using the alkaline comet assay. It was noted that the extent of repair was generally higher in the radioresistant cell lines and lower in the radiosensitive cell lines. However, the more radioresistant cell lines have lower level of immediate comet formation, which if indicative of lower levels of initial damage would presumably facilitate more rapid repair. On the other hand, the more radiosensitive cell lines had higher levels of immediate comet formation, which if indicative of higher levels of damage would presumably pose a greater burden to repair. Therefore, it could be argued that the extent of repair for the majority of cell lines studied is proportional to the level of initial damage, with the radioresistant cells (having low levels of immediate damage) exhibiting more complete repair, whilst the radiosensitive cells (possessing high levels of immediate damage) exhibit incomplete

repair. Indeed it can be clearly seen that irradiation of the most radioresistant cell line RT4 with a higher dose of radiation 6 Gy, to induce a greater level of immediate damage, leads to incomplete repair and a significant level of residual damage remaining after 30 minutes; this being similar to the extent of repair for the radiosensitive cell line J82 after irradiation with just 2.5 Gy. This strongly support notion that the extent of repair is proportional to the level of initial damage induced.

It was noted that the extent of repair (as assessed by measures of residual damage for five of the six cell lines) correlates well with SF2. The high degree of correlation between the extent of repair and SF2 may reflect the inclusion and contribution of the SSB components of DSBs (and other complex lesions) in the ACA assessment of damage at these time points. Many studies have shown that a greater abundance of residual DSBs, after a period of repair, is associated with a higher cellular radiosensitivity e.g. (Zhou *et al.*, 1998). However, for one cell line, T24, the measures of repair did not correlate with (SF2); there being higher level of residual damage than expected during repair incubation. This may to some extent be due to the change from resistant to sensitive phenotype for this cell line at doses of >2 Gy; at higher doses the T24 cells suffer proportionally greater levels of damage. Also, the relatively slower repair of single strand breaks plus alkali labile sites damage may reflect a deficiency in base excision repair, which at higher doses may contribute to the relative increase in radiation cell death seen with this cell line. Obviously, this scenario would seriously confound attempts to predict radiosensitivity from estimates of DNA damage repair determined by ACA. However, the fact that the notable dose dependent alteration in the phenotype of T24 was also reflected in the ACA response strongly supports the ability of one assay (ACA) to predict the other (survival). Furthermore, this phenotype alteration demonstrates the importance of conducting predictive tests using clinically relevant doses of radiation. Clearly, in this particular instance, the use of higher test doses yields a false impression of radiosensitivity at low dose.

Clearly, the results of the present study suggest that measures of initial comet formation, as determined by the alkaline comet assay, may be used to predict the radiosensitivity of individual bladder cancer cell lines. In an effort to delineate factors responsible for the different levels of immediate comet formation in bladder cancer cells of different radiosensitivity, a study was carried out to investigate the levels of

immediate radiation-induced comet formation in prepared nucleoid bodies and isolated nuclei of all the six cell lines studied, and these compared to the level of immediate comet formation in the corresponding intact cells. The observation of the level of immediate radiation-induced comet formation in the isolated nuclei and particularly in the prepared nucleoid bodies maintaining the same rank order as comet formation in the intact parent cell indicates that it is a feature of the nucleoid body that dictates the relative extent of radiogenic comet formation. This also includes the anomalous behaviour for T24; as noted for the intact cells, the irradiated nuclei and irradiated nucleoid bodies prepared from T24 cells exhibit a relatively low level of immediate comet formation at low dose 2 Gy but a higher relative level of immediate comet formation at higher doses. The greater level of comet formation of the nuclei compared to the intact parent cell is presumably due to the preparative loss of low-molecular weight and non-DNA associated nuclear components that would otherwise protect the DNA from damaging radiation-induced free radicals. Likewise, the greater radiation-induced comet formation of the nucleoid bodies compared to the intact parent cells is presumably due to the further removal of the protective histone proteins (during preparative lysis). Increases in radiation DNA damage sensitivity have been noted in studies in which cells and nuclei are treated with progressively greater concentrations of salt to sequentially remove histone and non-histone proteins (Elia *et al.*, 1992; Olive *et al.*, 1995; Oleinick *et al.*, 1994).

The initial level of radiation-induced comet formation in the isolated nuclei and prepared nucleoid bodies as determined by ACA exhibited a high degree of correlation with clonogenic survival for all six-bladder cell lines, with the nuclei and nucleoid bodies prepared from the most radiation sensitive cell line displaying the greatest measures of comet formation and the nuclei and nucleoid bodies prepared from the most radioresistant cell line displaying the least.

The observation that there is the same rank order for comet formation in the irradiated nucleoid bodies and irradiated nuclei as in the irradiated intact parent cells, presumably reflects differences in the organisation of the nuclear DNA within radiation resistant and sensitive cell lines. There is the possibility of larger supercoiled loops and/or weaker DNA attachment to the nuclear matrix proteins in radiation sensitive cell lines (Vaughan *et al.*, 1993; Malyapa *et al.*, 1994). Larger DNA loops

could lead to the DNA being more susceptible to radiation damage in the sensitive cell lines. However, in the present study there was no obvious correlation between the unirradiated size of nucleoid bodies Table 2.5 and the extent of comet formation with  $R^2$  values of 0.2087 and 0.061 for 2 and 6 Gy, respectively. A further factor that may influence the extent of comet formation is the nature of the DNA anchoring matrix associated regions (MARs). MARs allow the maintenance of contiguous looped regions of nuclear DNA with varying superhelical densities. Consequently, differences in the character of MARs may result in weaker DNA-matrix associations, thereby allowing additional release of the adjacent/contiguous loops of DNA resulting from the presence of radiation-induced DNA damage. Indeed, studies of radiation sensitive and resistant mutants have revealed differences in intrinsic radiosensitivity to correlate with DNA stability in the presence of DNA damage as measured by DNA loop rewinding ability, and which changes in nucleoid protein composition (Malyapa *et al.*, 1994; Malyapa *et al.*, 1996).

Cell line	Nucleoid size	Extent of comet formation at 2 Gy	Extent of comet formation at 6 Gy
J82	1.836	4.00 ± 0.54	21.50 ± 0.112
T24	1.245	2.34 ± 0.23	20.70 ± 0.84
RT112	1.258	3.93 ± 0.34	19.08 ± 0.94
UM-UC-3	1.045	3.26 ± 0.028	15.76 ± 0.54
HT1376	1.545	3.08 ± 0.33	11.32 ± 0.57
RT4	1.253	2.70 ± 0.13	9.341 ± 0.73

**Table 2.5.** The unirradiated nucleoid size for the six-bladder cancer cell lines studied.

The ‘apparent’ repair of radiation damage in prepared nucleoid bodies and isolated nuclei compared to the parent intact cell was examined using the alkaline comet assay. It was noted that the isolated nuclei experience compromised repair compared to the parent intact cell, with nuclei prepared from the most radioresistant cell line exhibiting the highest level of ‘repair’ and nuclei prepared from the most radiosensitive cell line exhibiting the lowest level of ‘repair’. One possibility that may account for the compromised repair in the isolated nuclei compared to the parent

intact cell, might be the loss of BER co-factors (*e.g.* XRCC1). For the prepared nucleoid bodies, a decrease in mean Olive tail moment was observed upon repair incubation. One possibility may be that the supercoiled DNA in the comet head is relaxed allowing more ethidium to bind, but that this relaxation is not due to strand breakage.

Epithelial cells isolated from human bladder cancer biopsies also reveal a range of radio-responses, as determined by the alkaline comet assay, at clinically relevant doses. From the above data for the bladder cancer cell lines, the differences in radiogenic immediate comet formation for the biopsies from epithelial cells are taken to reflect actual variations in tumour cell radiosensitivity; with biopsy 1 being of similar radiosensitivity to one of the most sensitive cell lines, RT112, and biopsy 4 being more radioresistant than either RT4 or HT1376. The observation of differing tumour cell radiosensitivity, in particular the observation of high degrees of radioresistance, is most significant as it may be a contributing factor to the high level of invasive bladder cancer radiotherapy failure (Shibley *et al.*, 1985; Ducan *et al.*, 1986).

The alkaline comet assay was also used to measure the extent of repair in epithelial cells prepared from invasive bladder tumour biopsies. It was generally noted that the extent of repair was higher in the predicted radioresistant biopsy cells and lower in the radiosensitive biopsy cells. The most radioresistant biopsy may have a lower level of immediate radiogenic damage, which presumably facilitates more rapid repair, whilst the more radiosensitive biopsy may have a higher level of immediate damage, which presumably possesses a greater difficulty to repair. However, the correlation between comet formation and extent of repair was not absolute, with one sample (1) exhibiting a higher level of residual damage than expected.

Previous studies have reported that many eukaryotic cells show defects in telomere maintenance. Most defects involve severe telomere shortening. McIlrath reported that there is a 7-fold reduction in telomere length for radiosensitive mouse lymphoma cells as compared with parental cells (McIlrath *et al.*, 2001). They found that the gradual telomere shortening may not be responsible for the loss of telomeric sequence. It seems more likely that a fast and severe telomere shortening may coincide with the

transformation of the radioresistant phenotype into radiosensitive phenotype. On the basis of these observations, it was reasoned that telomere length might be used as a marker for chromosomal radiosensitivity. A significant inverse correlation between telomere length and chromosomal radiosensitivity in lymphocytes from breast cancer patients seems to support this hypothesis (McIlrath *et al.*, 2001). In the present study we investigated the telomere length as a possible predictive test for chromosomal radiosensitivity in six bladder cancer cell lines. The telomere length measured for all six-bladder cell lines had no correlation with clonogenic survival. It was noted that the most radiation sensitive cell line as determined by the clonogenic assay did not exhibit the shortest telomere nor that the most radiation resistant cell line exhibited the longest telomere. In conclusion, the analysis of telomere length in these six cell lines failed to correlate chromosomal radiosensitivity.

## **CHAPTER THREE**

***MEASUREMENT OF CHEMOTHERAPEUTIC  
DRUG-INDUCED DNA DAMAGE IN BLADDER  
CANCER CELLS: EVALUATION OF THE  
ALKALINE COMET ASSAY AS A PREDICTIVE  
TEST OF BLADDER CANCER CELL  
CHEMOSENSITIVITY***

### **3.1 INTRODUCTION**

For more than a century investigations have addressed the potential role of systemic treatment in cancer therapy. However, not until the initial trials of alkylating agents by scientists and clinicians in 1944 at Yale University, was there actual proof that a systemically administered agent could cause the regression of a human tumour (Goodman *et al.*, 1946). In bladder cancer, the reason for combining early local therapy with systemic therapy is based on the theory that the combined treatment would result in a more effective outcome. However, in the treatment of some patients, local control is achieved without the use of cytotoxic chemotherapy and as such these patients do not benefit from this additional treatment.

Several attempts have been made to improve the outcome of the use of chemotherapy in cancer treatment. These attempts include the use of assays that can predict tumor sensitivity on an individual basis (Köberle *et al.*, 1997; Loprevite *et al.*, 2001; Hartley *et al.*, 1991; Blasiak *et al.*, 2000 and Huang *et al.*, 1998). The ability to predict tumor chemosensitivity is likely to improve the results of treatment centered on chemotherapy. An evaluation of the alkaline comet assays as a highly sensitive method for estimating chemosensitivity in bladder cancer is the ultimate objective of this second study. To evaluate the alkaline comet assay in such an endeavour it is important to understand the principles of chemotherapy and the specific features of commonly used agents. This Introduction aims to provide the necessary detailed information for understanding the action, toxicities, and the late side effects, as well as the conceptual basis for the use of cancer chemotherapeutic drugs.

### **3.2 CHEMOTHERAPY IN THE TREATMENT OF CANCER**

The ideal treatment for cancer is the killing of all cancer cells without affecting normal cells. Unfortunately such an ideal treatment dose not exist. Certain treatments are based on preventing tumour growth. However, since proliferation is such a general property of cells, methods designed to prevent growth will certainly have side effects. Consequently, the purpose of all current treatments is to maximise effects on the cancer whilst minimising adverse side effects on normal tissue. Surgical removal of the tumour mass is the best approach in eliminating cancer, but it has some limitations

(e.g. in the case of metastasis). Drug treatment is the most extensively used alternative therapy to surgery. The term chemotherapy is used to describe treatments based on drugs that affect cell processes such as DNA synthesis and cell proliferation. Chemotherapy is most effective against rapidly dividing cells and its effectiveness depends on the concentration of drug reaching the tumour and the time of its exposure. Chemotherapeutic drugs also have some side effects on normal cells. Side effects can be minimised by using combinations of drugs with different toxicities. Drug treatment can be given in three different settings. Primary or neoadjuvant chemotherapy describes its use as a first line treatment of local disease. Adjuvant chemotherapy uses agents following other treatments such as surgery and radiotherapy. Finally, chemotherapy can be used for treating advanced disease where surgery is not an option. In this case chemotherapy often does not cure a patient but its aim is to improve the quality of life and length of survival.

### **3.3 THE BASIS OF CANCER CHEMOTHERAPY**

Every phase of chemotherapeutic research, from drug discovery to clinical application, derives from the concepts of cancer cell biology. As such, chemotherapeutic drug research has evolved with the ever changing and expanding field of cancer cell biology. The essential properties of the cancer cell, properties that distinguish it from its normal counterparts and which form the basis of treatment, are excess proliferation, invasive capacity, ability to induce nutrient vessels, ability to escape immune surveillance, and the ability to metastasise. Each of these properties has been the starting point of drug discovery efforts. However, with few exceptions, the successful drugs have tackled only the first of these properties, proliferation.

#### **3.3.1 DRUGS THAT TARGET CELL PROLIFERATION**

Excessive proliferation of tumour cells is the consequence of many factors. The proliferative drive may result from sequential mutations that have several distinct effects. Some stimulate cell division (positive growth signals), whereas others free the cancer cell from normal cell cycle controls (loss of suppressor function) or inactivate cell death pathways. Other cancer causing mutations, such as the DNA repair defects and the loss of p53 function, increase the basic mutability of the genome. This enhanced mutability allows the generation of clones with inherent growth advantages.

In the past few years drug discovery efforts have targeted specific genetic defects. Among those defects are the activation of *Ras* in pancreatic, colon, and bladder cancer. The choice of targets is largely driven by the fact that it is exceedingly ambitious, if not impossible, to develop a product that replaces a missing suppressor gene product such as p53. On the other hand, inhibiting the function of a dominant gene product is a classic problem in drug discovery. In this case, drug design is a seemingly more straightforward task in which one identifies a key enzymatic process and develops a potent and specific inhibitor. Other pathways of great interest in modern cancer drug development are the Rb pathway (Sedlacek *et al.*, 1996), and protein kinase C (Dean *et al.*, 1994).

### **3.3.2 DRUGS THAT TARGET DNA SYNTHESIS AND MITOSIS**

Although much of the effort in modern cancer drug development is directed at targets that regulate growth signal transmission, most of the successful drugs in current clinical use act directly on the synthesis or integrity of DNA. These drugs inhibit synthesis of DNA or its precursors, block cell division, inhibit necessary changes in DNA topology, or covalently bind to DNA, causing strand breaks or miscoding. All such drugs affect the integrity of DNA and in the presence of the normal machinery for monitoring DNA integrity will induce programmed cell death (apoptosis). Unfortunately, they also tend to be cytotoxic toward normal cells. The reasons for selectively greater effects of cytotoxic drugs on malignant versus normal cells, as apparent in the cure of certain malignancies, are poorly understood, although the process of malignant transformation may enhance a cell's sensitivity to DNA damage (Lowe *et al.*, 1993). In designing clinical regimens, the relationship of drug action to the cell cycle is of particular importance, since this knowledge serves as the basis for developing drug combinations and sequences in clinical practice and influences their use in combination with radiation therapy.

The antimetabolites such as methotrexate, 5-fluorouracil, cytosine arabinoside, gemcitabine and the purine antagonists, require cells to be actively proliferating in order to be killed; therefore, they are cell cycle dependent. Many of these agents, including cytosine arabinoside, fludarabine phosphate, cladribine and 6-mercaptopurine, must be incorporated into DNA to be cytotoxic. Alternately, agents

such as etoposide and doxorubicin produce irreparable DNA strand breaks at any stage of the cell cycle, although these breaks become lethal only as the cell enters DNA synthesis. Still others, such as alkylating agents and platinum compounds, bind covalently to DNA and produce adducts, cross-links and strand breaks. Their toxicity seems less dependent on the stage of the cell cycle; indeed, some agents of this class, such as the nitrosoureas, are equally capable of killing nondividing and dividing cells (Hall 2000). Finally, a separate class of drugs, the antimetabolic agents, block the formation or dissociation of the mitotic spindle through their effects on microtubules and thereby prevent separation of chromosomes to the daughter cells. Drugs of this class are therefore most effective against cells that enter the mitotic phase of the cell cycle (Gunderson *et al.*, 2000).

Once damaged through its encounter with a cytotoxic drug, the cancer cell has several options and its eventual viability depends on which pathway it takes. If the normal monitors for genomic integrity (including most prominently the *p53* gene) are intact, the cell may halt further progression in the cell cycle while its DNA is repaired. If the damage is sufficiently severe, an intact functioning *p53* may initiate apoptosis (programmed cell death). If, however, the *p53* function is absent, cell cycle progression may continue despite drug-induced DNA damage and the cell may prove viable. In most, but not all, experimental settings lack of wild-type *p53* is associated with drug and radiation resistance, but with certain drugs loss of this checkpoint function actually sensitises cells to drug action (Li *et al.*, 1996).

The interaction of a chemotherapeutic agent with DNA may result in a hydroxyl, alkyl, aralkyl, or arylamine-modified nucleotide, which can cause mis-incorporation of the wrong base in the opposite strand during DNA replication. Alternatively, interaction of chemotherapeutic agent into the DNA strand can cause a frameshift mutation, and strand breaks may cause sequence additions or deletions. The fixation of the mutation in the genome depends on a dynamic equilibrium between cell replication and DNA repair processes. Prior to repair, normal DNA replication can produce mismatches where DNA adducts occur. These adducts have been used as an indicator of response to chemotherapeutic drugs. The concentration of adducts measured in the DNA is a consequence of an equilibrium between rates of adduct formation, repair, cell death and DNA replication. These adducts can be repaired if the amount of DNA damage

does not exceed the ability of the cell to repair. Cells defective in DNA repair tend to accumulate excess DNA damage, leading to apoptosis. It is conceivable, but not yet proven, that certain DNA repair defects, such as commonly found in mismatch repair (which is known to be involved in colon cancer), excision repair (ovarian cancer), or alkylation repair (gliomas), may be associated with greater sensitivity or, paradoxically, with reduced sensitivity to drug action (Fink *et al.*, 1997). From a theoretical viewpoint, it is understandable that rapidly dividing tumour cells, as found in leukemias, lymphomas and choriocarcinomas, might be exquisitely sensitive to antimetabolites and cell-cycle specific drugs.

### **3.4 DRUG ADMINISTRATION**

Cancer drugs are given in repetitive cycles of administration interrupted by rest periods that facilitate the recovery of normal tissues. The theoretical basis for cyclic chemotherapy was established by the work of Skipper and Schabel (Skipper *et al.*, 1964) who showed in animal models that for any given drug schedule and dose, a specific fraction of tumour cells is killed. Repeated cycles of treatment are therefore required for curative therapy. The fractional cell kill hypothesis must be modified to incorporate concepts of drug resistance. In practice, the response to an initial cycle of chemotherapy may be much greater than the subsequent doses and in many solid tumours, such as breast cancer and lung cancer, the cancer may regrow rapidly after an initial response. The explanation for this finding lies in the problem of drug resistance and the ability of cancer drugs to select for drug resistant cells. Although many examples of drug resistance have been characterized in model systems, the understanding of resistance in clinical practice remains incomplete. A likely reason for the development of resistance is that at the time of clinical recognition, tumours are significantly heterogeneous because of their underlying genetic instability (Hryniuk *et al.*, 1986; Gunderson *et al.*, 2000). Only the sensitive fraction of cells is killed with each dose of drug. Drug resistant mutants already exist at the time of initial treatment and under the selective pressure of therapy, expand in numbers in the presence of the drug.

Based on the experience of antibiotic treatment of bacterial infection, it was logical to combine drugs that had different mechanisms of resistance. Goldie and colleagues

have argued convincingly that the best strategy for chemotherapy is to employ as many non-cross resistant agents as early as possible in the treatment regimen (Goldie *et al.*, 1982).

With few exceptions, single agent treatment for cancer produces only temporary responses; multi agent therapy being more effective in the treatment of most human cancers. In general, the choice of drug combination therapy is made on the basis of mechanistic, pharmacokinetic, and toxicologic considerations. There are numerous examples of clinical regimens which combine agents with different mechanisms of action and different toxicity. Often these choices are dictated by impressive single agent activity in the tumour being treated and the uncertainty of knowledge about clinical mechanisms of drug resistance.

### **3.5 CHEMOTHERAPY IN THE TREATMENT OF BLADDER CANCER**

The use of chemotherapy in transitional cell carcinoma of the bladder has evolved from the use of single agent to combination chemotherapy. The evolution of chemotherapy has been such that it has clearly shown an impact on survival relative to that of single-agent therapy and that long-term disease free survival can be achieved in selective series. In view of its demonstrable effect on metastatic disease, it is logical to assume that chemotherapy given earlier in transitional cell carcinoma in combination with local modalities would result in improved cure results. The logic for combining early therapy with systemic therapy is based on the reasoning that the combination treatment of small volume disease would result in a more effective tumour cell kills. However, in the treatment of early disease, a portion of patients will survive without the use of cytotoxic chemotherapy and as such these patients do not benefit from this additional treatment, and may suffer associated short-term morbidity and long term risk.

Chemotherapy as a monotherapy achieves a clinical complete response in only 25% to 37% of bladder cancer patients (Maffezzini *et al.*, 1991). This is more frequently reported in early stage, small tumours and papillary tumours. Chemotherapy has been used with TURBT in an attempt to further spare the bladder. In a highly selected patient population, 5-years local control rates of up to 48% have been reported.

Pathological complete response rates after chemotherapy and TURBT range from 45% to 48% (Shearer *et al.*, 1988). These findings suggest that addition of chemotherapy to TURBT confers an advantage in terms of complete response rate (Shearer *et al.*, 1988; Scher *et al.*, 1989). Chemotherapeutic agents can also be used following partial cystectomy in a highly selected patient population (Herr *et al.*, 1994).

Chemotherapy in conjunction with radiotherapy is another approach for bladder cancer preservation. Many cytotoxic agents, in particular *cis*-platin and 5-fluorouracil were capable of sensitising tumour tissue to radiation and increase cell killing in a synergistic fashion. The term radiosensitisation can be defined as the application of an agent that when given concomitantly with ionising radiation, increases the lethal effects of ionising radiation (Shenoy *et al.*, 1992). Chemoradiation significantly improved complete response rates compared with complete response rates noted after the use of chemotherapy alone. After two cycles of CMV [*Cis*-platin, methotrexate and vinblastine], the complete response rate for patients with T2 disease was 62% and with T3 or T4 disease, 41%. After adding a course of concurrent chemoradiation, the complete response rate increased to 81% and 64% respectively (Gunderson *et al.*, 2000).

Chemotherapy in transitional cell carcinoma of the bladder can be applied after (adjuvant) or preceding (neoadjuvant) local therapy. Neoadjuvant chemotherapy has been used in two general settings: before radical cystectomy to enhance local control and in conjunction with radiotherapy for bladder preservation. However, the theoretical advantages of a neoadjuvant approach are that it provides a systemic therapy at the earliest opportunity and chemotherapy sensitivity can be judged on a case-by-case basis by evaluating the response of the primary tumour in the bladder.

*Cis*-platin is currently the most widely used and probably the most active agent in transitional cell carcinoma of the bladder. Overall response rates with *cis*-platin ranged from 21% to 31%; complete response was observed in 0% to 10% of patients (Fagg *et al.*, 1984). Other drugs that have been investigated in the treatment of bladder cancer include carboplatin, methotrexate, ifosfamide, doxorubicin, 5-fluorouracil, vinblastine, and mitomycin-C. Carboplatin achieves response rates of

14%, and a complete response in 2% of patients (Motter-Auselo *et al.*, 1993). Methotrexate achieves response rates in 29% and a complete response in less than 10% of patients (Oliver *et al.*, 1986). Other single agents drugs under investigation include ifosfamide with a response rate 28% (Witte *et al.*, 1993), doxorubicin (17%), 5-fluorouracil (15%), vinblastine (16%) and mitomycin-C (13%) (Gunderson *et al.*, 2000).

Numerous randomised, controlled trials clearly exhibit the superiority of multitarget regimens over a single drug regimen in the treatment of invasive bladder cancer in terms of both overall and complete response rates. Three and four-drug regimens such as MAVC [methotrexate, adriamycin, vinblastine and *cis*-platin] (Sternberg *et al.*, 1988), CMV [*cis*-platin, methotrexate and vinblastine] (Harlcer *et al.*, 1985), *cis*-platin and methotrexate, and CISCA [*cis*-platin, cyclophosphamide, and adriamycin] (Logothetis *et al.*, 1989) can produce significantly improved results in terms of survival for invasive bladder cancer, with response rates of 70%, of which 50% are complete responses.

### **3.6 CHEMOTHERAPEUTIC AGENTS USED IN THE TREATMENT OF BLADDER CANCER**

Most generally used chemotherapeutic agents can be divided into four different classes: alkylating agents, antitumour antibiotics, antimetabolites and miscellaneous. Some are phase specific like the antimetabolites, while others are equally active against non-dividing cells like the alkylating agents and the platinum derivatives. Mitomycin-C and *cis*-platin are important drugs used in the treatment of superficial and invasive bladder cancer, respectively, and both have been investigated. Therefore, it is essential to understand the principles and the specific features of these agents.

#### **3.6.1 MITOMYCIN-C**

The mitomycins are a group of potent antibiotics that were discovered in the 1950s. One member of the family is mitomycin-C (Figure 3.1), which has been used for clinical cancer chemotherapy since the 1960s because of its broad-spectrum of activity against solid tumours. Mitomycin-C is sometimes used in combined

chemotherapy in breast, lung, and prostate cancer. It has some activity against colorectal cancer, and is the drug of choice for intravesical administration in superficial bladder cancer (Carter *et al.*, 1979). In addition to its antitumour activity, mitomycin-C has a variety of specific biological effects in mammalian cells, including selective inhibition of DNA synthesis, recombination, chromosome breakage, sister chromatid exchange and induction of DNA repair in bacteria (Carrano *et al.*, 1979).

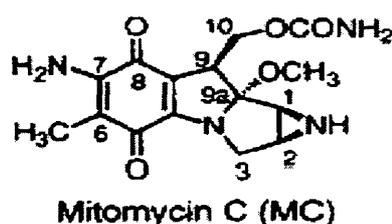


Figure 3.1. Structure of mitomycin-C.

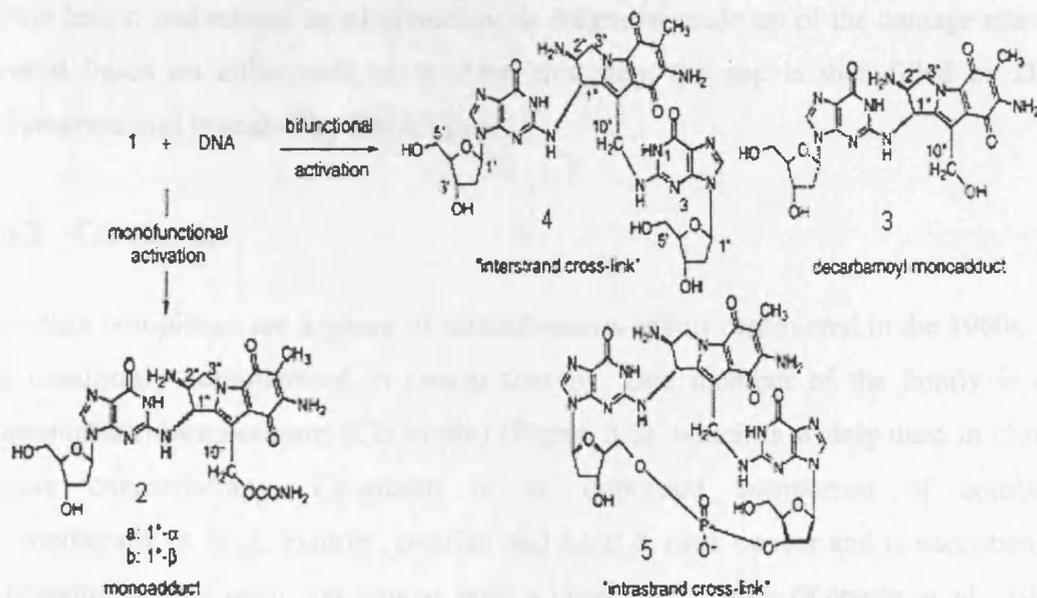
Early studies of the molecular pharmacology of mitomycin-C revealed an extraordinary property of this class of antitumour antibiotics; mitomycin-C was found to cross-link the complementary strands of DNA (Lyer *et al.*, 1963). It has been demonstrated that mitomycin-C exerts its antiproliferative activity primarily as a DNA replication inhibitor, and there is much evidence indicating that the mitomycin-C induced cross-links are fundamentally responsible for this inhibition (Tomasz *et al.*, 1997).

Mitomycin-C is an extremely toxic antitumour antibiotic; it is activated *in vivo* to a bifunctional alkylating agent (Tomasz *et al.*, 1997). Mitomycin-C is always administered intravenously. It is cleared from the plasma with a half-life of 10 to 15 minutes, primarily by metabolism in the liver. Mitomycin-C is known to induce DNA damage in cells in the form of DNA cross-links and monofunctional DNA alkylation (attachment of the drug molecule to only one DNA strand). It has been demonstrated that a single cross-link per genome was sufficient to cause death in bacterial cells (Szybalski *et al.*, 1964). Bifunctional alkylation of DNA by cross-linking agents has been shown to be profoundly more cytotoxic than alkylation by a corresponding monofunctional agent (Pratt *et al.*, 1994). As a result, DNA interstrand cross-links are regarded as a highly lethal type of DNA damage, and it has been hypothesized that

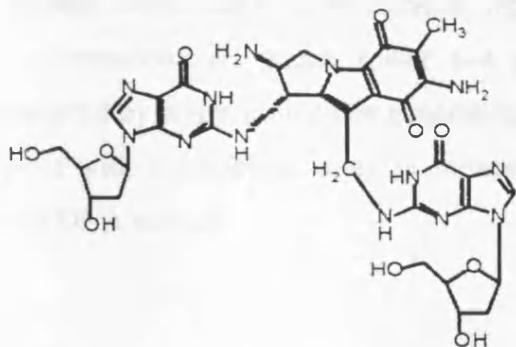
cross-links at replication forks may pose a more severe impediment to replication than the corresponding monofunctional drug DNA adduct. An alternative hypothetical rationale for the high cytotoxicity of cross-links is that the cellular repair mechanism for cross-linked DNA is less efficient than that of monofunctional lesions. Mitomycin-C possesses unique antitumour selectivity against hypoxic regions of solid tumours, indicating that oxygen can decrease the DNA damage produced by this drug in intact cells (Palom *et al.*, 2001).

A unique feature of the molecular mechanism of mitomycin-C action is that DNA cross-linking and alkylating activity requires the reduction of the quinone moiety, which transforms the drug to a highly reactive alkylator (Tomasz *et al.*, 1997). The mechanism begins with the C-1 aziridine and the C-10 carbamate groups of the mitomycin-C. These are two masked alkylating functions that become “allylic” and therefore activated as a result of reduction of the quinone and consequent spontaneous elimination of methanol from the 9- and 9a position. Their subsequent displacement by two nucleophiles in DNA results in a mitomycin-C DNA cross-link (Lyer *et al.*, 1964). The structure of all the major mitomycin-C DNA adducts formed in cell free systems is shown in Figure 3.2. Under reductive conditions mitomycin-C alkylates only the 2-amino group of guanine nucleosides in the minor groove of DNA. The distribution of the various adducts depends on the reductive activating conditions employed. Under monofunctional activation, only the monoadducts 2a (major) and 2b (minor) are formed, while bifunctional activation gives rise to monoadduct 3 and bisadducts 4 and 5; these are the interstrand and intrastrand cross-links adducts (Figure 3.3 & 3.4), respectively.

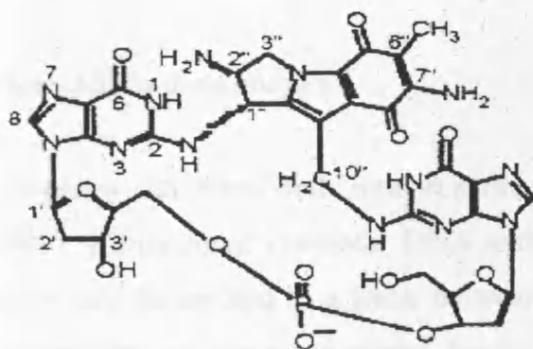
Mitomycin-C adducts are believed to be repaired by NER (Leadon *et al.*, 1996). NER is fully described in Section 2.5.5 and Figure 2.11; briefly in NER, a group of enzymes, termed *structure specific endonucleases*, commence the excision repair process. These do not recognise the specific lesion, but they are thought instead to recognise more generalised structural distortions in DNA that necessarily accompany a larger base adduct. The endonucleases incise the affected DNA strand on both sides



**Figure 3.2.** Mitomycin-C DNA adducts formed with reductively activated mitomycin-C (Tomasz *et al.*, 1997).



**Figure 3.3.** Mitomycin-C interstrand cross-links.

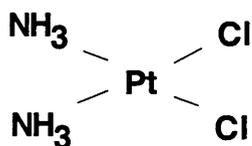


**Figure 3.4.** Mitomycin-C intrastrand cross-links.

of the lesion and release an oligonucleotide fragment made up of the damage site and several bases on either side of it. After this step, the gap is then filled by DNA polymerase and is sealed by DNA ligase.

### 3.6.2 CIS-PLATIN

Platinum complexes are a group of miscellaneous agents discovered in the 1960s, and are commonly administered in cancer therapy. One member of the family is *Cis*-diamminedichloroplatinum (*Cis*-platin) (Figure 3.5), which is widely used in clinical cancer chemotherapy. *Cis*-platin is an important component of combined chemotherapy in lung, bladder, ovarian and head & neck cancer and is exceptionally successful against testicular cancer with a cure rate of 95% (Köberle *et al.*, 1997). *Cis*-platin is administered intravenously. It is kept in its inactive form in the blood stream because the chloride concentration is high (~100 mM) and therefore platinum remains coordinated to its chloride ligands. Upon entering the cell, where chloride concentrations are much lower (~4 mM), the chloride ligands of *cis*-platin are replaced by water molecules generating a positively charged aquated species that can react with nucleophilic sites in intracellular macromolecules to form protein, RNA and DNA adducts.

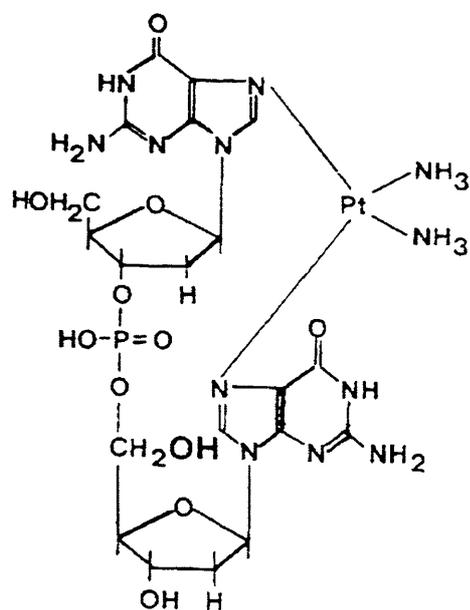


**Figure 3.5.** *Cis*-platin structure.

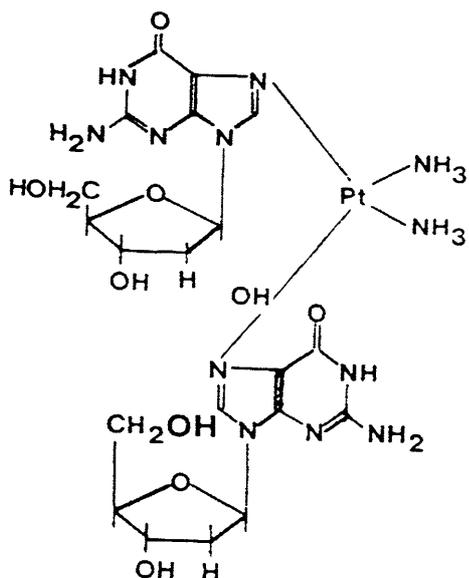
*Cis*-platin can form both monofunctional and bifunctional adducts (Försti *et al.*, 1989). Bifunctional *cis*-platin DNA adducts can be formed between two adjacent nucleotide bases (and to a lesser extent between two nucleotides separated by one or more nucleotides) in the same strand forming intrastrand cross-links, or between two nucleotides in different DNA strands to form interstrand cross-links. The antitumour activity of *cis*-platin is thought to be associated with its ability to form intrastrand cross-links (Reedijk *et al.*, 1985). Adducts that are not removed by DNA repair may

block DNA replication and transcription. Cells deficient in DNA repair are more sensitive to *cis*-platin than the corresponding wild type, indicating that DNA adducts are the key toxic lesions formed by this agent. There is an inverse correlation between DNA adduct levels and survival after *cis*-platin treatment in cell culture (Reed *et al.*, 1993) and a direct correlation between DNA adducts in blood cells of cancer patients and clinical response has been found (Schellens *et al.*, 1996).

*Cis*-platin preferentially binds to the N7 position in purines and forms two major adducts, *Cis*-Pt(NH<sub>3</sub>)<sub>2</sub> d(pGpG) and *Cis*-Pt(NH<sub>3</sub>)<sub>2</sub> d(pApG), both giving rise to intrastrand cross-links (Figure 3.6) between neighbouring bases, and a very small percentage of interstrand cross-links (Figure 3.7) and monofunctional adducts (Försti *et al.*, 1989). *Cis*-platin treatment results in arrest at the G2 phase of the cell cycle leading to apoptosis. *Cis*-platin is a highly toxic agent and it is cell cycle non-specific. *Cis*-platin adducts are repaired by NER (Leadon *et al.*, 1996). NER is fully illustrated in Section 2.5.2 and Figure 2.12.



**Figure 3.6.** *Cis*-platin intrastrand cross-links.



**Figure 3.7.** Cis-platin interstrand cross-links.

### 3.7 METHODS TO ASSESS CHEMOTHERAPY DRUG CROSS-LINK FORMATION AND CYTOTOXICITY

DNA damaging agents have been commonly used in cancer chemotherapy for many years with varying degree of success in the treatment of cancer. A spectrum of lesions is induced in DNA by drugs. Particular types of lesions are recognised for each class of agents. Alkylators cause covalent binding of alkyl groups preferentially to the O<sup>6</sup> and N<sup>7</sup> positions of guanine bases to form drug adducts. Monofunctional agents will lead to monofunctional adducts (which is the presence of an adduct on one reactive group), however, the action of a bifunctional agent leads to interstrand or intrastrand DNA-DNA cross-links. Several commonly used clinical agents, such as mitomycin-C and *Cis-platin*, are bifunctional. DNA interstrand cross-links (ISC) formed in cells are clearly critical cytotoxic lesions and the formation of DNA interstrand cross-links has been shown to correlate with cytotoxicity *in vitro* (Sunters *et al.*, 1992).

The identification of the biologically significant lesions can be achieved by correlating cell killing with the production of different lesions within one cell type or/and by comparing the levels of damage produced in different cells of varying

sensitivity to the agent. Cell killing is defined as the loss of reproductive capacity and is directly assessed by clonogenic assay and indirectly by the MTT assay. DNA interstrand cross-link formation and repair can be specifically measured by alkaline elution, agarose gel and single cell-gel electrophoresis (comet assay). All these assays for cell killing and cross-link formation and repair are discussed in detail in the following section.

### **3.7.1 ASSAYS OF CYTOTOXICITY**

#### **3.7.1.1 Clonogenic Assay**

The clonogenic assay is the method of choice for measuring the cytotoxicity of cells exposed to chemotherapeutic agents. The assay is described in Section 2.6.2 and Figure 2.17. Briefly, the cytotoxicity is measured by the loss of reproductive integrity, that is the inability to sustain proliferation indefinitely. The basis of the clonogenic assay is to assess the ability of the cell to grow to form a large colony, which can be seen by the naked eye, as proof that the cell had retained its reproductive integrity after treatment with chemotherapeutic agents (Hall 2000).

Clonogenic assay has been used in the past to measure cytotoxicity in testicular and bladder cancer cell lines exposed to *cis*-platin (Köberle *et al.*, 1997). Köberle demonstrated that cells derived from the testis and the bladder showed differences in their response to *cis*-platin, and that the cells could be divided based on their response to *cis*-platin. This assay indicates that the chemosensitivity may be a useful predictor to improve the results of treatment centered on chemotherapy. Nevertheless, the time scale and the inability of some sampled biopsies to yield colonies are the disadvantages of this assay.

#### **3.7.1.2 MTT Assay**

The MTT assay is a widely used semi-automated method. It is a colorimetric assay in which the response is measured by a colorimetric reaction. The MTT assay was developed to estimate cell survival. It quantifies viable cells through their ability to reduce 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltertazolium bromide, a soluble yellow

tetrazolium salt to a blue formazan precipitate. This process requires active mitochondrial function because it utilises the activity of mitochondrial enzyme, succinate dehydrogenase. The MTT assay has been described in Section 2.11.3.

The MTT assay has been used extensively for measuring drug cytotoxicity *in vitro*. Loprevite demonstrated that the MTT assay could be used as a suitable assay for estimating the chemosensitivity in a panel of lung cancer cell lines using *cis*-platin (Loprevite *et al.*, 2001). In that study, differing drug dose response curves were created indicating that the MTT assay can be used as a useful predictor of cellular chemosensitivity. However, this assay is insensitive at low doses and the need to optimise the assay conditions for every cell line limits its potential clinical application as a predictive test.

### **3.7.2 ASSAYS OF DNA CROSSLINKS FORMATION**

#### **3.7.2.1 Alkaline Elution**

The alkaline elution method employ filters to distinguish DNA single strand sizes in mammalian cells. The filters do not adsorb DNA under the conditions employed, but rather act mechanically to impede the passage of long DNA strands. Variations of the method can be used to measure single strand breaks, alkali labile sites, DNA protein cross-links, and interstrand cross-links. The sensitivity of the measurement is of the order of one DNA lesion per  $10^7$  nucleotides. The assay is conducted by placing the cells on filters prior to lysis with a detergent solution. Most of the cell protein and RNA can be washed through the filter leaving the DNA intact on the filter. An alkaline solution of about pH 12 is then slowly pumped through the filter and the rate of elution of the DNA is measured. Single strand breaks are measured on the basis of an increase in DNA elution rate. Cross-links are gauged on the basis of reductions in elution rates due to an effective increase in strand length in the case of interstrand cross-links, or due to an adsorption of protein to the filter in the case of DNA protein cross-links (Kohn *et al.*, 1981). The effects of cross-links on DNA elution rate are amplified by introducing into the DNA an appropriate frequency of single strand breaks. This is accomplished by subjecting the cells to a standard dose of X-rays prior to carrying out the elution procedure. Cross-links reduce the DNA elution that would

otherwise be produced by the X-ray dose alone. Although sensitive enough to measure interstrand cross-links at pharmacologically relevant doses, alkaline elution requires a relatively large number of cells and a radiolabelling step prior to performing the assay, which make it hard to adapt toward *in vivo* studies (Spanswick *et al.*, 2002).

### 3.7.2.2 Agarose Gel Electrophoresis

The agarose gel electrophoresis method is a simple method of detecting DNA interstrand cross-link formation in plasmid DNA *in vitro*. This is accomplished by complete denaturation of the labelled DNA. The presence of an interstrand cross-link results in renaturation to double stranded DNA. The single and double stranded bands are separated on an agarose gel and can be accurately measured by densitometry of the autoradiograph produced from the dried gel. The method is particularly suitable for detailed time course experiments of cross-link formations in particular, following removal of free drug, the “second-arm” of cross-link reaction. This method is sensitive to follow the formation of cross-links by slow and inefficient cross-linking agents (Hartley *et al.*, 1991). However, it is only suitable for *in vitro* studies.

### 3.7.2.3 Comet Assay

The comet assay is introduced in Section 2.6.9 with the alkaline version of the comet assay described in detail in Section 2.6.9.2. DNA interstrand cross-link formation produced by chemotherapeutic agents and the repair of these cross-links can be measured easily using a modified version of the alkaline comet assay. Interstrand cross-links prevent DNA strands from separating in alkali, so as the number of cross-links increases, the effective size of the DNA molecules increase and the amount of DNA able to migrate is reduced. To identify the presence of cross-links induced by some chemotherapeutic drugs, cells can be exposed to an increasing duration of electrophoresis to such an extent that the DNA of control cells exhibit significant migration or by using a secondary agent, such as hydrogen peroxide or ionising radiation (Blasiak *et al.*, 2000), to induce a fixed level of random DNA strand breakage. The presence of DNA cross-links will retard the migration of the irradiated

DNA during electrophoresis, giving a reduced tail moment compared to the non-cross-linked irradiated control (Figure 3.8 and 3.9) (Olive *et al.*, 1995).

Many studies have examined the ability of the alkaline comet assay to detect cross-links. Listed below are some of the major studies. The alkaline comet assay was successful in detecting genotoxicity caused by platinum anticancer drugs *in vivo* (Blasiak *et al.*, 2000). In that study, *cis*-platin formed cross-links with DNA in lymphocytes, and these lesions were slowly removed through repair. The genotoxicity was determined by the inability of the lymphocytes to repair the DNA cross-links formed by *cis*-platin at a higher concentration. The alkaline comet assay was also able to detect DNA interstrand cross-links formation and repair in plasma cells from myeloma patients treated with melphalan. In this study, the *in vitro* sensitivity to melphalan of plasma cells was found to correlate with interstrand cross-links repair (Spanswick *et al.*, 2002). Other studies indicate that DNA interstrand cross-link formation correlates with cytotoxicity *in vitro* (Sunters *et al.*, 1992). Further studies investigated the ability of the alkaline comet assay to detect multi drug resistance *in vitro* and *in vivo* (Huang *et al.*, 1998). Finally, a study by McKenna (McKenna *et al.*, 2003) revealed the ability of the alkaline comet assay to detect mitomycin-C induced interstrand cross-links in one bladder cancer cell line, RT4.

### **3.8 RATIONALE FOR THE STUDY**

In the on-going search for effective local control with improved survival in bladder cancer, chemotherapeutic agents have been used with local modalities. Nevertheless, in the treatment of some patients, local control is accomplished with out the use of cytotoxic chemotherapy and as such these patients do not benefit from this additional treatment. Numerous attempts have been made to improve the outcome for muscle invasive bladder cancer following treatment. These improvements would ideally include the use of an assay that can predict tumor sensitivity on an individual basis.

Many chemotherapeutic agents are limited in their application due to the severe side effects. However, in the search for reducing adverse side effects and improving clinical effectiveness of these chemotherapeutic agents, it is desirable that a reliable test be established to measure the response of tumour cells to chemotherapy which

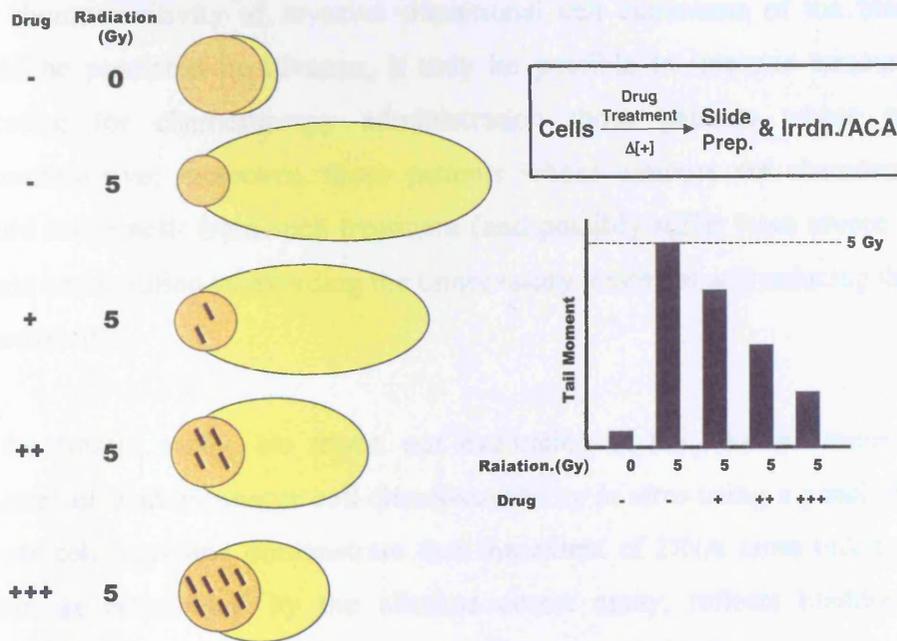


Figure 3.8. Schematic depicting the detection of crosslink formation by the alkaline comet assay.

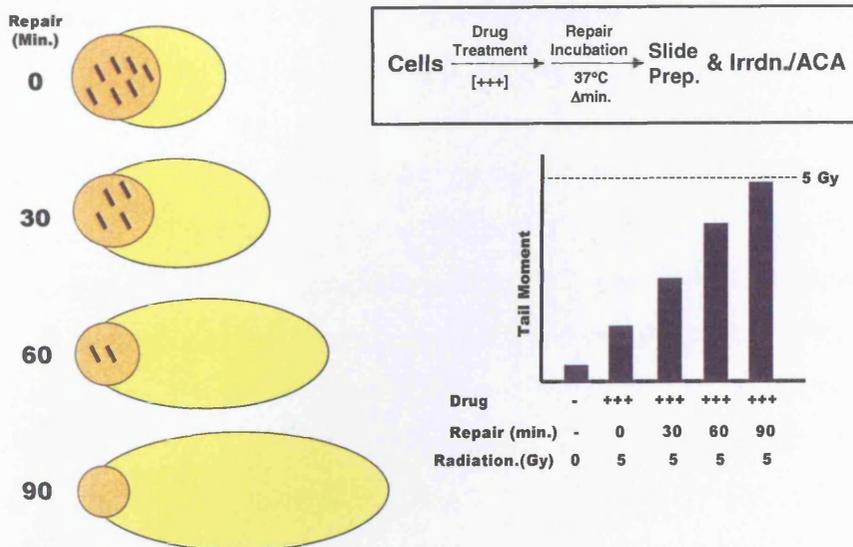


Figure 3.9. Schematic depicting the detection of crosslink repair by the alkaline comet assay.

would be able to distinguish between chemosensitive and chemoresistant tumours. If the chemosensitivity of invasive transitional cell carcinoma of the bladder (TCC) could be predicted in advance, it may be possible to improve tumour control by selecting for chemotherapy administration those patients whose tumours are chemosensitive; moreover, those patients whose tumours are chemoresistant, who would not benefit from such treatment (and possibly suffer from severe side effects) would be identified so avoiding the unnecessary treatment and reducing the associated genotoxicity.

In the present study, we report our evaluation of the alkaline comet assay, as a measure of bladder cancer cell chemosensitivity *in vitro* using a panel of six bladder cancer cell lines and demonstrate that the extent of DNA cross-link formation and repair, as determined by the alkaline comet assay, reflects bladder cancer cell chemosensitivity.

## 3.9 MATERIALS AND METHODS

### 3.9.1 CELL LINES AND CULTURE CONDITIONS

Six bladder cancer cell lines derived from high grade transitional cell carcinoma, namely J82, T24, RT112, UM-UC-3, HT1376 and RT4, were used in the study. Culture conditions were as described in Section 2.11.1.

### 3.9.2 CLONOGENIC ASSAY

Cells from exponential growing culture are prepared into suspension by the use of trypsin as described in Section 2.11.2. The number of cells per unit volume of this suspension was counted and adjusted to  $1 \times 10^5$  cells/ml. The cells were then treated with *cis*-platin using seven different drug concentrations: 0, 10, 15, 20, 25, 30, 35 and 40  $\mu\text{M}$  in 1 ml of culture medium. For treatment with mitomycin-C, cells were treated with 0, 2, 4, 6, 8, 10, 12, and 15  $\mu\text{M}$  in 1 ml of culture medium. Cells were incubated for one hour at 37°C with occasional agitation and then washed three times using PBS. The cells were then seeded in appropriate number in 60 x 15 mm petri dishes with 10 ml of corresponding culture medium. The number of cells seeded per dish is adjusted based on the plating efficiency of each cell line, so that a countable number of colonies will result. The plating efficiencies are given in Section 2.11.2.

For J82 and HT1376, control cells and the 10, 15 and 25  $\mu\text{M}$  *cis*-platin treated cells were seeded at 500 cells/dish. At the higher drug doses of 30, 35 and 40  $\mu\text{M}$  the numbers of cell seeded were increased to 800 and 1000 cells/dish, respectively. For RT112 control cells and the 10, 15 and 20  $\mu\text{M}$  *cis*-platin treated cells were seeded at 200 cells/dish. For 25 and 30  $\mu\text{M}$ , the numbers of cells seeded were increased to 500 cells/dish and for 35 and 40  $\mu\text{M}$  the number of cells seeded were 1000 cells/dish. For T24 and UM-UC-3, control cells and the 10, 15 and 25  $\mu\text{M}$  *cis*-platin treated cells were seeded at 100 cells/dish. For higher drug concentrations of 30, 35 and 40  $\mu\text{M}$  the numbers of cells seeded were increased to 500, 800 and 1000 cells/dish, respectively. Finally, for RT4, control cells and the 10, 15 and 25  $\mu\text{M}$  *cis*-platin treated cells were seeded at 300 cells/dish. For the 30, 35 and 40  $\mu\text{M}$  treatments the numbers of cells seeded were increased to 500, 600 and 800 cells/dish, respectively.

For mitomycin-C treatment, different drug concentrations were used due to the high cytotoxicity of this drug. Based on the plating efficiency of the six bladder cancer cell lines, the numbers of cells seeded were as follows. For J82 and HT1376, control cells and the 2, 4 and 6  $\mu\text{M}$  mitomycin-C treated cells were seeded at 500 cells/dish. At the higher drug doses of 8, 10, 12 and 15  $\mu\text{M}$  the numbers of cells seeded were increased to 800, 1000, 1500 and 5000 cells/dish, respectively. For RT112, control cells and the 2, 4 and 6  $\mu\text{M}$  mitomycin-C treated cells were seeded at 200 cells/dish. For 8 and 10  $\mu\text{M}$ , the numbers of cells seeded were increased to 500 cells/dish, and for 12 and 15  $\mu\text{M}$ , the numbers of cells seeded were 1000 cells/dish. For T24 and UM-UC-3, control cells and the 2, 4 and 6  $\mu\text{M}$  mitomycin-C treated cells were seeded at 100 cells/dish. For higher drug concentrations of 8, 10, 12 and 15  $\mu\text{M}$ , the numbers of cells seeded were increased to 500, 800, 1000 and 1500 cells/dish, respectively. Finally, for RT4, control cells and the 2, 4 and 6  $\mu\text{M}$  mitomycin-C treated cells were seeded at 300 cells/dish. For 8 and 10  $\mu\text{M}$  treatments the numbers of cells seeded were increased to 500 cells/dish, and for the 12 and 15  $\mu\text{M}$  treatments the number of cells seeded were 1000 cells/dish.

Dishes were subsequently incubated, fixed, the colonies counted and the surviving fraction determined as described in Section 2.11.2.

### **3.9.3 MICROTETRAZOLIUM ASSAY (MTT)**

Cells from exponential growing culture were harvested into a single cell suspension by the use of trypsin as described in Section 2.11.1. The number of cells per unit volume of this suspension was counted and adjusted to  $1 \times 10^5$  cells/ml. The cells were then treated with mitomycin-C or *cis*-platin using four different drug concentrations (15, 50, 100 and 200  $\mu\text{M}$ ) in 1 ml of culture medium; untreated cultures were processed in parallel. Cells were incubated for one hour at 37°C with occasional agitation and were then washed three times using PBS. The MTT assay was then performed on the six bladder cancer cell lines as described in Section 2.11.3.

### **3.9.4 ALKALINE COMET ASSAY**

The alkaline comet assay is a relatively simple, rapid and sensitive method for measuring DNA strand breaks at the level of single cells. Numerous alterations to the original comet assay have been developed to increase its applications. One such alteration permits the measurement of DNA cross-links by assessing the relative reduction in DNA migration induced by a strand-breaking agent.

#### **3.9.4.1 Drug Treatment and Alkaline Comet Assay Analysis**

Cells from exponential growing culture were harvested into a single cell suspension by the use of trypsin as described in Section 2.11.1. The number of cells per unit volume of this suspension is counted and adjusted to  $1 \times 10^5$  cells/ml. Cell suspensions were then treated with 0, 50, 100 and 200  $\mu\text{M}$  of mitomycin-C or *cis*-platin in 1 ml of culture medium. The cells were placed at 37°C for one hour with occasional agitation. After drug treatment, the eppendorfs were centrifuged at 1500rpm for 5 minutes and each cell pellet is washed three times with 1 ml PBS and processed for the alkaline comet assay as described in Section 2.11.5, except that prior to lysis the prepared cell slides were irradiated with 5 Gy as described in Section 2.11.5.2. For repair studies, cells were treated with 50  $\mu\text{M}$  of drug and incubated at 37°C for one hour with occasional agitation. After treatment, cells were centrifuged at 1500rpm for 5 minutes and the pellets were resuspended in growth medium (RPMI supplemented with 20% FCS) and allowed to repair for 30, 60 and 90 minutes with occasional agitation. Cells were washed three times with PBS as described above, and processed for the alkaline comet assay as described in Section 2.11.5, except that prior to lysis the prepared cell slide were irradiated with 5 Gy as described in Section 2.11.5.2. Additional non-drug treated cells were prepared by incubating them at 30, 60 and 90 minutes at 37°C in repair media. These cells are then processed in parallel with the drug treated cells for the alkaline comet assay as described in Section 2.11.5.

#### **3.9.4.2 Comet Image Capture and Analysis**

50 cells per slide were analysed to obtain a result representative of the population of cells. The comet image capture analysis was undertaken as described in Section

2.11.5.5. The percentage decrease in mean Olive tail moment due to cross-link formation is calculated using the following formula:

$$\% \text{ Decrease in Mean Olive tail moment} = \left(1 - \frac{(\text{TMdi} - \text{TMcu})}{(\text{TMci} - \text{TMcu})}\right) \times 100$$

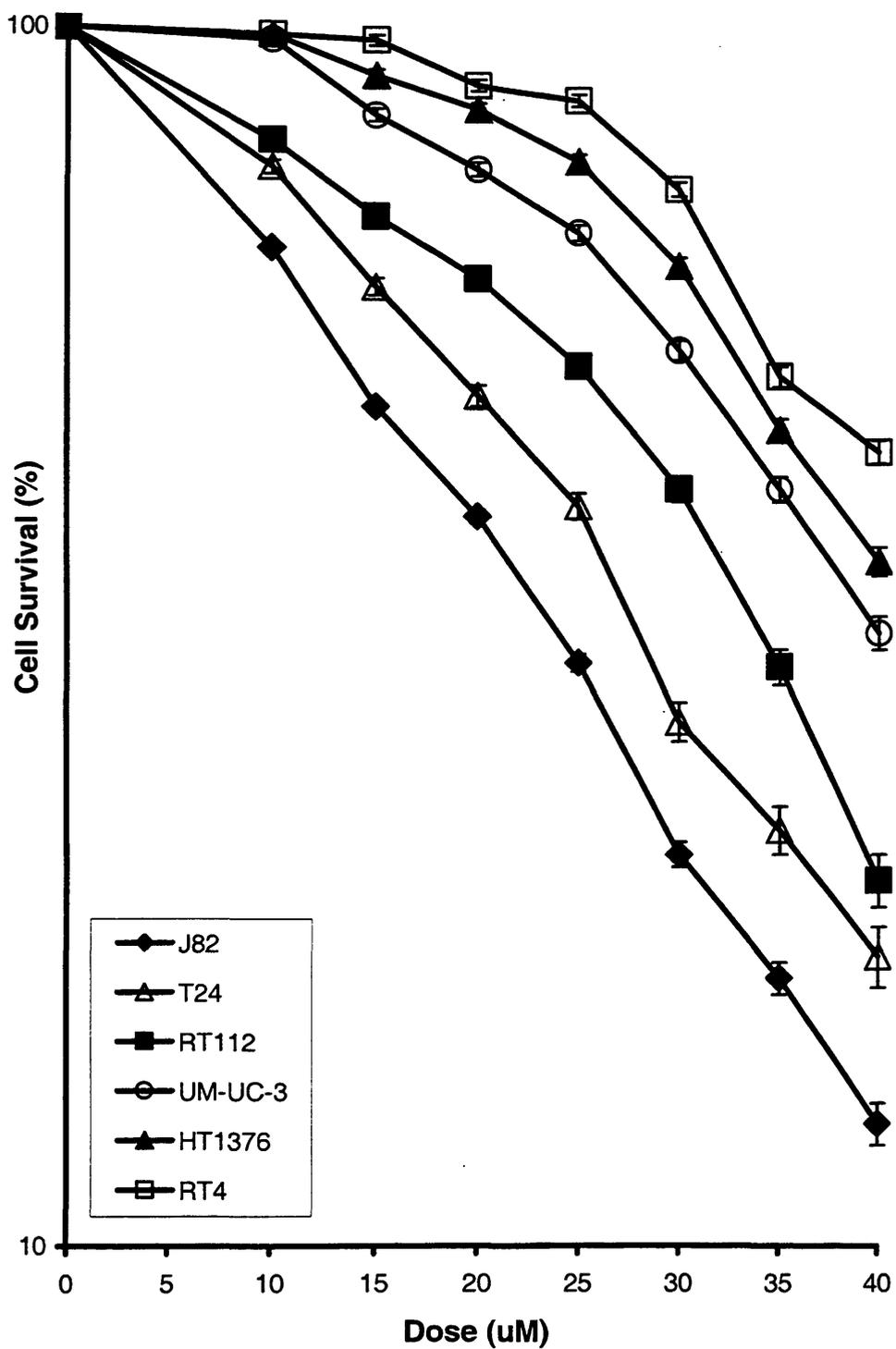
Where TMdi is the mean Olive tail moment of drug treated irradiated sample, TMcu is the mean Olive tail moment of untreated unirradiated control and TMci is the mean Olive tail moment of untreated irradiated control. The percentage decrease in tail moment is proportional to the level of DNA crosslinking.

### 3.10 RESULTS

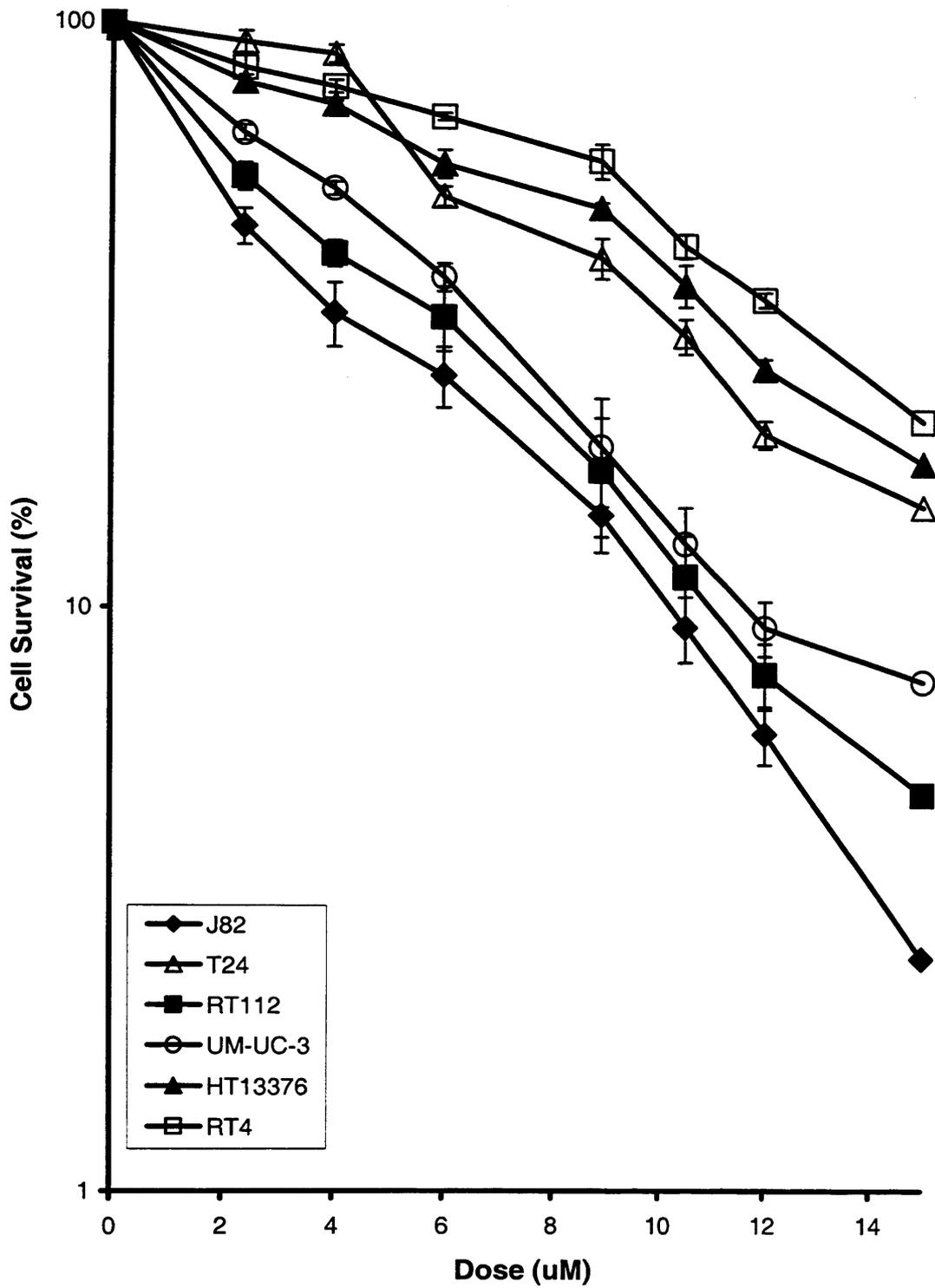
Survival responses have been determined for the six-bladder cancer cell lines investigated over a dose range of 0-40  $\mu\text{M}$  *cis*-platin and 0-15  $\mu\text{M}$  mitomycin-C using the clonogenic assay. The results are shown in Figures 3.10 & 3.11. These cell lines encompass a range of chemosensitivities, with J82 and T24 being the most sensitive to *cis*-platin-induced cell killing, and HT1376 and RT4 being the most resistant to *cis*-platin induced-cell killing. For mitomycin-C, it was observed that J82 is the most sensitive to mitomycin-C induced-cell killing, followed closely by RT112 and UM-UC-3 cell lines, with HT1376 and RT4 being the most resistant to mitomycin-C induced-cell killing. Notably, one cell line, T24, demonstrated a chemoresistant response at low doses (2, and 4  $\mu\text{M}$  of mitomycin-C) but a more chemosensitive response at higher doses.

Cell viability has been measured for the six-bladder cancer cell lines over a dose range of 0-200  $\mu\text{M}$  *cis*-platin and mitomycin-C using the MTT assay; the results are shown in Figures 3.12 & 3.13, respectively. The cell lines encompass a range of chemo-responses, with J82 being the most sensitive cell line to *cis*-platin and mitomycin-C-induced loss of cell viability, and HT1376 and RT4 being the most resistant. Interestingly, the T24 cell line shows a resistant response at low treatment doses (50  $\mu\text{M}$ ) of mitomycin-C but a sensitive response at the higher doses.

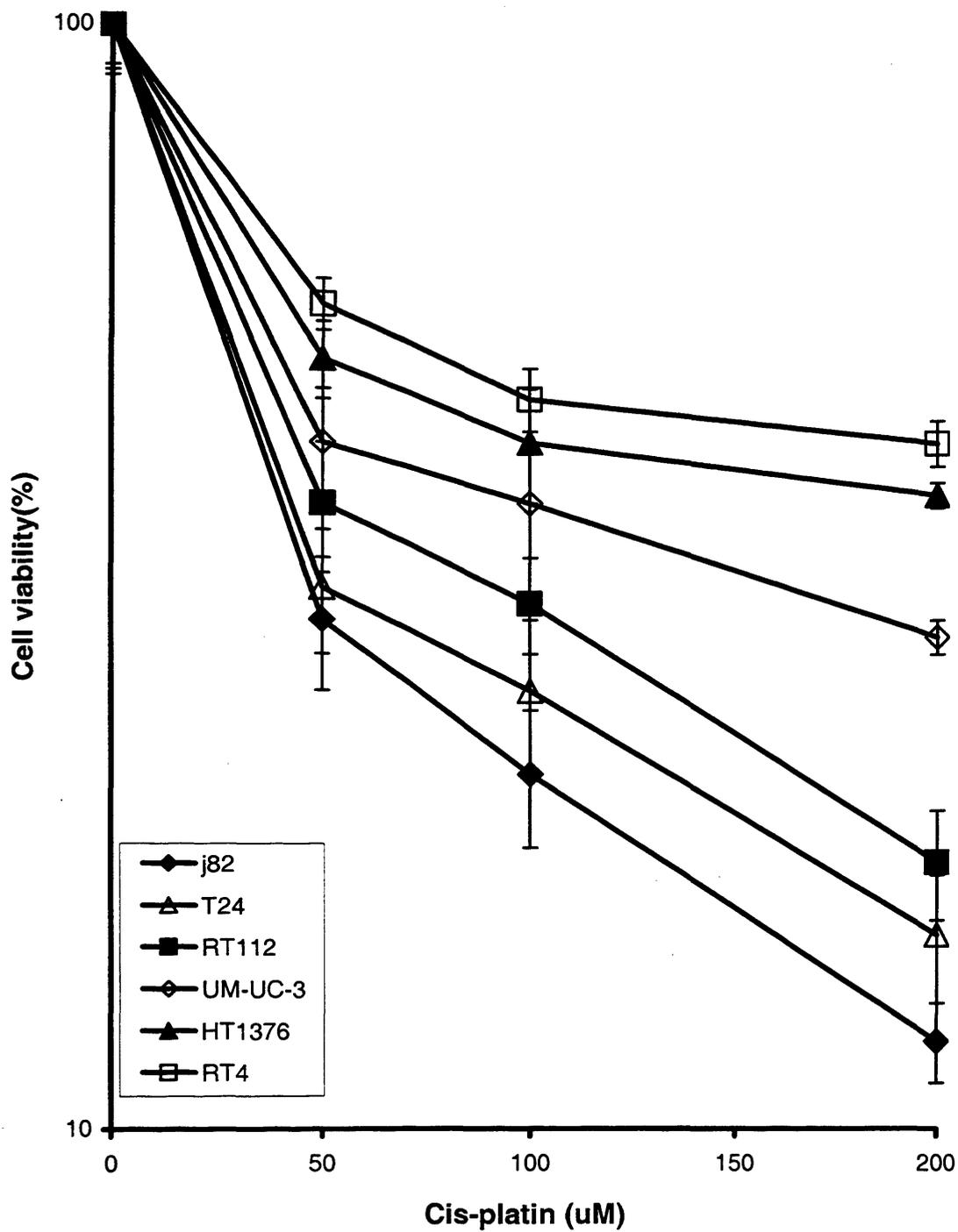
The level of DNA cross-link formation was measured for the six bladder cancer cell lines, over a dose range of 0-200  $\mu\text{M}$  *cis*-platin using the alkaline comet assay. The results are shown in Figure 3.14. Cross-link formation was assessed by the relative reduction in DNA migration after exposure to 5 Gy X-irradiation. The mean Olive tail moment for each cell line was measured and clear dose response curves were obtained, with the chemosensitive cell line J82 displaying the highest level of *cis*-platin induced cross-link formation, and the most chemoresistant cell lines RT4 and HT1376 exhibiting the lowest level of *cis*-platin induced cross-link formation. The rank order for cross-link formation matches the rank order of cell killing for all six-cell lines. Furthermore, at all drug concentrations studied, the rank order of cell viability as determined by the MTT assay agrees with the level of cross-link



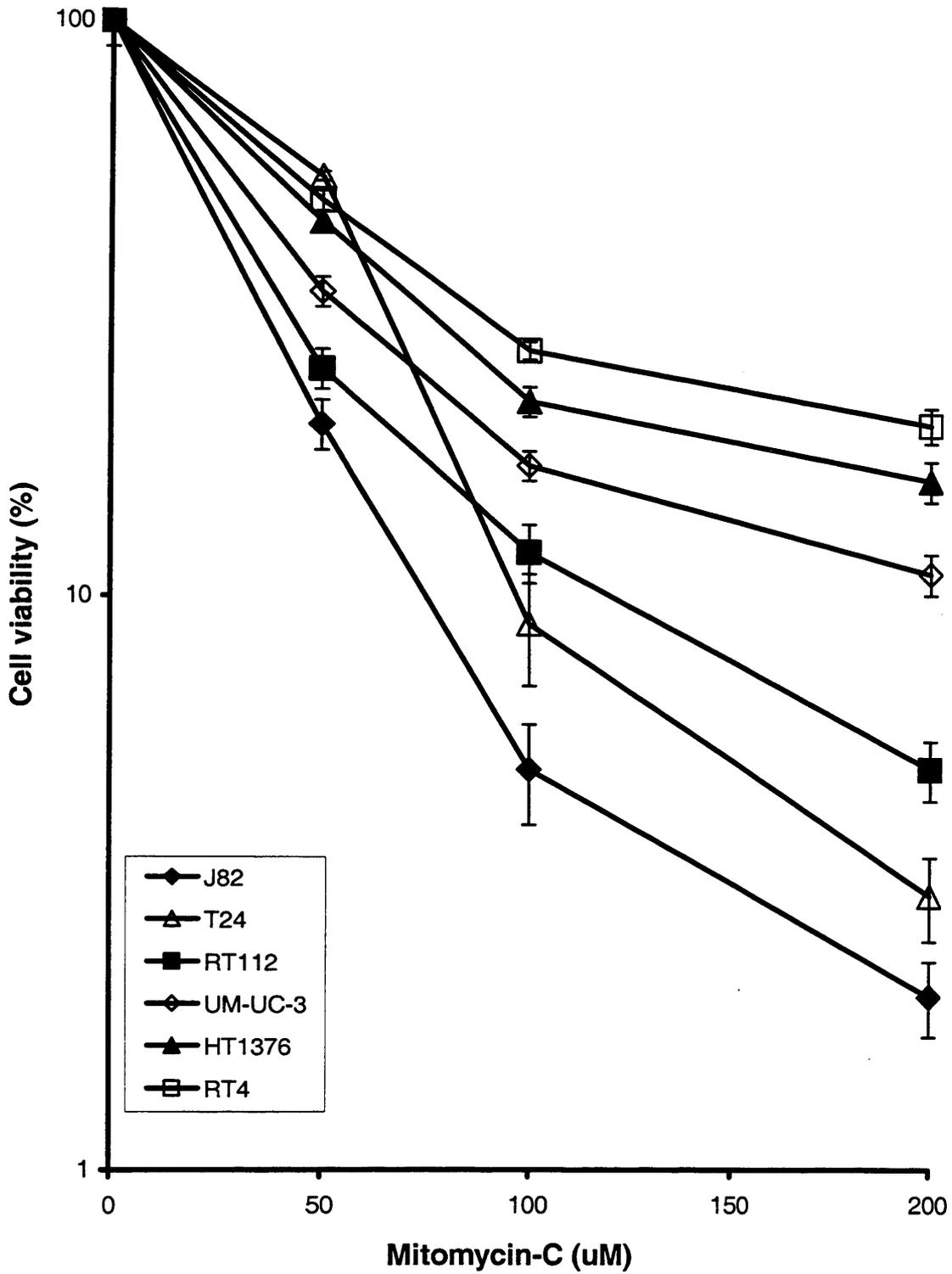
**Figure 3.10.** *Cis*-platin survival curve responses for six-bladder cancer cell lines investigated, over a dose range of 0- 40  $\mu$ M/ml, as determined by the clonogenic assay. Survival was determined as the number of colonies formed following *Cis*-platin treatment. Each data point is the mean of three independent experiments  $\pm$  SE.



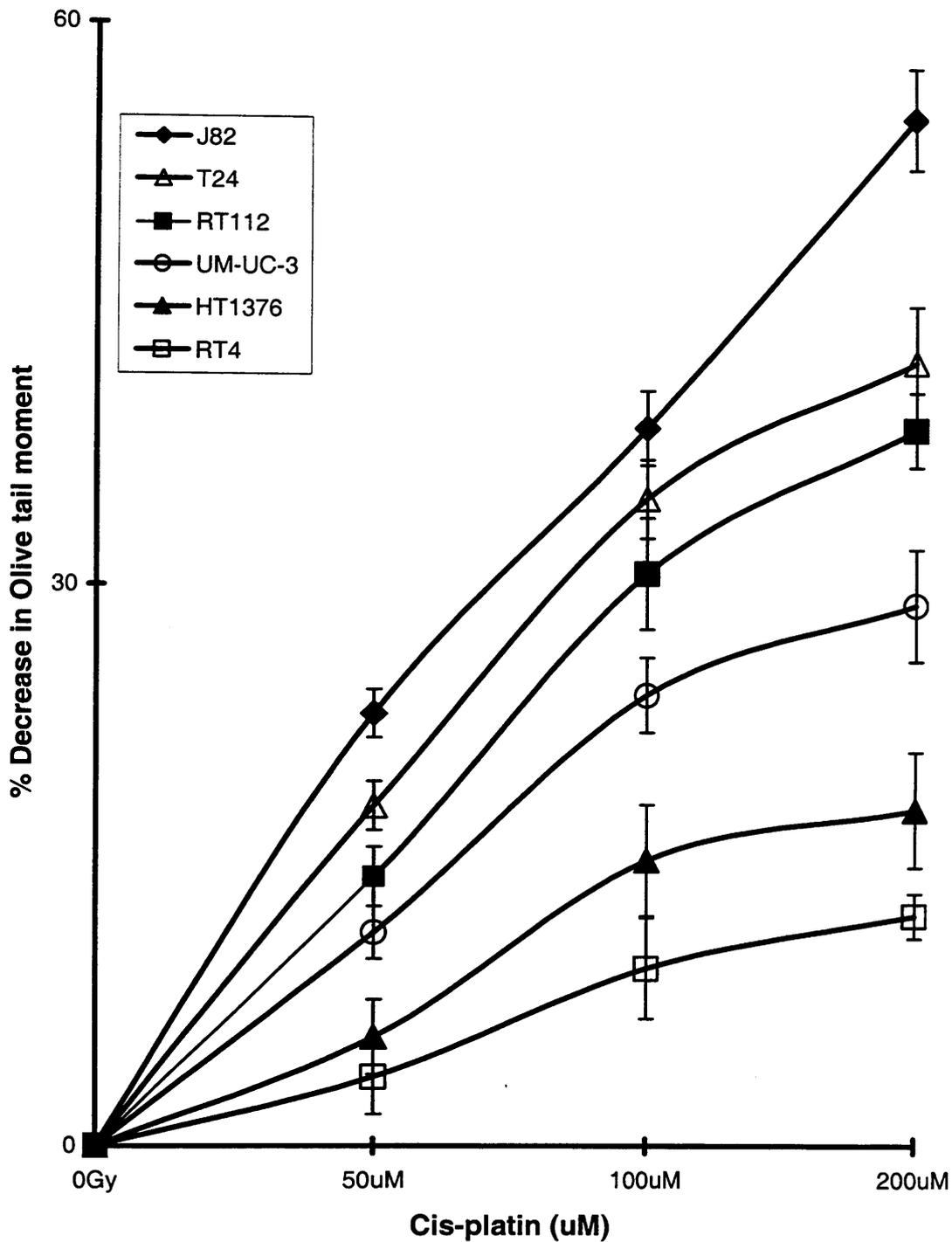
**Figure 3.11.** Mitomycin-C survival curve responses for six-bladder cancer cell lines investigated, over a dose range of 0- 15  $\mu$ M/ml, as determined by the clonogenic assay. Survival was determined as the number of colonies formed following mitomycin-C treatment. Each data point is the mean of three independent experiments  $\pm$  SE.



**Figure 3.12.** *Cis-platin* response for six-bladder cancer cell lines investigated, over a dose range of 0-200  $\mu\text{M}/\text{ml}$ , as determined by the MTT assay. Survival was determined, as the number of viable cells remains following *Cis-platin* treatment. Each data point is the mean of three independent experiments  $\pm$  SE.



**Figure 3.13.** Mitomycin-C response for six-bladder cancer cell lines investigated, over a dose range of 0- 200  $\mu\text{M}/\text{ml}$ , as determined by the MTT assay. Survival was determined, as the number of viable cells remains following mitomycin-C treatment. Each data point is the mean of three independent experiments  $\pm$  SE.

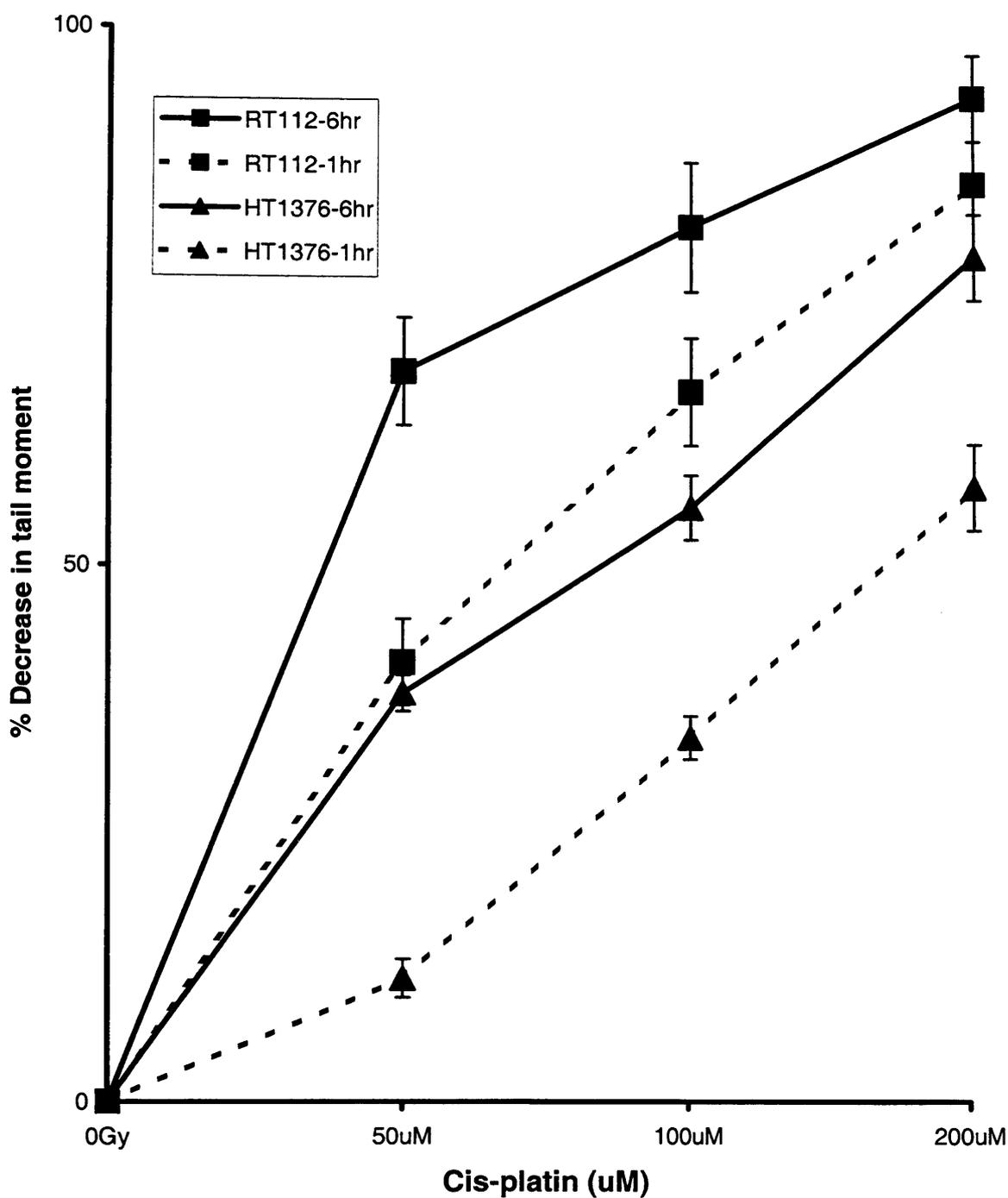


**Figure 3.14.** The level of *Cis-platin* induced cross-link formation for six-bladder cancer cell lines, as determined by the alkaline comet assay. Cells were treated with three different concentrations of *Cis-platin* 50  $\mu\text{M}/\text{ml}$ , 100  $\mu\text{M}/\text{ml}$  and 200  $\mu\text{M}/\text{ml}$ ; cells were subsequently irradiated with 5 Gy. DNA cross-linking damage was measured as reduction in mean Olive tail moment, each data point of three independent experiments  $\pm$  SE.

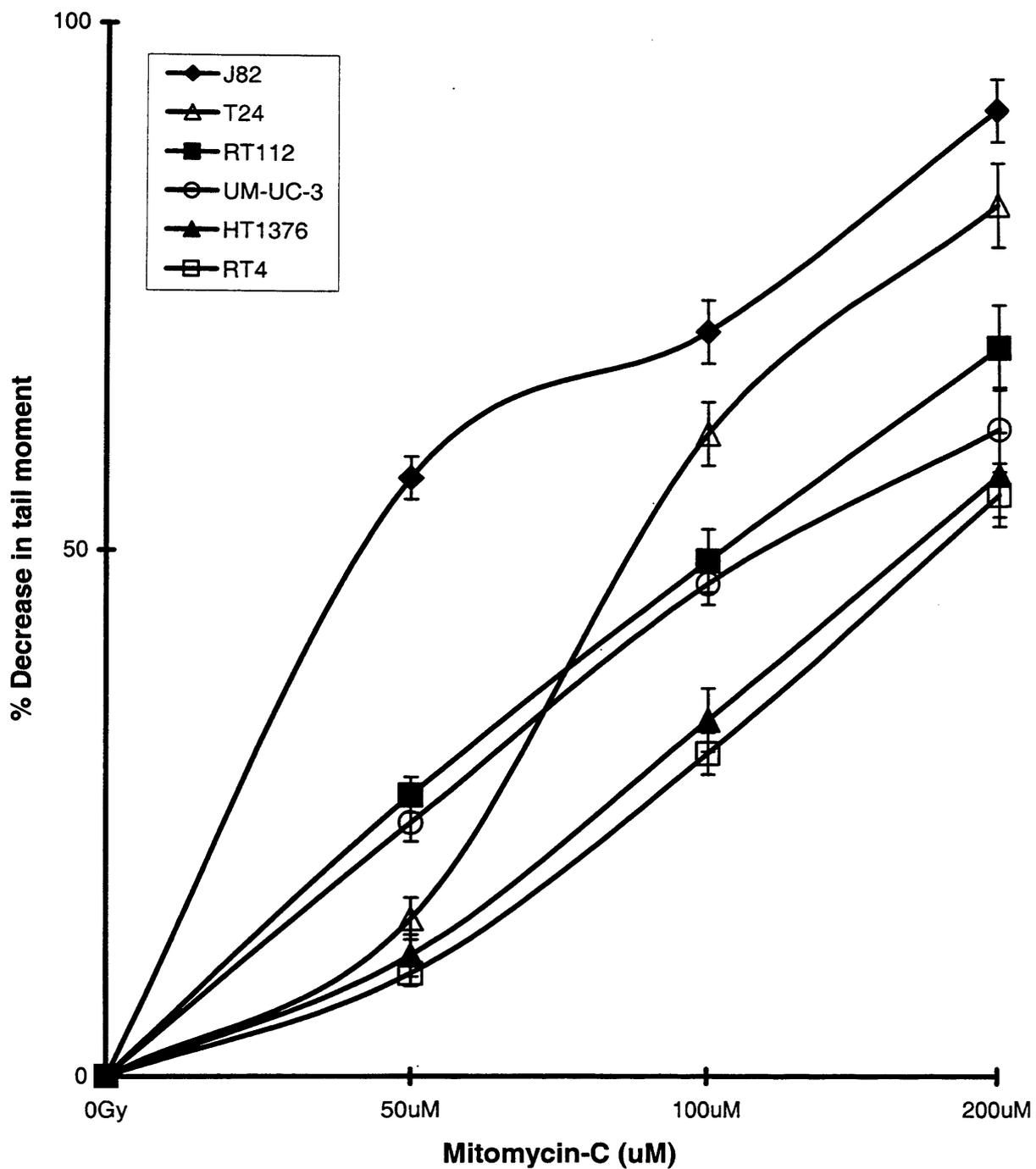
formation as determined by ACA. These results show clearly that there is a difference in DNA cross-link formation for cell lines of different chemosensitivity.

The level of DNA cross-link formation after one hour and six hours exposure to *Cis*-platin was measured for the RT112 and HT1376 cell lines over a dose range of 0-200  $\mu\text{M}$  of *cis*-platin using the alkaline comet assay. The results are shown in Figure 3.15. The reduction in the mean Olive tail moment for RT112 and HT1376 was calculated and a dose response curve was created, with the chemosensitive cell line RT112 displaying the highest level of *cis*-platin induced cross-link formation, and the chemoresistant cell line HT1376 exhibiting lowest level of *cis*-platin induced cross-link formation after both one hour and six hours exposure. As the exposure time to *cis*-platin increases the level of cross-links formation increases in both chemosensitive and chemoresistant cell lines. It can be clearly seen that incubating the cells with *cis*-platin for a longer time, 6 hours, induces a greater level of cross-links. The cross-link profile of the most chemoresistant cell line HT1376 after 6 hours was similar to the extent *cis*-platin induced cross-link for the chemosensitive cell line RT112 after one-hour incubation.

The level of DNA cross-link formation was measured for the six bladder cancer cell lines over a dose range of 0-200  $\mu\text{M}$  of mitomycin-C using the alkaline comet assay. The results are shown in Figure 3.16. Cross-link formation was assessed by the relative decrease in DNA migration after exposure to 5 Gy X-irradiation. The mean Olive tail moment for all cell lines was measured and clear dose response curves were obtained, with the chemosensitive cell line, J82, showing the highest level of mitomycin-C induced cross-link formation, and the most chemoresistant cell lines RT4 and HT1376, displaying the lowest level of mitomycin-C induced cross-link formation. At the highest drug concentration studied by clonogenic survival (*e.g.* 10, 12 and 15  $\mu\text{M}$ ), the rank order of cell killing dose matches the levels of cross-link formation as determined by the ACA at the lowest dose (50  $\mu\text{M}$ ) (J82> RT112> UM-UC-3> T24> HT1376> RT4). Furthermore, at all drug concentrations studied the rank order of cell viability as determined by the MTT assay broadly agrees with the level of cross-link formation as determined by the ACA. This includes the noted behaviour of the T24 that is relatively resistant at low doses and relatively more sensitive at high



**Figure 3.15.** The level of *Cis-platin* induced cross-link formation for RT112 and HT1376 cell lines, as determined by the alkaline comet assay. Cells were treated with three different concentrations of *Cis-platin* 50  $\mu\text{M/ml}$ , 100  $\mu\text{M/ml}$  and 200  $\mu\text{M/ml}$  for 1 hour and 6 hours respectively; cells were subsequently irradiated with 5 Gy. DNA cross-linking damage was measured as reduction in mean Olive tail moment, each data point of three independent experiments  $\pm$  SE.



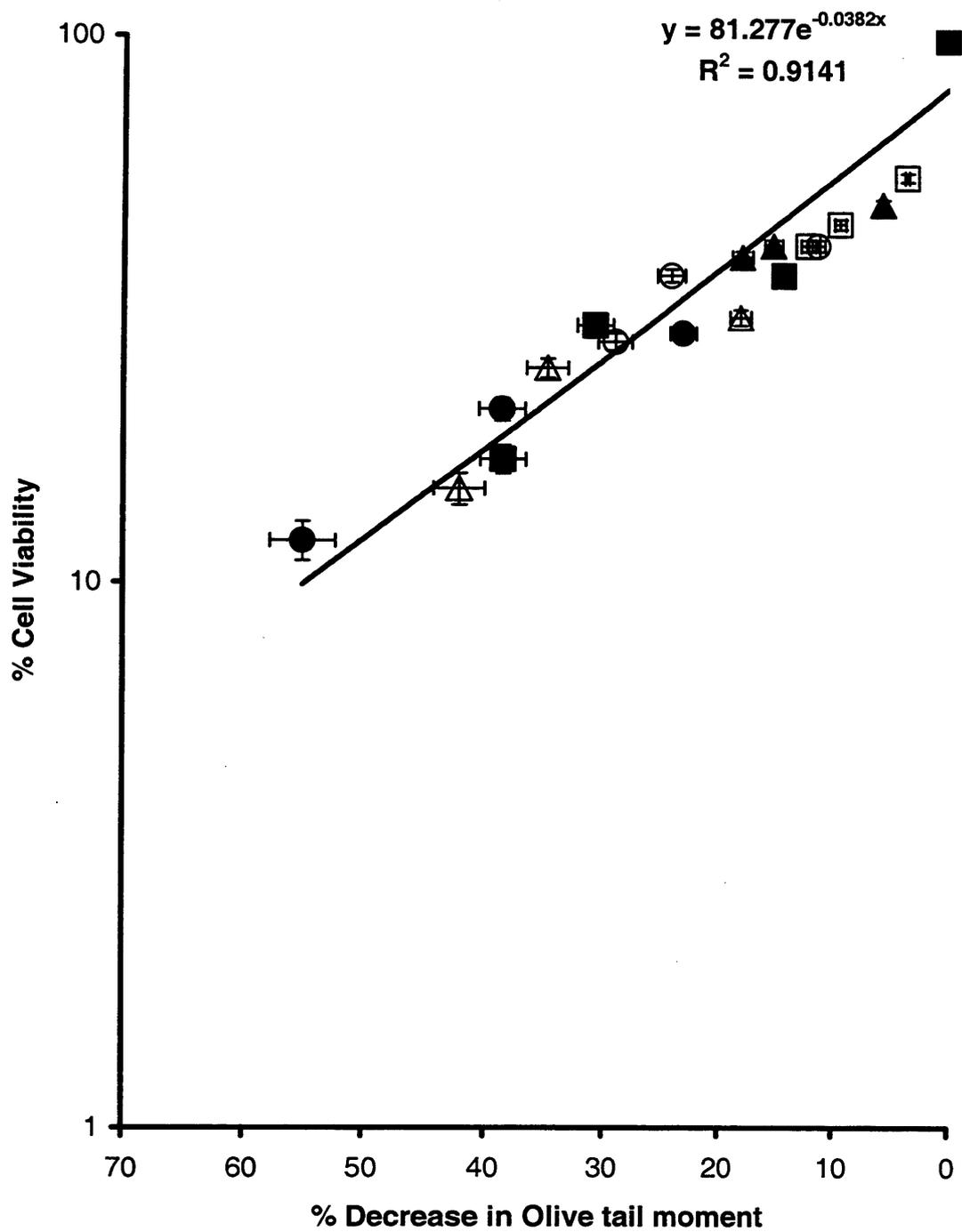
**Figure 3.16.** The level of mitomycin-C induced cross-link formation for six-bladder cancer cell lines, as determined by the alkaline comet assay. Cells were treated with three different concentrations of mitomycin-C 50  $\mu$ M/ml, 100  $\mu$ M/ml and 200  $\mu$ M/ml; cells were subsequently irradiated with 5 Gy. DNA cross-linking damage was measured as reduction in mean Olive tail moment, each data point of three independent experiments  $\pm$  SE.

doses, matching the low level of cross-links at lower doses and the higher level of cross-links at higher doses.

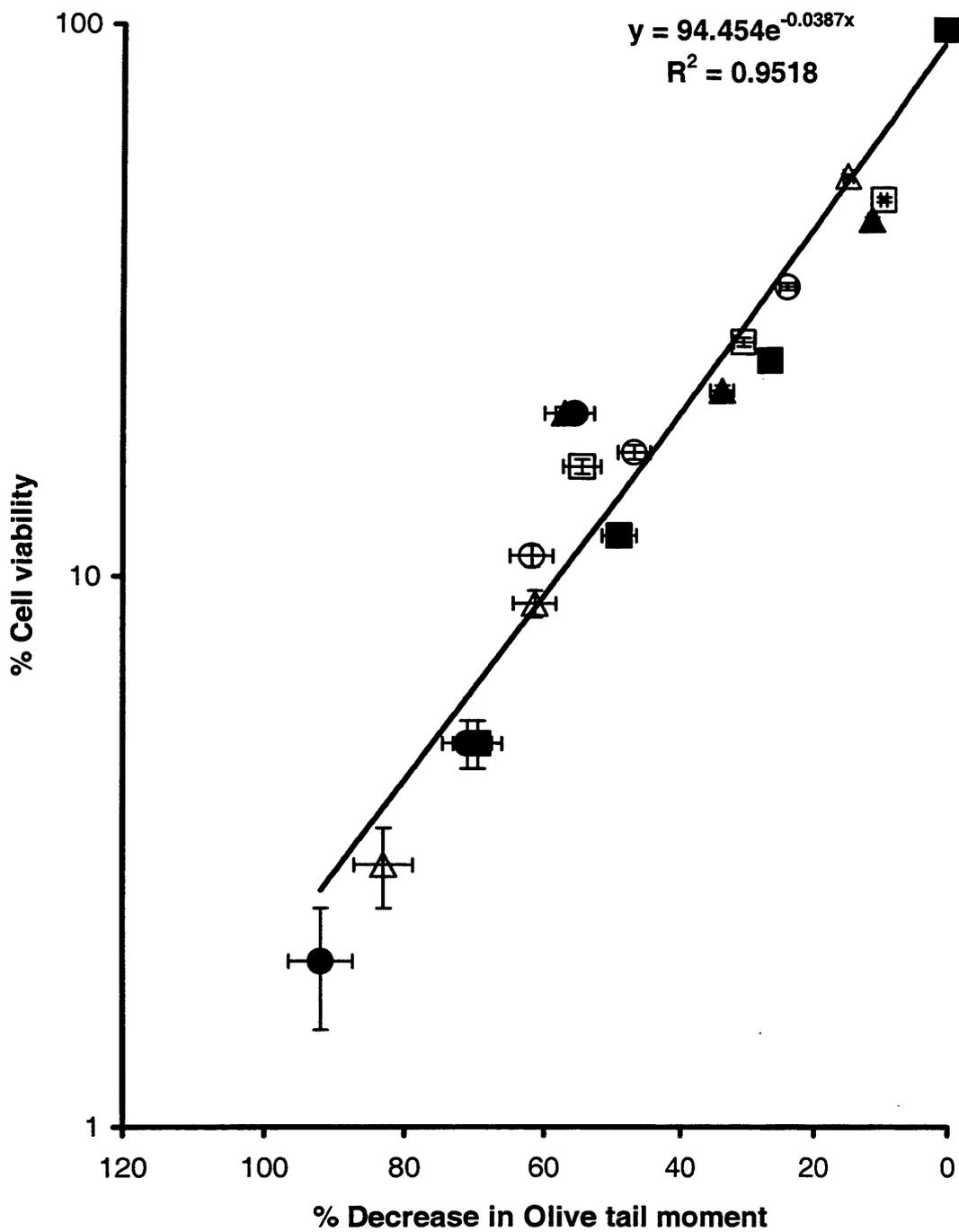
The relationship between the measures of cross-link formation, and the measures of loss of cell viability for all six-bladder cancer cell lines at 0, 50, 100 and 200  $\mu\text{M}$  is illustrated in Figures 3.17 and 3.18 for *cis*-platin and mitomycin-C, respectively. A high degree of correlation exists between the two measures for all six-bladder cancer cell lines ( $R^2 = 0.9141$  for *cis*-platin and  $R^2 = 0.9518$  for mitomycin-C) indicating that the measure of cross-links (% decrease in Olive tail moment) reflects the loss of cell viability for these six cell lines over the dose range studied. Equally, for each individual cell line the relationship between the measures of initial cross-link formation, and measures of cell viability at 0, 50, 100 and 200  $\mu\text{M}$  of *cis*-platin and mitomycin-C is illustrated in Figures 3.19 and 3.20. A high degree of correlation exists between cross-link formation and cell viability for each individual cell line. The single correlation coefficients ( $R^2$ ) for each cell line are given in Table 3.1 for *cis*-platin and in Table 3.2 for mitomycin-C. They vary between 0.9671-0.812 for *cis*-platin and between 0.9921-0.886 for mitomycin-C, with the values of the individual slopes compared to the collated value varying by no more than ~20% for *cis*-platin for 5 of the 6 cell lines (the exception being RT4), and by no more than 10% for mitomycin-C (again the exception being RT4). The high degree of correlation for each cell line reveals that one measure (cross-link formation) accurately reflects the other (cell viability).

Cell line	Slope	$R^2$
J82	-0.0377	0.9671
T24	-0.0414	0.9312
RT112	-0.041	0.9204
UM-UC-3	-0.0394	0.8822
HT1376	-0.0457	0.812
RT4	-0.0649	0.8576
Collated	-0.0382	0.9141

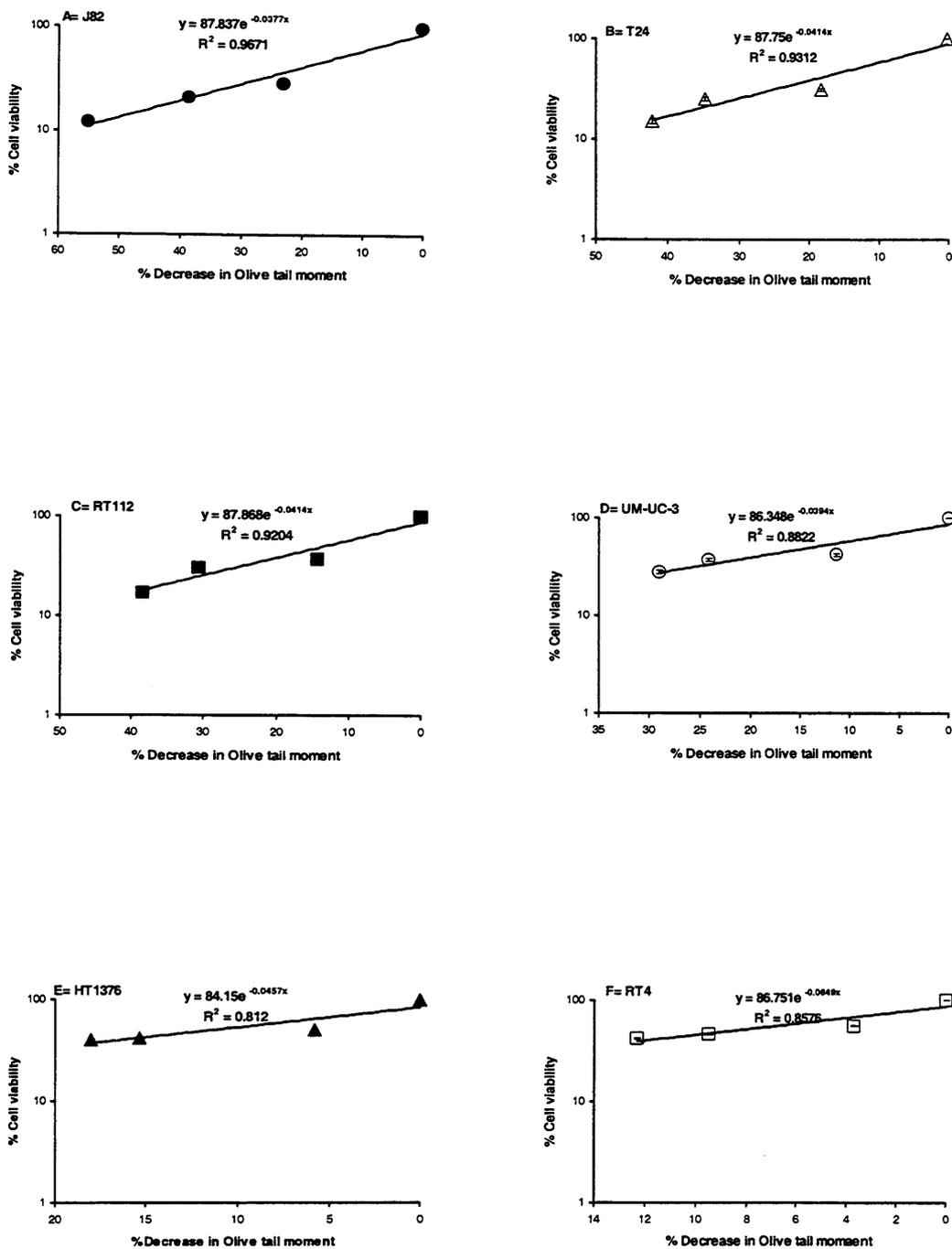
**Table 3.1.** Correlation between cell viability and % decrease in Olive tail moment for *cis*-platin-induced cross-link formation.



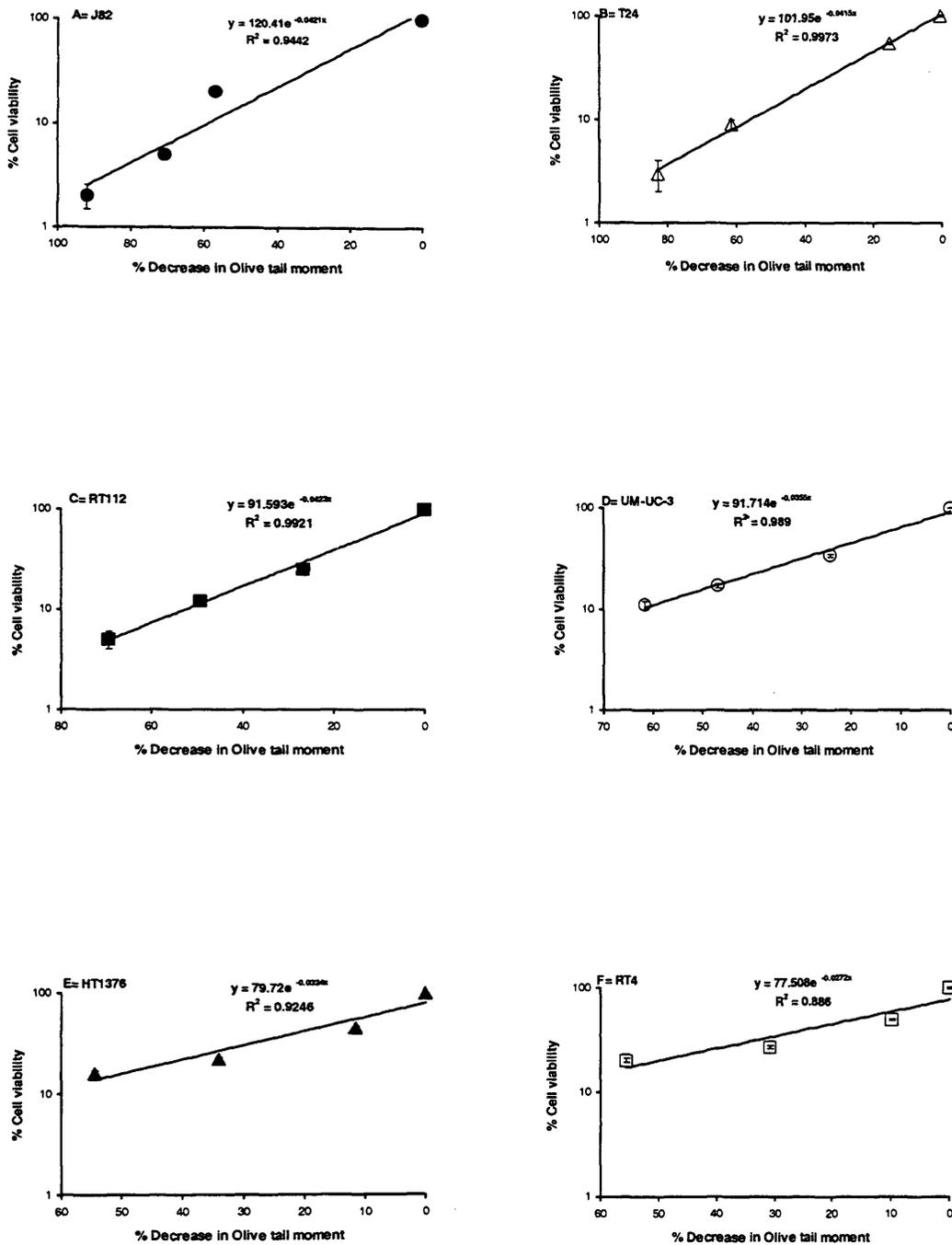
**Figure 3.17.** The relationship between the measures of % decrease in Olive tail moment for initial *cis*-platin-induced cross-link formation, as detected by the alkaline comet assay, and the measures of MTT cell viability, for all six bladder cell lines at 0, 50, 100, 200  $\mu$ M of *cis*-platin.



**Figure 3.18.** The relationship between the measures of % decrease in Olive tail moment for initial mitomycin-C-induced cross-link formation, as detected by the alkaline comet assay, and the measures of MTT cell viability, for all six bladder cell lines at 0, 50, 100, 200  $\mu$ M of mitomycin-C.



**Figure 3.19.** The relationship between the measures of % decrease in Olive tail moment for *cis*-platin-induced cross-link formation, as detected by the alkaline comet assay, and the measures of MTT cell viability, for each cell line at 0, 50, 100 and 200  $\mu$ M.



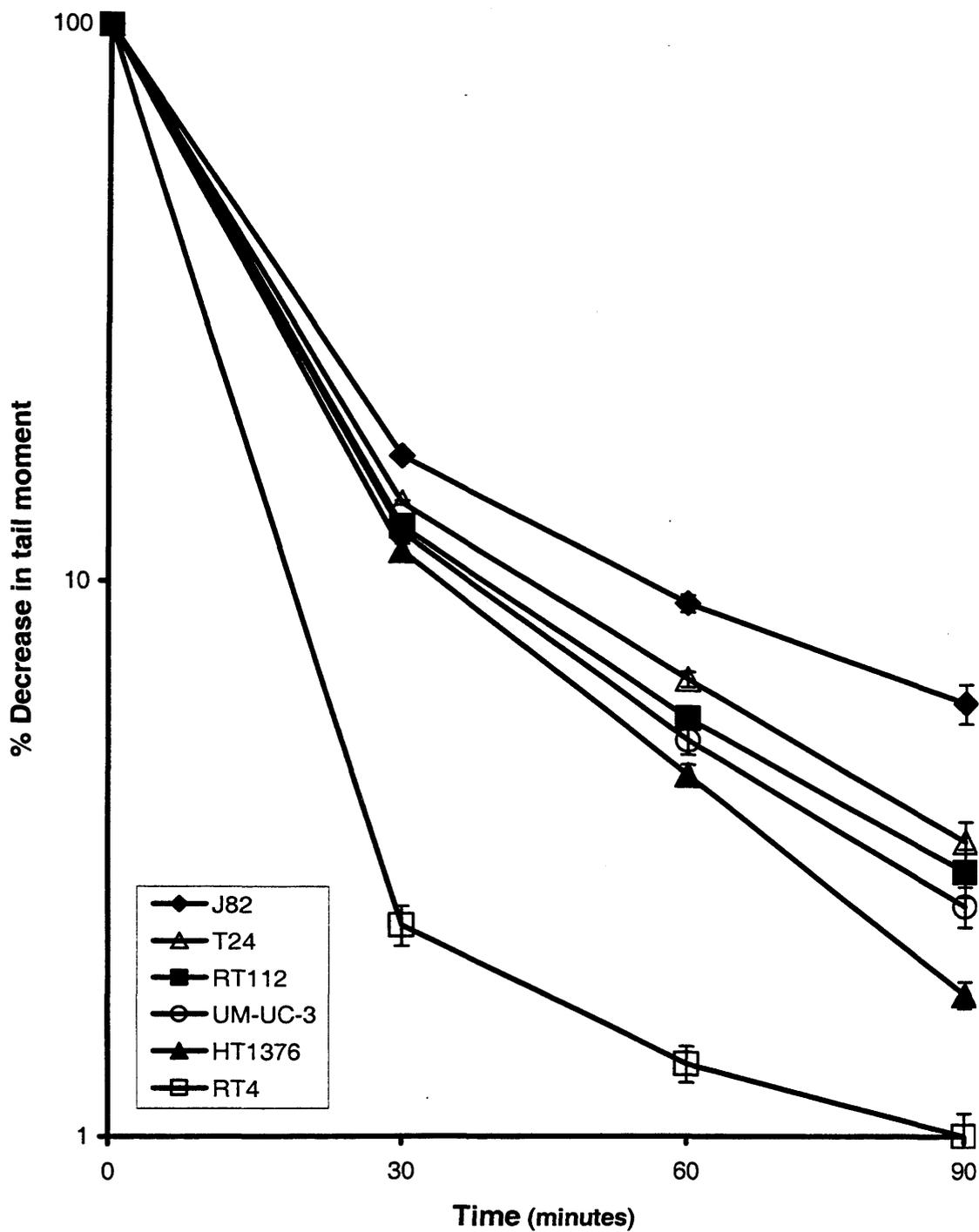
**Figure 3.20.** The relationship between the measures of % decrease in Olive tail moment for mitomycin-C-induced cross-link formation, as detected by the alkaline comet assay, and the measures of MTT cell viability, for each cell line at 0, 50, 100 and 200  $\mu$ M.

Cell line	Slope	R <sup>2</sup>
J82	-0.0421	0.9442
T24	-0.0415	0.9973
RT112	-0.0423	0.9921
UM-UC-3	-0.0355	0.989
HT1376	-0.0324	0.9246
RT4	-0.0272	0.886
Collated	-0.0387	0.9518

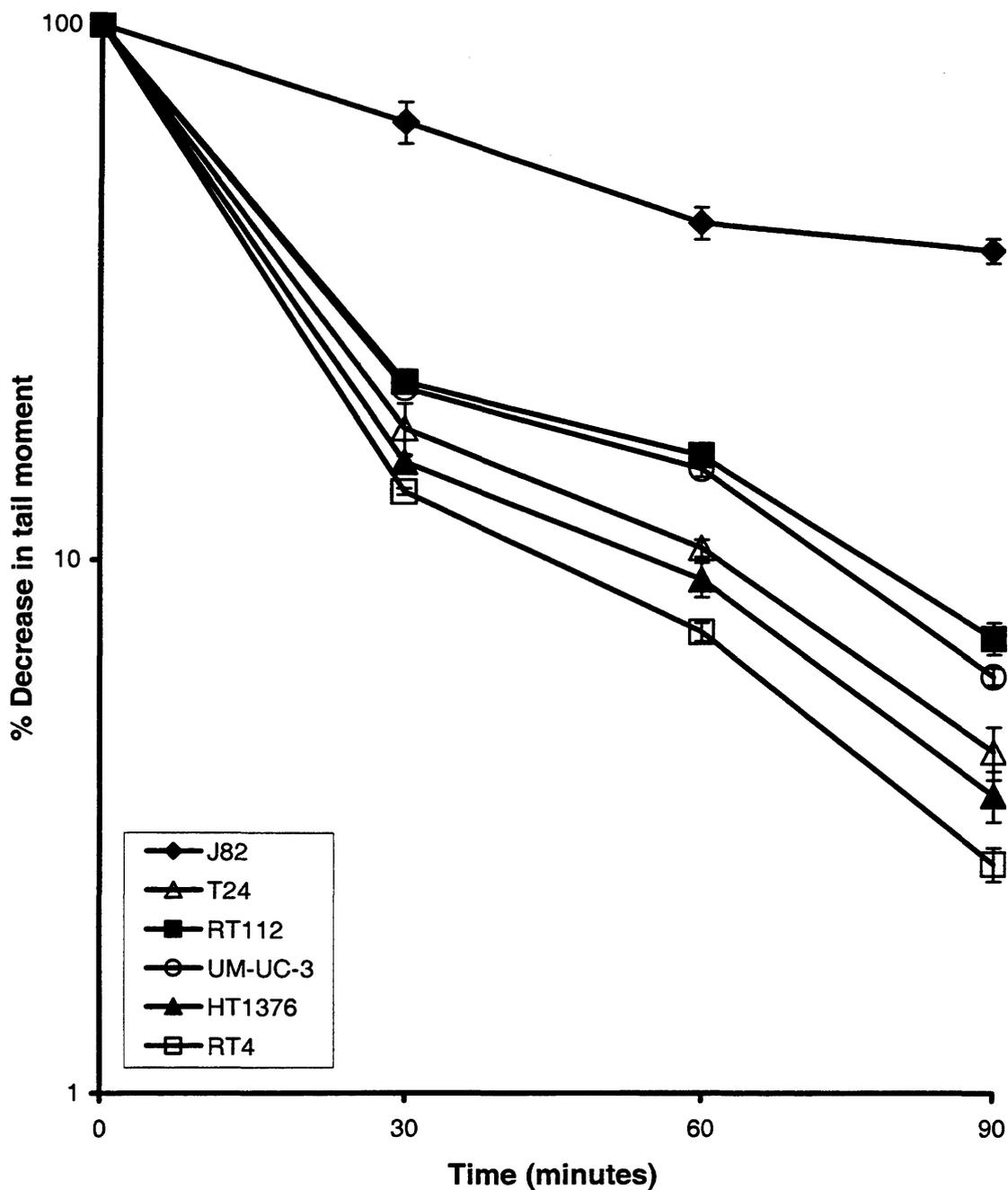
**Table 3.2.** Correlation between cell viability and % decrease in Olive tail moment for mitomycin-C-induced cross-link formation.

The repair of *cis*-platin and mitomycin-C induced cross-links was determined by incubating the cells at 37°C (following 50 µM drug treatment) for 30, 60 and 90 minutes, prior to 5 Gy X-irradiation and alkaline comet analysis for cross-link formation. The results are shown in Figures 3.21 & 3.22 for *cis*-platin and mitomycin-C, respectively. As *cis*-platin and mitomycin-C cross-links were allowed to repair prior to irradiation, the radiation-induced damage in the DNA became identifiable as an increase in comet tail. In the *cis*-platin repair studies it was observed that the most chemoresistant cell lines RT4 and HT1376 displayed the highest level of repair, while the most chemosensitive cell lines J82 and T24 displayed a lowest level of repair. For mitomycin-C repair studies, the chemosensitive cell line J82 showed very little repair, but the chemoresistant cell lines RT4 and HT1376 exhibited a higher level of repair.

As *cis*-platin and mitomycin-C induced cross-links were allowed to repair, the relative mean Olive tail moment values increased with increasing repair time and the level of damage observed in these cell lines after the repair was similar to the level of radiation-induced damage after 5 Gy only, indicating that most cross-linking damage had been repaired. Additional non-drug treated, non-irradiated cells were also prepared, these cells are then analysed in parallel with the drug treated cells. The final analysis was made by subtracting the mean Olive tail moment of the control cell at each time point from the mean Olive tail moment of the irradiated and treated sample at the same time point.



**Figure 3.21.** *Cis*-platin cross-links repair for six-bladder cancer cell lines, as determined by the alkaline comet assay. Cells were treated with 50  $\mu\text{M}/\text{ml}$  of *Cis*-platin and allowed to repair for 30, 60 and 90 minutes, cells were then irradiated with 5 Gy. DNA cross-links repair was manifested as an increase in the mean Olive tail moment to a level greater than the control level (5 Gy + 50  $\mu\text{M}$ ), each data point of three independent experiments  $\pm$  SE.



**Figure 3.22.** Mitomycin-C cross-links repair for six-bladder cancer cell lines, as determined by the alkaline comet assay. Cells were treated with 50  $\mu\text{M}/\text{ml}$  of mitomycin-C and allowed to repair for 30, 60 and 90 minutes, cells were then irradiated with 5 Gy. DNA cross-links repair was manifested as an increase in the mean Olive tail moment to a level greater than the control level (5 Gy + 50  $\mu\text{M}$ ), each data point of three independent experiments  $\pm$  SE.

### 3.11 DISCUSSION

The development of a fast and reliable predictive test for tumour cell chemosensitivity would be a significant contribution towards improving the treatment outcome of chemotherapy. If a predictive test could provide appropriate information based on the individual cell chemosensitivity, this would permit treatment to be designed specifically for each individual patient. A great deal of effort has been expended to develop ways to assess which agents are likely to be effective for a particular tumour. Prediction of patients most likely to benefit from chemotherapy is a worthwhile goal. If patients who are resistant to chemotherapy could be identified before treatment, this would avoid the unnecessary toxic treatment of this patient group and the benefit of chemotherapy could be better evaluated in the remaining chemosensitive group. In bladder cancer the potential for chemotherapy is to improve the cure rate. However, because of the likelihood of serious side effects and resistance, such treatments are limited in their efficiency. Numerous attempts have been made to improve the outcome of muscle invasive bladder cancer following treatment. These improvements could include the development and validation of an assay to distinguish between chemosensitive and chemoresistant tumours.

Over the years, several assays have been developed to predict tumour cell chemosensitivity. However, few have been found to be of value. Previously, the clonogenic assay has been used to measure survival after exposure to chemotherapeutic agents (Köberle *et al.*, 1997). Nevertheless, the timescale and the inability of some primary cell lines to undergo colony formation, seriously disadvantage this assay. Other faster and more automated assays have been developed; the MTT assay quantifies the viable fraction of cells after chemotherapeutic treatment (Loprevite *et al.*, 2001). However, the need to optimise the assay conditions for each individual cell line and its low sensitivity limit the clinical utility of this protocol.

Another route to determine the chemosensitivity of tumour cells is by measuring the level of damage produced in each individual cell. DNA interstrand cross-links can be measured quite easily using an agarose gel based assays or alkaline elution assays

(Hartley *et al.*, 1991). Still, these assays are disadvantaged by the low sensitivity and their inability to be readily adapted for use in *in vivo* studies (Spanswick *et al.*, 2002).

Since its discovery by Ostling and Johanson in 1984 (Ostling *et al.*, 1984), the comet assay has proven to be a versatile and widely used method. Several modifications to the original comet assay have been developed to increase its applications. One such modification allows the measurement of DNA cross-links by assessing the relative reduction in DNA migration induced by a strand-breaking agent. DNA cross-link formation produced by chemotherapeutic agents and the repair of these cross-links can be measured easily using the alkaline comet assay. In order to measure the level of DNA cross-linking using the alkaline comet assay, cells are treated with the cross-linking agent of interest and then DNA strand breaks are subsequently induced using standard agents such as ionising radiation. The level of strand breakage induced is easily measured by the comet assay and the relative reduction in the migration of DNA containing strand breaks gives an indication of how much DNA cross-linking has occurred. Since the extent of cross-linking will determine how fast the DNA migrates during electrophoresis, it is possible to obtain a relative value for the level of cross-linking within a given cell.

*Cis*-platin and mitomycin-C cell survival curves and cell viability measures for all six bladder cancer cell lines have been obtained using the clonogenic assay and the MTT assay, respectively. The results show differences between the cell response curves produced by the clonogenic assay and the MTT assay. Several attempts were made to obtain survival curves using the high doses required to measure cross-link formation, however, due to the toxicity of these chemotherapeutic agents only low doses of *cis*-platin and mitomycin-C could be used in the study. For the MTT assay higher doses of *cis*-platin and mitomycin-C were used in the study. The response curves produced by the MTT assay showed that the most chemosensitive cell line displays the lowest level of cell viability and the most chemoresistant cell line exhibit the highest level of viability. These results demonstrate that the clonogenic assay is more sensitive in determining survival at lower doses than the MTT assay is in determining cell viability.

A modified version of the alkaline comet assay was demonstrated to be capable of measuring the formation and repair of *cis*-platin and mitomycin-C in six-bladder cancer cell lines. For *cis*-platin studies, clear dose response curves were obtained with the chemosensitive cell line displaying the highest level of *cis*-platin induced cross-link formation, and the chemoresistant cell line exhibiting the lowest level of *cis*-platin induced cross-link formation. For *cis*-platin the rank order for cross-link formation matches the rank order of cell killing and loss of cell viability for all six-cell lines. Therefore, it is suggested that the extent of sensitivity for the majority of the cell lines studied is proportional to the level of initial damage, with the resistant cell line (having low levels of crosslinks formation), and the sensitive cell line (possessing high levels of crosslinks). Indeed it can be clearly seen that incubating the most resistant cell line RT4 for 6 hours with *cis*-platin, induces a greater level of crosslinks; this being similar to the extent of crosslink formation in the most sensitive cell line J82 after 1 hour incubation with *cis*-platin.

For mitomycin-C studies, dose response curves for cross-link formation were also obtained, with, generally, the chemosensitive cell lines demonstrating the highest level of mitomycin-C induced cross-link formation and the chemoresistant cell lines exhibiting the lowest level of mitomycin-C induced cross-link formation. The rank order for cell killing and the loss of cell viability did match the rank order for cross-link formation at the most equivalent concentrations. This included the notable change for T24 from a resistant (low level of mitomycin-C induced cross-link formation) to a sensitive (high level of mitomycin-C induced cross-link formation) phenotype observed in the MTT analysis with increasing dose of mitomycin-C.

At similar doses it was observed that the level of cross-links produced by mitomycin-C is greater than the level of cross-links produced by *cis*-platin for all six-bladder cancer cell lines. This is in agreement with the known mechanisms of the drugs, with mitomycin-C giving higher levels of interstrand crosslinks than *cis*-platin (Tomasz *et al.*, 1997).

Overall, in the present study, the alkaline comet assay was demonstrated to be capable of predicting cell viability after chemotherapeutic treatment of a panel of six-bladder cancer cell lines. The initial level of cross-link formation as determined by the

alkaline comet assay maintain the same rank order as cell survival and had a strong inverse correlation with cell viability for all six-bladder cell lines, with the most sensitive cell line displaying the greatest measure of cross-link formation and the most resistant cell line displaying the least. Furthermore, for one cell line, T24, the notable change of resistant to sensitive phenotype with increasing dose of mitomycin-C was mirrored in both the alkaline comet assay and the MTT assay.

The repair of *cis*-platin and mitomycin-C cross-links was observed using the alkaline comet assay. When the cells are allowed to repair cross-linking damage, the DNA containing radiation-induced damage is free to migrate and this is revealed as an increase in the mean Olive tail moment as repair time increases. It was noted that the extent of repair was higher in the chemoresistant cell lines and lower in the chemosensitive cell lines. However, the chemoresistant cells had lower levels of initial damage, which presumably facilitates more rapid repair, whilst chemosensitive cells had a higher level of damage that presumably possess a greater burden to repair. After 90 minutes, the *cis*-platin and mitomycin-C induced DNA cross-links have been sufficiently removed for the cells (from the chemoresistant in particular) so that they displayed comparable levels of damage to that shown by cells that received 5 Gy radiation only. Generally the repair of the mitomycin-C induced cross-links was slower than the repair of the *cis*-platin induced cross-links for all six-bladder cancer cell lines.

The results presented here indicate that there are different levels of drug-induced DNA cross-linking damage in cell lines of different chemosensitivity, as measured by the alkaline comet assay, and that this matches the determined measures of cell viability and survival. The alkaline comet assay is useful in studying the mechanism of action and the biological significance of chemotherapeutic cross-linking agents and it may be possible to apply this method to assess the effectiveness of chemotherapeutic agents for individual patients.

**CHAPTER FOUR**

***SUMMARY & CONCLUSIONS***

## 4.1 INTRODUCTION

A diagnosis of cancer creates a critical crossroad in life. In the UK 12,500 new cases of bladder cancer are diagnosed every year. Approximately 30% of these patients will have muscle-invasive bladder cancer at initial presentation. Of the remaining 70% who initially present with superficial disease, 10%-15% will progress to invasive disease. There are many treatment options for bladder cancer patients, some have long been in use, and some are still considered experimental. Each case is unique, and there are many factors to consider before deciding which approach is best for the individual.

Radical cystectomy with pelvic lymph node dissection is considered to be the standard treatment for patients with invasive bladder cancer in many countries, particularly in the USA. Radiation therapy is a second approach for the treatment of muscle invasive bladder cancer and is primarily considered for patients who are unfit for cystectomy based on age, comorbid conditions and extent of disease. A more recent advancement in the treatment of invasive bladder cancer has occurred with the advent of effective chemotherapy. However, the role of systemic chemotherapy, as either an adjuvant or a neoadjuvant treatment, continues to evolve and its impact on survival remains the subject of investigation.

Of the two principle treatment modalities for muscle invasive bladder cancer, that is surgery and radiotherapy based treatment, each has only a 50% chance of success. This might be due to the uncertainty in selecting patients that should go for radical cystectomy or radical radiotherapy with adjuvant or neoadjuvant chemotherapy. The inadequacy of the precystectomy prognostic factors to distinguish reliably between patients who will benefit from cystectomy and those who benefit less is the main reason for obtaining poor survival in patients that undergo total cystectomy. Equally, the primary reason for the lack of success of bladder preserving radiotherapy is the lack of information regarding patients' response to radiation, in particular, the inability to distinguish between patients with radiosensitive and radioresistant tumours.

In the on-going search for effective local control with improved survival, chemotherapeutic agents have been used with surgery and radiotherapy. The case for combining early therapy with systemic therapy is based on the supposition that the combined treatment would result in a more effective outcome. Nevertheless, in the treatment of some patients, local control is accomplished without the use of cytotoxic chemotherapy. As such, these patients do not benefit from this additional treatment, but are exposed to the associated short and long term complications of chemotherapy treatment.

Numerous attempts have been made to improve the outcome of muscle invasive bladder cancer following treatment. One such improvement would be the development and exploitation of assays that can predict tumour sensitivity on an individual basis.

The development of a fast and applicable prognostic test for tumour cell radiosensitivity and chemosensitivity would make an important contribution towards improving the efficiency of bladder cancer treatment. If a predictive test could provide appropriate information based on an individual's tumour cell radiosensitivity and chemosensitivity, this would permit treatment to be designed specifically for each individual patient. A great deal of effort has been expended to develop ways to assess which treatment is likely to be effective for a particular tumour. Prediction of patients most likely to benefit from radiotherapy or chemotherapy is a worthwhile goal. If patients who are resistant to radiotherapy or chemotherapy could be identified before treatment, this would prevent the unnecessary toxic treatment of this group, and the benefit of radiotherapy and chemotherapy could be better evaluated in the remaining radiosensitive and chemosensitive group.

If tumour radiosensitivity and chemosensitivity could be predicted in advance, it would be possible to significantly improve bladder tumour control rate by selecting for immediate radiation therapy and chemotherapy those patients whose tumours are radiation sensitive and chemosensitive; additionally, those patients who would benefit from initial surgery (those whose tumours are radiation resistant or chemoresistant) would be identified earlier, reducing the risk of metastatic spread and so improving rates of survival.

The development of predictive tests to determine tumour cell radiosensitivity and chemosensitivity could further promote bladder preservation protocols in the treatment of muscle invasive disease. Over the years, several assays have been developed to predict tumour cell radiosensitivity and chemosensitivity, however, few have been found to be of clinical value. A mechanism proposed to account for differences in intrinsic sensitivity suggests that it is due to differences in the extent to which DNA damage is induced in different cells; with higher levels of damage being noted in the more sensitive cells. Recently, the alkaline comet assay has been established as a highly sensitive method for assessing DNA damage formation and repair at the individual cell level (Singh *et al.*, 1988) and is particularly suitable for the measurement of radiogenic damage (Olive *et al.*, 1999) and chemotherapeutic drug cross-link formation and cross-link repair (Olive *et al.*, 1995).

In the present study, evaluation of the alkaline comet assay is reported, as a rapid predictive assay for the measurement of bladder cancer cells radiosensitivity (Chapter 2) and chemosensitivity (Chapter 3) *in vitro* using a panel of six bladder cancer cell lines. The extent of DNA damage formation and repair as determined by the assay, reflects bladder cancer cell radiosensitivity and chemosensitivity.

#### **4.2 EVALUATION OF THE ALKALINE COMET ASSAY AS A PREDICTIVE TEST OF BLADDER CANCER CELL RADIOSENSITIVITY**

The alkaline comet assay was demonstrated to be capable of predicting radiation cell survival for a panel of six bladder cancer cell lines (Chapter 2). The initial level of radiation-induced DNA comet formation as determined by the alkaline comet assay had a strong inverse correlation with clonogenic survival for all six-bladder cell lines, with the most radiation sensitive cell line displaying the greatest measure of comet formation and the most radioresistant cell line displaying the least. The results presented here possibly suggest that there are different levels of immediate radiogenic DNA damage as measured by the alkaline comet assay, which may determine the radiosensitivity of individual bladder cancer cell lines.

For the repair studies, which includes measures of residual single strand breaks and alkali labile sites-damage at various repair time points, there was good correlation

with survival (SF2) for five of the six-bladder cancer cell lines investigated, with the extent of repair being greater in the radioresistant cell lines and lower in the radiosensitive cell lines. However, the excellent correlation of radiation clonogenic survival with the extent of immediate radiation-induced comet formation for all six-bladder cancer cell lines studied, as compared to the correlation of survival with the extent of damage repair, deems that immediate damage formation best predicts survival for the six cell lines.

Clearly, the results of the present study suggest that parameters of initial damage formation, as determined by ACA, may be used to predict the radiosensitivity of individual bladder cancer cell lines. In an effort to delineate factors responsible for the different levels of immediate damage formation in bladder cancer cells of different radiosensitivity, the level of immediate radiation-induced comet formation in prepared nucleoid bodies and isolated nuclei of the six cell lines were investigated. The observation of the level of immediate comet formation in the isolated nuclei and particularly in the prepared nucleoid bodies maintaining the same rank order as in the intact parent cell indicates that it is a feature of the nucleoid body that dictates the relative extent of comet formation. This also includes the anomalous increase in comet formation noted for T24. The observation of the same rank order for comet formation in the irradiated nucleoid bodies and nuclei as in the irradiated intact parent cells, presumably reflects differences in the organisation of the nuclear DNA within radiation resistant and sensitive cell lines; with there potentially being weaker DNA attachment to the nuclear core matrix protein in radiation sensitive cell lines (Vaughan, Anderson *et al.*, 1993; Malyapa, Wright *et al.*, 1994). This could lead to the DNA being more unstable towards radiation-induced damage in the sensitive cell lines; several studies have shown that differences in nuclear matrix protein composition and the stability of DNA-matrix protein attachments may have an influence on intrinsic cellular radiosensitivity (Malyapa, Wright *et al.*, 1994; Malyapa, Wright *et al.*, 1996).

Finally, studies were conducted to evaluate the alkaline comet assay using clinical samples. Epithelial cells isolated from invasive bladder cancer biopsies also reveal a range of radio-responses, as determined by the alkaline comet assay, at clinically relevant doses. The differences in radiogenic immediate comet formation in the

biopsy-derived epithelial cells were taken to reflect actual variations in tumour cell radiosensitivity. The observation of differing tumour cell radiosensitivity, in particular the observation of high degrees of radioresistance, is most significant as it may be a contributing factor to the current high level of invasive bladder cancer radiotherapy treatment failure (Shiple *et al.*, 1985; Duncan and Quilty 1986). Overall, the results obtained suggest that there are different predicted radiosensitivities for tumour cells derived from differing muscle invasive tumours as measured by the alkaline comet assay. This observation strengthens the possible clinical utility of this assay.

#### **4.3 EVALUATION OF THE ALKALINE COMET ASSAY AS A PREDICTIVE TEST OF BLADDER CANCER CELL CHEMOSENSITIVITY**

The second part of this study (Chapter 3) was concerned with evaluating a modified version of the alkaline comet assay capable of measuring the formation and repair of *cis*-platin and mitomycin-C DNA damage in six-bladder cancer cell lines. For studies with *cis*-platin, clear dose response curves were obtained, with the chemosensitive cell lines displaying the highest level of *cis*-platin induced cross-link formation, and the chemoresistant cell lines exhibiting the lowest level of *cis*-platin induced cross-link formation. The rank order for cross-link formation matches the rank order of cell killing and loss of cell viability for all six-cell lines. For studies with mitomycin-C, dose response curves were also obtained, with generally the chemosensitive cell lines demonstrating the highest level of mitomycin-C induced cross-link formation, and the chemoresistant cell lines exhibiting the lowest level of mitomycin-C induced cross-link formation. The rank order for cross-link formation matches the rank order for cell killing at the most equivalent dose. Furthermore, at all drug concentrations studied the rank order for loss of cell viability as determined by the MTT assay broadly agrees with the level of cross-link formation as determined by the ACA. This includes the noted behaviour of the T24 that is relatively 'resistant' at low doses (having both low level of mitomycin-C induced cross-link formation as determined by ACA, and a low loss of cell viability as determined by the MTT assay) and relatively more 'sensitive' at high doses (having a high level of mitomycin-C induced cross-link formation as determined by ACA, and a high loss of cell viability as determined by the MTT assay).

The repair of *cis*-platin and mitomycin-C cross-links was observed using the alkaline comet assay. It was noted that the extent of repair was higher for the chemoresistant cell lines and less for the chemosensitive cell lines. As at the later time points of repair the cells display comparable levels of damage to that shown by cells that received 5 Gy radiation only, particularly the chemoresistant cell lines.

The results presented here prove that there are clear differences in the level of DNA cross-link formation for cell lines of different chemosensitivity, as measured by the alkaline comet assay, and that the alkaline comet assay is a highly sensitive method for measuring the effect of cross-linking agents.

#### **4.4 CONCLUDING REMARKS**

From the results presented in this thesis, it was observed that the cell line with the purposed highest level of radiogenic DNA damage was also the cell line with the highest level of DNA cross-link formation, and the cell line with the purposed lowest level of radiogenic DNA damage was also the cell line with lowest level of DNA cross-link formation as measured by the alkaline comet assay (excluding the T24 cell line), demonstrating that the rank order of radiation damage sensitivity was the same as the rank order of drug damage sensitivity. Consequently, these results, to a certain extent, indicate that one measure (radiosensitivity) can indeed predict the other (chemosensitivity).

The development and validation of a predictive test to determine radiosensitivity and chemosensitivity would make an important contribution toward improving the efficiency of radiotherapy and chemotherapy in cancer treatment, as this would permit treatment to be designed specifically for each individual patient. In the present study, it has been demonstrated that the ACA can predict bladder cancer cells radiosensitivity and chemosensitivity *in vitro*. Further studies of clinical samples have revealed the clinical potential of this assay. The ACA is attractive as a possible clinical test as it is simple, inexpensive, rapid, sensitive, and the assay requires only low numbers of cells for measuring DNA damage formation and repair.

The results of the *in vitro* studies and the preliminary human data indicate that a study to fully evaluate ACA as a predictive assay of the radiotherapy treatment of invasive bladder cancer is warranted. In this study, the ultimate outcome to be established is whether predicted radiosensitivity, as determined by the ACA, positively correlates with tumour clearance by radiotherapy. These studies are now undertaken in Leicester and Ulster.

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## **PUBLICATIONS**

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## **ABSTRACTS:**

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