Repair of DNA interstrand cross-links induced by anti-cancer agents in mammalian cells

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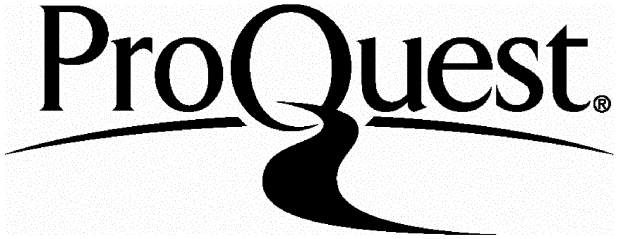

ABSTRACT

Bifunctional agents, which produce DNA interstrand cross-links (ICLs), are among the most effective anitumour agents in clinical use. The cellular mechanisms that act to eliminate DNA ICLs in mammalian cells are still not well understood. Studies in bacteria and yeast indicate that both nucleotide excision repair (NER) and recombination are required for ICL removal and that double-strand breaks (DSBs) are produced as repair intermediates in yeast cells. To elucidate the mechanism of ICL repair in mammalian cells, repair of ICLs induced by major groove binding bifunctional anti-cancer agents nitrogen mustards, cisplatin and the novel pyrrolobenzodiazepine (PBD) based minor groove binding agent SJG-136 was investigated. The role of NER, homologous recombination (HR) and non-homologous end joining (NHEJ) pathways in the repair of ICLs was studied using a panel of Chinese Hamster Ovary (CHO) mutant cell lines.

Results presented indicate that there is no unique mechanism for the repair of DNA ICLs, but the pathway involved depends on the type of ICL. Nitrogen mustard induced ICLs are incised or 'unhooked' by XPF and ERCC1 which are involved in NER and some types of recombination, but other NER factors are not required. DSBs are induced during processing of these ICLs and are subsequently repaired by a homologous recombination pathway involving the XRCC2 and XRCC3 genes. In contrast, unhooking of cisplatin induced ICLs required other NER factors in addition to XPF and ERCC1, DSBs were not induced and repair was dependent on functional XRCC2 and XRCC3. Compared to major groove binding agents SJG-136 was highly cytotoxic in the parent CHO cell line AA8, and the ICLs induced were poorly repaired. Repair of SJG-136 induced ICLs required the whole NER system in addition to XPF and ERCC1 and DSBs were not induced. XRCC2 and XRCC3 appear to play a role in the elimination of these adducts. The NHEJ pathway did not seem to play a major role in the repair of DNA lesions induced by any of the drugs studied.

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Finally many thanks to my husband Mahesh and my parents, without whose continued love and support this work would not have been possible.

This thesis is dedicated to my parents.

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ABBREVIATIONS

4MU	4-methylumbelliferone (7-hydroxy-4-methylcoumarin)
6-4 PP	Pyrimidine 6-4 pyrimidone photoproducts
8-oxoG	7,8-dihydro-8-oxoguanine
AP-site	Apurinic/apyrimidinic site
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BER	Base excision repair
CHEF	Contour-clamped homogeneous field electrophoresis
CHO	Chinese hamster ovary
CPD	Cyclobutane pyrimidine dimers
CS	Cockayne's syndrome
DAC	2'-deoxy-5-acacytidine
DMSO	Dimethyl sulfoxide
DNA-PK	DNA protein kinase
DSBs	Double strand breaks
ELISA	Enzyme Linked Immunosorbant Assay
EMS	Ethyl methanesulfonate
ERCC	Excision repair cross complementing
FA	Fanconi anemia
FCS	Foetal calf serum
GG-NER	Global genome nucleotide excision repair
HMG	High mobility group
HN1	2-dimethylaminoethylchloride hydrochloride
HN2	Mechlorethamine
HNPCC	Hereditary nonpolyposis colon cancer
HR	Homologous recombination
ICLs	Interstrand cross-links
MGMT	O^6-methylguanine DNA methyltransferase
MMC	Mitomycin C
MMR	Mismatch repair

MPG/AAG	3-methyladenine DNA glycosylase
MUG	methylumbelliferyl ß-galactoside
NA-AAF	N-acetoxy-2-acetylaminofluorene
NBP	4-(4-nitrobenzyl) pyridine
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
OD	Optical density
Ogg1	8-oxoguanine DNA glycosylase
PBD	pyrrolo[2,1-c][1,4]benzodiazepines
PFGE	Pulse field gel electrophoresis
RCG	Rodent complimenting group
RF-C	Replication factor C
SRB	Sulforhodamine B
SSA	Single strand annealing
TBE	Tris-borate-EDTA buffer
TCA	Trichloro Acetic Acid
TCR	Transcription coupled repair
TDG	Thymine DNA glycosylase
TM	Tail Moment
TTD	Trichothiodistrophy
UDG	Uracil DNA glycosylase
XP	Xeroderma pigmentosum
XRCC	X-ray repair cross complementing

CHAPTER 1: INTRODUCTION

Deoxyribonucleic acid (DNA) is the single most important molecule in living cells and contains all of the information that specifies cellular properties. Thus, the integrity of this molecule is vital for living cells. However, DNA is continuously challenged by the damaging effect of numerous chemical and physical agents. DNA damage can arise from three main causes. Firstly, products from normal cellular metabolism, such as oxygen species constitute a permanent threat to DNA structure from within. Secondly, some chemical bonds in DNA tend to spontaneously disintegrate under physiological conditions. Hydrolysis of nucleotide residues leaves non-instructive abasic sites. Spontaneous or induced deamination of cytosine, adenine, guanine or 5-methylcytosine converts these bases to miscoding uracil, hypoxanthine, xanthine and thymine, respectively. Finally, environmental agents such as the ultraviolet (UV) component of sunlight, ionizing radiation and numerous chemicals such as alkylating agents cause various alterations in DNA structure. To ensure genetic stability, all organisms have evolved an intricate network of complementary DNA repair systems that are capable of removing most (but not all) types of DNA damage.

The consequences of DNA damage are diverse. The cell cycle apparatus has the ability to sense the genome injury and arrest at specific checkpoints in G1, S, G2, and M to allow repair of the lesions, before they are converted into permanent mutations. Lesion detection may occur by blocked transcription, replication or specialised sensors. When

damage is too significant, a cells may opt for the ultimate mode of rescue by initiating cell death, apoptosis.

1.1. DNA damaging anticancer agents

Many chemotherapeutic drugs such as alkylating agents used to combat cancer act principally by damaging DNA. DNA damaging alkylating agents such as nitrogen mustards melphalan, chloambucil, cyclophosphamide and ifosfamide, the platinum drugs cisplatin and carboplatin, chloroethylnitrosoureas 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and CCNU, the alkylalkanesulphonate busulfan and the natural product mitomycin C are among the most widely administered and effective anticancer agents used at present.

Many cancer patients treated with these agents ultimately die of progressive disease, which has become resistant to the drug. There is mounting evidence that DNA repair processes play a role in the development of drug resistance by tumour cells and this subject is becoming an increasingly active area of research. Quantitative analysis of drug-DNA interactions and repair of these lesions provides a basis for understanding the biology of therapeutic kill as a rational strategy for optimising and predicting tumour response.

1.1.1. Alkylating agents

The alkylating agents were the first non-hormonal drugs to be used effectively in the treatment of cancer. These agents act through covalent bonding of alkyl groups to

cellular molecules. Recognition of the anti-tumor effects of these compounds evolved from the clinical observations that mustard gas, di(2-chloroethyl)sulphide (sulfur mustard), used in the First World War caused death through massive damage to the haematopoietic system. Sulfur mustard was found to produce bone marrow suppression, lymphoid aplasia, vesicant action to the skin, conjuctiva and respiratory tract and was evaluated as an antitumour agent (Karnofsky, 1958). Subsequently, the closely related, but less toxic, nitrogen mustards were selected for further study. Trials in patients with lymphoma demonstrated regression of tumours, with relief of symptoms (Jacobson *et al.*, 1946, Goodman *et al.*, 1946). These results encouraged the search to find other alkylating agents with antitumour activity leading to the wide variety of antitumour drugs in use today.

1.1.1.2. Chemistry of alkylating agents

Chemically, an alkylating agent is defined as a compound capable of replacing a proton in another molecule by an alkyl cation. As shown in figure 1.1, an alkylation reaction can occur by two mechanisms; S_N1 (nucleophilic substitution, first order) or S_N2 (nucleophilic substitution, second order). In the S_N1 reaction, there is an initial formation of a highly reactive carbonium ion intermediate, followed by the rapid reaction of this intermediate with a nucleophile. The rate limiting step in this reaction is the initial formation of the reactive intermediate and this type of reaction exhibit first order kinetics. S_N2 reaction represents a biomolecular nucleophillic displacement and the rate of reaction depends on the concentration of both the alkylating agent and the target nucleophile. This type of reaction follows second order kinetics (reviewed in

Hartley, 2001). The clinically useful agents include drugs that alkylate through an SN_1 mechanism (e.g. nitrogen mustards and nitrosoureas), and drugs that alkylate through SN_2 mechanism (e.g. busulfan), and some compounds that alkylate through reactions with characteristics of both an SN_1 and an SN_2 mechanism (Hartley, 2001, Tew *et al.*, 1996).

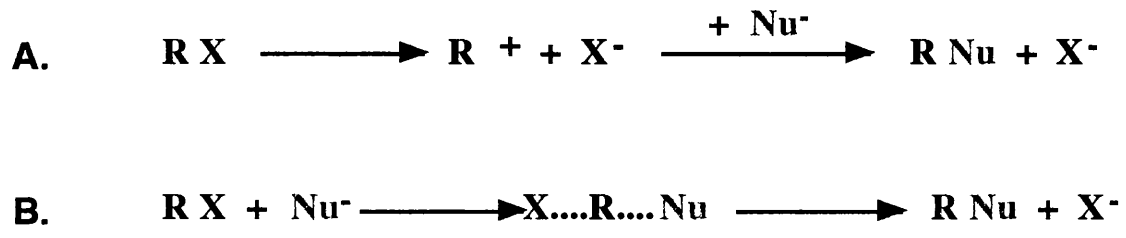


Figure 1.1. SN_1 (A) and SN_2 (B) reactions.

Although alkylating agents can bind a wide variety of biological molecules (Skipper *et al.*, 1951) including nucleic acids, protein, aminoacids and nucleotides, cytotoxicity of these agents is believed to be due to alkylation of DNA. One of the consistently observed biochemical effects of alkylating agents at cytotoxic levels is the inhibition of DNA synthesis (Wheeler *et al.*, 1962). It is believed that the cytotoxic effect of these agents is due to alkylation of DNA bases. The N-7 position of guanine is the most electronegative site anywhere within the bases of DNA and is usually the most readily alkylated. Other sites of alkylation includes the O-6 position of guanine and N-1 and N-3 positions of adenine (Hartley, 2001). These alkylation sites are shown in figure 1.2.

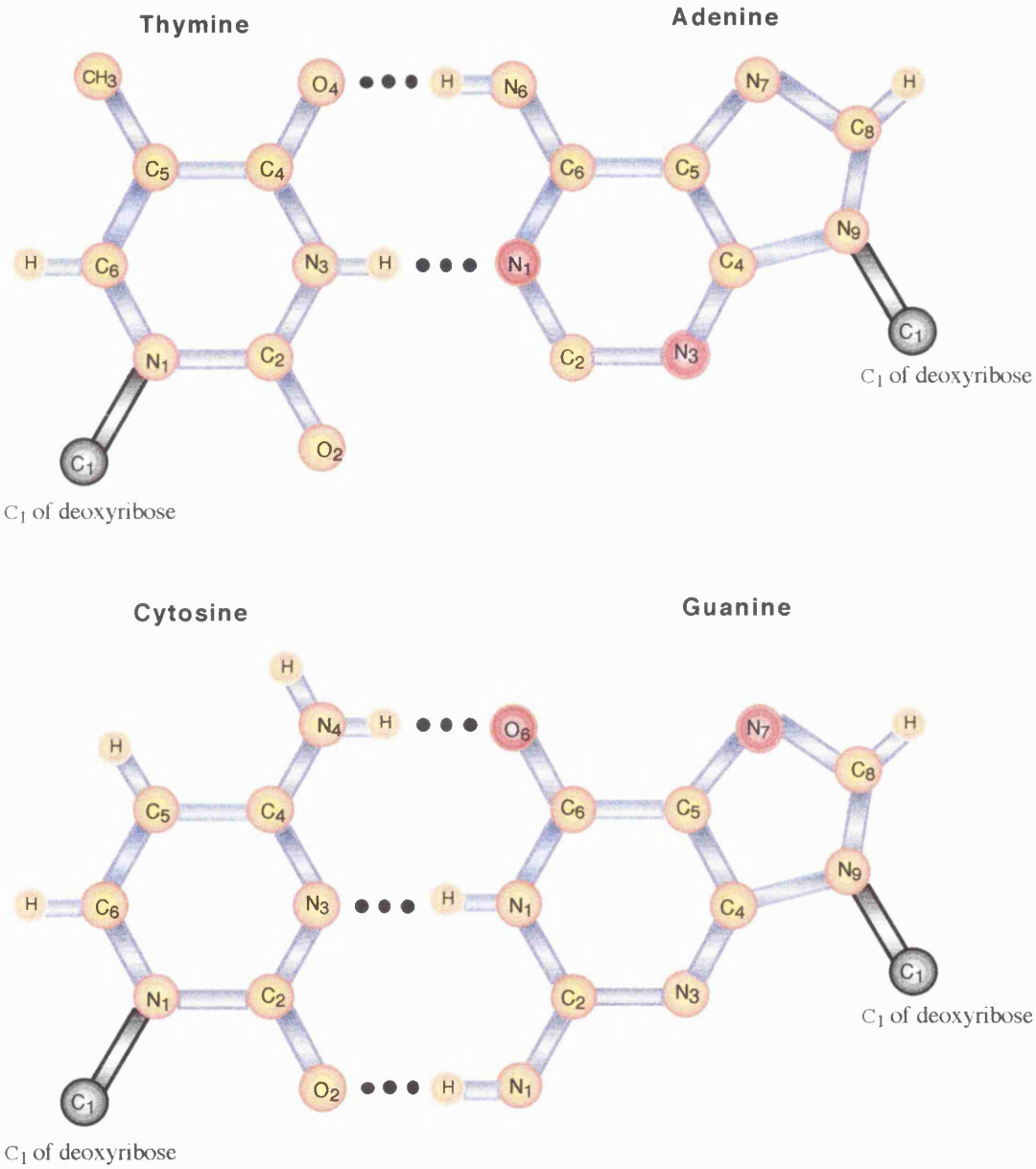


Figure 1.2. Alkylation sites of DNA bases. The reactive sites of guanine and adenine are highlited in red ( ).

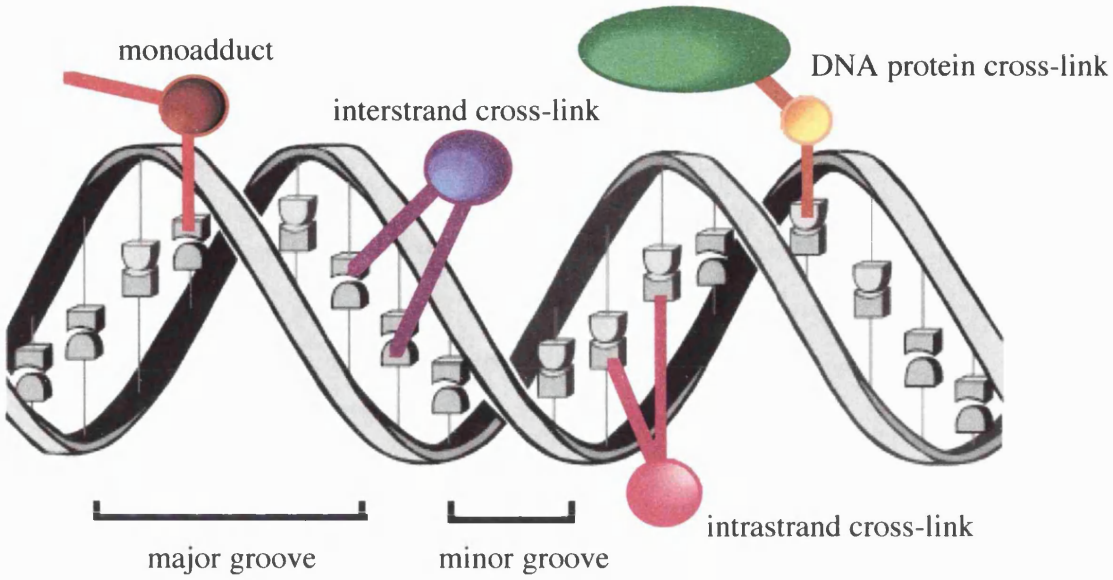


Figure 1.3. Different type of DNA adducts formed by alkylating agents.

Alkylating agents can be classified as mono-functional and bifunctional, depending whether the molecule has one or two reactive groups. Reaction of mono-functional agents with DNA results in mono-adducts. Bifunctional agents, in addition form crosslinks with DNA. The resulting adducts therefore include mono-adducts, DNA intra- and interstrand crosslinks (ICLs) and DNA-protein cross-links (figure 1.3). In the 1940s Ross, Haddow and co-workers demonstrated that bifunctionality was an essential prerequisite for potent cytotoxicity of alkylating agents. Most attention has focused on interstrand crosslinking as the major cytotoxic lesion induced by bifunctional alkylating agents. The formation of crosslinks requires two steps. The first functional group reacts to form a mono-adduct followed by reaction of the second functional group to form a crosslink. Not all mono-adducts form crosslinks. For many

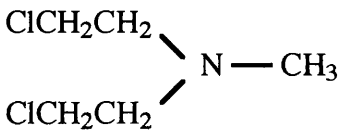
agents, the ratio of mono-adducts to cross-links is at least 20:1 and often much higher (Brendel *et al.*, 1984).

Alkylating agents can also be classified as major groove and minor groove binding agents. Nitrogen mustards and platinum drugs such as cisplatin and carboplatin act by binding covalently to the major groove resulting in a variety of lesions. Recent studies have focused the attention on the minor groove as a target for alkylation. Novel anticancer drugs have been developed recently which bind in the minor groove of DNA in a sequence selective manner. These include pyrrolobenzodiazepine (PBD)-based compounds (Bose *et al.*, 1992, Smellie *et al.*, 1994).

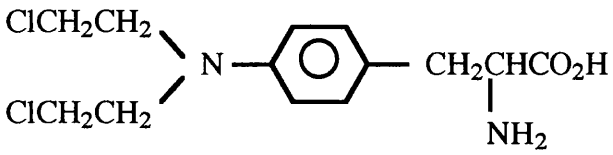
1.1.1.3. Nitrogen Mustards

The first nitrogen mustard to be used extensively in the clinic was mechlorethamine (figure 1.4A), sometimes referred to by its original code name HN2 or by the term nitrogen mustard. The mechanism of alkylation by the nitrogen mustards is illustrated in figure 1.4B. In the initial step, chlorine is lost and the β-carbon reacts with the nucleophilic nitrogen atom to form a positively charged very reactive aziridinium ion intermediate. Reaction of this aziridinium ring with a nucleophile yields the initial alkylated product. Formation of the second aziridinium by the remaining chloroethyl group allows formation of a second alkylation, which produces a crosslink between the two alkylated nucleophiles (Tew *et al.*, 1996, Hartley, 2001).

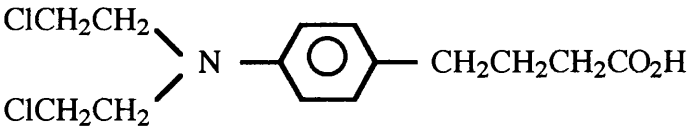
A.



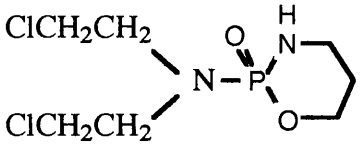
Mechlorethamine



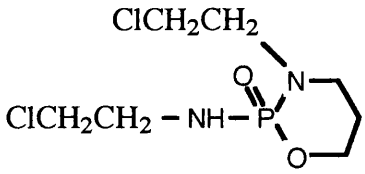
Melphalan



Chlorambucil



Cyclophosphamide



Ifosfamide

B.

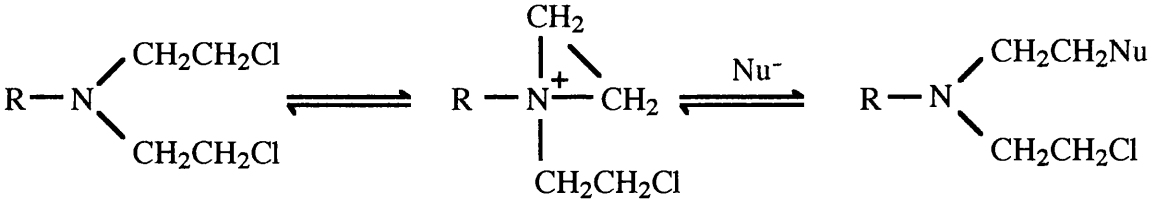


Figure 1.4. Structures of nitrogen mustards (A) and their alkylation reaction mechanism (B).

After introduction of HN2, many analogues have been synthesised in which the methyl group was replaced by a variety of chemical groups. Most of these compounds demonstrated less anitumour activity than HN2, but four derivatives, melphalan (L-phenylalanine mustard), chlorambucil, cylclophosphamide and ifosfamide have shown a higher therapeutic index and broader range of clinical activity. The latter two drugs do not posses alkylating activity and must be metabolised to produce alkylating compounds (Hartley, 2001).

1.1.1.3.1. Mechlorethamine or HN2

HN2 is a bifunctional alkylating agent which forms a variety of DNA adducts, including mono-adducts and cross-links. The vast majority of adducts are mono-adducts (~90%), and inter- and intra-strand cross-links comprise only a small fraction of total lesions (Povirk *et al.*, 1994). Mono-adducts form principally at the N7 position of guanines and to a lesser extent at the N3 position of adenine. Inter- and intra-strand cross-links form between guanine N7 positions, primarily in the sequence 5'-GNC-3' / 3'-CNG-5', and between DNA and proteins (Ojwang *et al.*, 1989). Although ICLs comprise only minority of the lesions, there is good evidence that it is the major cytotoxic lesion induced by nitrogen mustards (Sunters *et al.*, 1993.). Because of its high cytoxicity profile, HN2 is not frequently used in the clinic and is restricted almost solely to the treatment of Hodgkin's disease.

1.1.1.3.2. Melphalan

Melphalan (figure 1.4A) is the amino acid phenylalanine derivative of HN2. This alkylating agent readily forms N7 adducst with guanine. In addition, melphalan also forms a relatively high proportion of thermolabile adenine adducts, and those properties are consistent with alkylation at the N3 position (Povirk *et al.*, 1994). Since guanine N-7 faces into the major groove while adenine N-3 faces into the minor groove this adduct makes much greater demands on DNA conformation than HN2. Melphalan--induced ICLs occur with the same GNC specificity as those induced by HN2, but are formed more slowly and are considered to be more stable to depurination (Povirk *et al.*, 1994). Melphalan has been widely used in the treatment of ovarian cancer, multiple myeloma, and carcinoma of the breast (Tew *et al.*, 1996).

1.1.1.3.3. Chlorambucil

Chlorambucil (figure 1.4A) is an aromatic derivative of HN2 and is closely related in structure to melphalan. It is the slowest acting and generally least toxic of the alkylating agents. Alkylation of DNA results in breaks in the DNA molecule as well as cross-linking. The half-life of chlorambucil in the circulation is around 90 minutes and it is rapidly converted to its metabolites aminophenylacetic acid and phenylacetic acid mustard. Chlorambucil is used for the treatment of chronic lymphocytic leukemia, low-grade lymphomas, Hodgkin's disease and multiple myelomas. (Hartley, 2001).

1.1.1.3.4. Cyclophosphamide

Cyclophosphamide (figure 1.4A) has been the most widely used antitumour alkylating agent. It is an inactive prodrug which would become activated to its reactive form by phosphoamidase enzymes, which are over-expressed in a variety of solid tumours (Hartley, 2001, Tew *et al.*, 1996). Cyclophosphamide is initially metabolised in the liver by P450 to produce 4-hydroxycyclophosphamide which then enters the circulation and tissues. 4-hydroxycyclophosphamide exists in equilibrium with aldophosphamide. Aldophosphamide can either be deactivated by the enzyme aldehyde dehydrogenase to form carboxyphosphamide, or it can be broken down spontaneously to phosphoramide mustard and acrolein. It is believed that phosphoramide mustard is the active bifunctional agent that appears to be responsible for the biologic effects of cyclophosphamide. This ani-cancer agent is used in combination chemotherapy for the treatment of many types of cancers including Hodgkin's disease, non-Hodgkin's lymphoma Burkitt's lymphoma and cancers of the breast, bladdder, lung, cervix and ovary (Hartley, 2001, Tew *et al.*, 1996).

1.1.1.3.5. Ifosphamide

Like cyclophosphamide, ifosfamide (figure 1.4A) is another prodrug which undergoes hepatic activation by hydroxylation. This process occurs at a slower rate for ifosfamide than for cyclophosphamide resulting in a longer plasma half-life for the parent compound. Ifosfamide has a broad anitumour activity and it is particularly important in the treatment for soft tissue sarcomas and testicular cancer (Hartley, 2001, Tew *et al.*, 1996).

1.1.2. Platinum Complexes

The biological activity of platinum complexes was discovered by Rosenberg *et al.*, in 1965 during studies of the effect of an electric current on *Escherichia coli*. It was found that cell division was inhibited not by the electric current but by the production of the platinum complex cis-diamminedichloroplatinum(11) (cis-DDP or cisplatin) from the platinum electrodes. This inhibitory effect on cell division suggested that cisplatin (figure 1.5A) might have potential as an anti-cancer agent. Cisplatin was approved for clinical use in 1979 (Loehrer & Einhorn, 1984) and is now one of the most powerful anticancer drugs in use. It is widely used for the treatment of many malignancies, including ovarian, head and neck, bladder, lung, but it is most strikingly effective in testicular cancer (Loehrer & Einhorn, 1984). Following introduction of cisplatin, several other clinically effective platinum analogues have emerged, such as carboplatin, oxaliplatin and tetraplatin.

1.1.2.1. Cisplatin

The platinum class of agents are designated as Pt(II) or Pt(IV) depending on the oxidation state of the platinum. In Pt(II) complexes, the platinum atom has four coordination complexes and in Pt(IV) complexes there are six. Because these coordination complexes are fixed and not readily exchangeable, they have distinct *cis*- and *trans* isomers. The *cis* isomer is cisplatin and *trans*-isomer is termed transplatin (figure 1.5A), which has much lower cytotoxicity and no anti-tumour activity (Hacker *et al*, 1984).

A.

Cisplatin Transplatin

B.

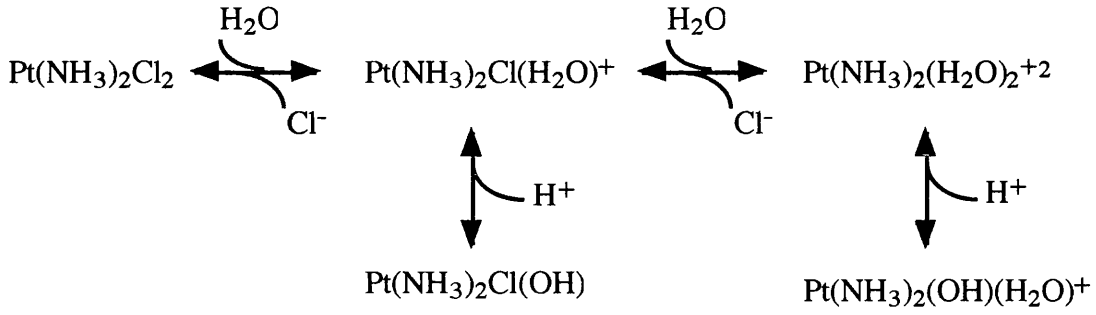


Figure 1.5. Structure of cisplatin and transplatin (A) and activation of cisplatin in aqueous solution (B).

The biological actions of Pt(II) agents are due to displacement reactions. As shown in figure 1.5B, in an aqueous environment, the chloride ions of cisplatin are displaced to allow the formation of an electrophilic aquated species, which is the reactive form of the compound (Chu, 1994). Activated cisplatin is a potent electrophile that will react with any nucleophile, including proteins and RNA, but the most important intracellular target is DNA. These complexes are bifunctional in that it can form, by successive displacement reactions, two stable bonds under physiologic conditions to produce a covalent cross-link. In this respect Pt(II) complexes are similar to bifunctional alkylating agents such as nitrogen mustard (Reed *et al.*, 1999).

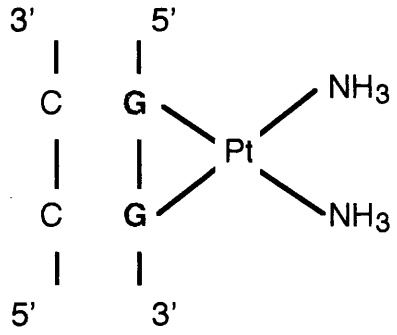
Although the basis of therapeutic effectiveness is still not completely understood, there is strong evidence to suggest that cisplatin exerts its cytotoxic effects by damaging DNA (Rosenberg, 1985). The DNA adducts of cisplatin are proposed to mediate cytotoxicity by inhibiting DNA replication and transcription and ultimately by activating programmed cell death, apoptosis. Cisplatin has been noted to bind to all DNA bases, but shows preferential binding to the N-7 positions of purine bases (guanine and adenine) due to the high nucleophilicity of the imidazole ring, particularly at the N-7 position (Pinto *et al.*, 1985, Johnson *et al.*, 1980).

Cisplatin forms a variety of DNA adducts, including DNA mono-adducts, inter- and intra-strand cross-links and DNA protein cross-links. It binds to DNA in two successive steps. First, monofunctional adducts are formed by binding to N-7 positions of purine bases, which are exposed in the major groove of the DNA double helix. Subsequently, the remaining electrophillic centre will either react with a nearby purine on the same strand of the DNA to form a bifunctional intra- strand cross-link or it will react with a purine on the complementary DNA strand to form an ICL (Trimmer *et al.*, 1999).

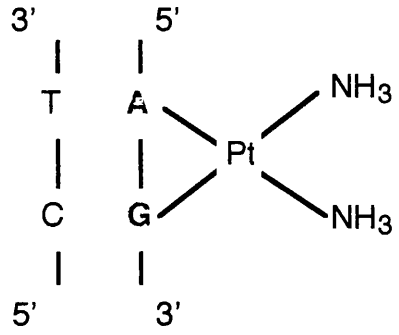
The major DNA cisplatin-adducts are shown in figure 1.6. Quantitative *in vitro* studies have shown that 1,2-intra-strand d(GpG) adducts between adjacent guanines comprise 65% of the adducts, 1,2-intra-strand d(ApG) adducts between adjacent adenine and guanine make 25% of the adducts, and 1,3-intra-strand d(GpXpG) adducts between guanines separated by an intervening nucleotide make 6% of adducts (Fichtinger-

Schepman *et al.*, 1985). ICLs which form between guanines at d(G*pC)/d(G*pC) sites (where asterisks indicate sites of platinum binding) and monofunctional adducts occur at very low frequency, 1-2%. (Fichtinger-Schepman *et al.*, 1985). Although cisplatin has been studied for many years, the types of DNA lesions primarily responsible for the cytotoxicity is still not clear. Several studies have shown that cytotoxicity of cisplatin against cells in culture co-relates closely to 1,2-intra-strand d(GpG) (Chu *et al.*, 1994), 1,2-intra-strand d(ApG) (Fichtinger-schepman *et al.*, 1995), ICLs (Zwelling *et al.*, 1979) and total platinum binding to DNA (Reed *et al.*, 1996).

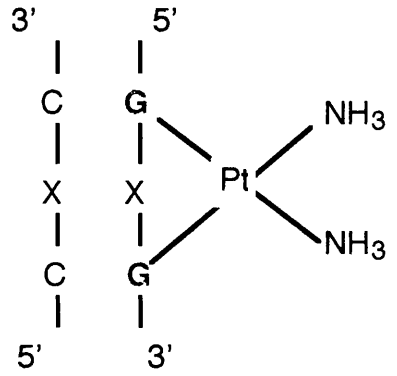
The adduct spectrum of therapeutically inactive transplatin is different to that induced by cisplatin. In naked DNA, transplatin primarily forms 1,3-intra-strand d(GpXpG) adducts (40%) between guanine residues separated by at least one base and ICLs (Eastman, 1988, Brabec and Leng, 1993). The rate of induction of ICLs by transplatin is slower than that induced by cisplatin, but transplatin forms about double the quantity of ICLs over long period in comparison with cisplatin. However, these ICLs occur between complementary guanine and cytosine residues in contrast to ICLs formed between guanine residues by cisplatin (Brabec and Leng, 1993). Although transplatin induce high proportion ICLs in naked DNA, only cisplatin produces substantial ICLs in mammalian cells (Zwelling *et al.*, 1979). 1,2-Intra-strand cross-links between adjacent bases are not induced by transplatin and therefore it has been suggested that the differences in antitumour activity of cisplatin and transplatin may arise from the different nature of intra-strand cross-links induced by these agents (Brabec and Leng, 1993).



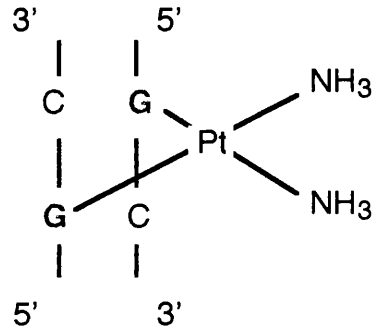
1,2-intra-strand d(GpG) adduct



1,2-intra-strand d(ApG) adduct



1,3-intra-strand d(GpXpG) adduct



Interstrand cross-link

Figure 1.6. Bifunctional adducts formed by cipslatin

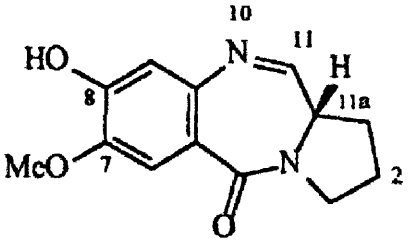
Analysis of mammalian cellular extracts revealed the existence of proteins that bind specifically to the major adducts of cisplatin, but not to DNA modified by transplatin (Bruhn *et al.*, 1992, Hughes *et al.*, 1992, Kartalou and Essigmann, 2001). These proteins share a common structural element, the high mobility group (HMG) domain. HMG proteins recognise and further bend DNA distorted by 1,2-intra-strand cisplatin-DNA adducts, but not the 1,3-intra-strand cross-links (Pil and Lippard, 1992, Chow *et*

al., 1994). In vitro excision repair assays demonstrate that HMG proteins specifically inhibit excision repair of the 1,2-d(GpG) and 1,2-d(ApG) intra-strand cross-links but not of the 1,3-d(GpXpG) adducts (Zamble *et al.*, 1996, Huang *et al*, 1994). The mechanism by which HMG proteins might mediate cisplatin cytotoxicity has not yet been elucidated, but it has been proposed that specific binding to HMG proteins to platinated DNA could shield the adducts from DNA repair proteins (Kartalou and Essigmann, 2001).

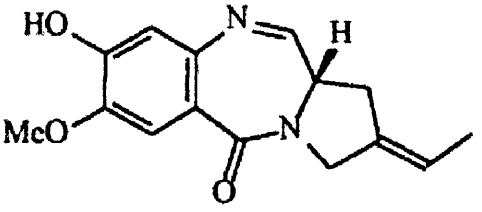
1.1.3. Novel minor groove binding sequence specific alkylating agents

The nitrogen mustards and platinum agents discussed above are conventional anti-cancer agents, which bind in the major groove of DNA. Recent studies have focused attention on anticancer agents that bind in the minor groove of DNA. These includes CC-1065, distamycin and the pyrrolo[2,1-c][1,4]benzodiazepines (PBDs). PBD molecules are a family of naturally occurring antitumour antibiotics derived from various *Streptomyces* species which form covalent bonds to specific base sequences of DNA. Well-known members of this family include DC-81 and tomamycin (figure 1.7) (Thurston, 1993).

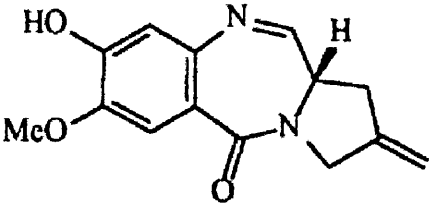
The cytotoxic activity of these molecules is attributed to their ability to form covalent DNA adducts between their electrophillic N10-C11-position and the exocyclic C2-NH2 group of a guanine base in the minor groove of DNA (Thurston, 1993). These molecules span three DNA base pairs with preference for purine-gunanine-purine.



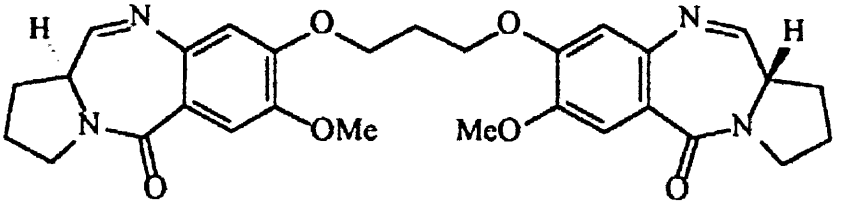
DC-81



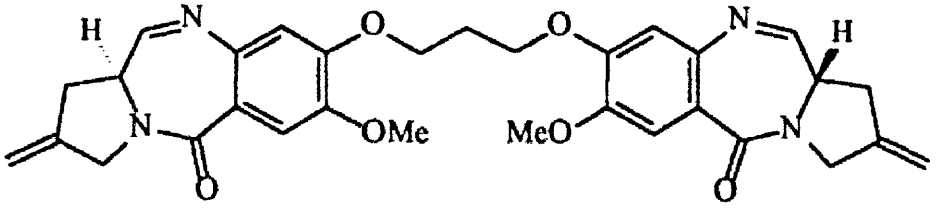
Tomamycin



MMY



DSB-120



SJG-136

Figure 1.7. Structures of PBD monomers; DC-81, tomamycin, MMY and PBD dimers; DSB-120 and SJG-136.

PBD molecules are currently of considerable interest due to their ability to recognise and subsequently form covalent bonds with DNA in a sequence selective manner.

1.1.3.1. PBD related ICL agents

Recently, two PBD molecules have been joined together through their C-8 positions via a linker to create a novel bifunctional agent with the ability to alkylate two guanines on opposite strands of DNA in a sequence selective manner to form an ICL. DSB-120 was the first such a compound to be synthesised with a linker consisting of three methylene groups between two DC-81 molecules (figure 1.7) (Bose *et al.*, 1992). This agent has a high affinity for DNA and forms irreversible DNA ICLs. It spans six DNA base pairs in the minor groove and forms a symmetric crosslink between spatially separated internal guanines and with active recognition of an embedded 5'-(pu/py)GATC(py/pu) site. Studies have shown that DSB-120 is approximately 50 and 300 fold more efficient at producing ICLs in naked DNA than the major-groove cross-linking agents, cisplatin and melphalan respectively. (Bose *et al.*, 1992). However, *in vivo* studies with DSB-120 in murine models has been disappointing as low therapeutic index was observed (Walton *et al.*, 1996). It was thought that this is partly due to reaction of DSB-120 with cellular thiols before reaching the tumour site.

PBD monomers with unsaturation at the C2 position are known to be biologically more potent than their C2-saturated members. C2-unsaturation can lead to lower electrophilicity at the N10-C11 positions which may allow greater availability of the agent at the target DNA due to lower level of deactivation by cellular nucleophiles. In

an attempt to further extend potency of DSB-120, the novel PBD dimer, SJG-136 (figure 1.7) has been developed recently. SJG-136 is a C2/C2' *exo*-methylene analogue of DSB-120 and it is synthesised by joining two C2-methylene DC-81 (MMY) monomers via a linker (Gregson *et al.*, 1999, 2001). DNA cross-linking studies have shown that SJG-136 is a more efficient cross-linking agent than DSB-120 (Gregson *et al.*, 2001). SJG-136 is exquisitely cytotoxic in a panel of human ovarian carcinoma cell lines and some cisplatin resistance sublines, and have demonstrated the lowest IC50 values so far obtained for any synthetic PBD monomer or dimer (Gregson *et al.*, 1999, 2001). In CH1 cell line, SJG-136 is approximately 275-fold more potent than DSB-120 and 1500-fold more potent than cisplatin (Gregson *et al.*, 2001). In addition to reduced cellular deactivation, the exquisite cytotoxicity of SJG-136 is thought to be due to more snug fit to the minor groove by the C2/C2'-methylene groups and the adducts formed may not be recognised by the DNA repair systems. SJG-136 has demonstrated a significant anti-tumour activity in a number of human tumour zenografts and is currently in pre-clinical development.

1.2. Drug resistance

Despite many advances in the management of cancer over recent decades, drug resistance remains a major reason for treatment failure with the result that cancer remains one of the major cause of death in the Western World. Drug resistance can be defined as the failure of a tumour to respond to chemotherapy or the continued growth of a tumour during treatment. There are two types of drug resistance. In the first, an initial response to chemotherapy is followed at relapse by the emergence of disease

refractory to the treatment (acquired drug resistance). In the second, low rate of initial response is seen due to intrinsic drug resistance (innate drug resistance).

A clearer understanding of the complex biological mechanisms involved in drug resistance would enable better design of treatment strategies in the future. Many mechanisms of drug resistance have been elucidated which may occur independently or act synergistically. These include reduced intracellular drug uptake, increased drug efflux, decreased drug activation and increased drug catabolism and detoxification (e.g. elevation in cellular thiols) (Hartley, 2001). Additionally enhanced ability of tumour cells to repair DNA damage induced by antitumour agents may also play a role in the development of resistance. For example, increased expression of O^6-methyguanine DNA methyl transferase (MGMT) has been reported in various tumours with decreased response to some alkylating agents (Preuss *et al.*, 1996, Yu *et al.*, 1999). In addition chronic leukaemia cells from patients who have acquired resistance to nitrogen mustards have enhanced ability to repair ICLs induced by melphlan *in vitro* compared to cells from sensitive or untreated patients (Torres-Garcia *et al.*, 1989). Also, a co-relation has been observed between the level of DNA-PK activity (which play a role in DNA double strand break repair) and the *in vivo* development of resistance to chlorambucil in chronic lymphocytic leukaemia patients (Muller *et al.*, 1998). A recent study in this laboratory has shown a marked elevation of ICL repair in plasma cells from patients with multiple myeloma who have relapsed melphalan therapy compared to cells from untreated patients (Spanswick *et al.*, 2002). These

studies highlight that DNA repair may play an important role in the development of resistance to alkylating antitumour agents.

1.3. DNA repair

DNA encounters various assaults on its native structure and sequence throughout the life span of a cell. No single repair process can cope with all kinds of damage. Instead, sophisticated, interwoven DNA repair systems have evolved that as a whole cover most types of DNA lesions. Damage from both endogenous sources, often products from cellular metabolism and exogenous sources such as environmental toxins, ionising radiation and chemotherapy are mainly corrected through five major repair pathways: 1) direct damage reversal, 2) mismatch repair, 3) base excision repair, 4) nucleotide-excision repair, 5) double strand break repair pathways; homologous recombination (HR), non-homologous end joining (NHEJ) and single strand annealing (SSA).

1.3.1. Direct damage reversal

Using specific enzymes, this repair pathway eliminates the abnormal chemical bonds between bases or between a nucleotide and an abnormal substituent. Examples include, repair of cylobutane pyrimidine dimers induced by ultraviolet light by DNA photolyase and repair of methylation damage at the O^6 position of guanine bases by O^6-methylguanine DNA methyltransferase (MGMT) (Sancar, 1996). MGMT levels in cancer cells often co-relate to their sensitivity to chemotherapeutic agents and as discussed earlier, elevated levels of MGMT have been reported in ovarian cancer,

pancreatic cancer, colon cancer, and brain tumours. Increased levels of MGMT generally but not always co-relates with the tumour's decreased response to alkylating agents like temazolomide, cyclophosphamide, BCNU and dacarbazine (Preuss *et al.*, 1996, Yu *et al.*, 1999).

Human hematopoietic cells contain a low or absent level of endogenous MGMT. Because the primary dose limiting toxicity of majority of alkylating agents is bone marrow suppression and secondary hematopoietic tumours occasionally result from the initial treatment of cancer, many laboratories are studying the use of over-expressed human MGMT in hematopoietic progenitor cells to protect the bone marrow from chemotherapeutic agent's toxicity (Jansen *et al.*, 2001, Wang *et al.*, 1996, Moritz *et al.*, 1995).

1.3.2. Mismatch repair (MMR)

This mechanism detects and repair mismatches which can occur in DNA due to incorrect incorporation by DNA polymerases, damage to the precursors in the cellular nucleotide pool or by some types of damage to DNA. (Hansen and Kelley, 2000, Hoeijmakers, 2001). Correction of the mismatch involves the removal of long tract of DNA followed by synthesis of a long repair patch (up to 1000 nucleotides).

Initially the genes involved in this pathway were discovered in bacteria and the major proteins involved include MutS, MutL, MutH, DNA polymerase, single stranded binding proteins and DNA ligase. Homologues of these genes have now been

identified in mammalian systems. In humans, there are six homologues of the *E.coli* MutS gene termed MSH (MSH1-6) genes, and at least 16 genes that code for homologues of the *E.coli* MutL gene, termed MLH genes (Hansen and Kelley, 2000).

The mechanism of MMR is outlined in figure 1.8. Firstly, the MSH2 protein in a complex with either MSH6 or MSH3 (depending on the type of damage), binds to the damaged DNA. MSH2-MSH6 focuses on mismatches and single-base loops, whereas MSH2-3 dimers recognise insertion, deletion loops. Then, MLH1 and PMS2 bind to each other and to the MSH2-MSH6 or MSH2-MSH3 complex. This complex produces a 3' or 5' nick followed by exonuclease activity resulting in a 100 - 1000 nucleotide gap in the newly synthesised strand with the incorrectly paired base. Repair is then completed by re-synthesis and ligation (Hansen and Kelley, 2000, Hoijmakers, 2001).

Defects in the MMR system cause replication errors in both coding and non-coding regions of the genome, leading to an accumulation of multiple mutations, a prerequisite of tumorigenesis. In humans, MMR is associated with a large fraction of hereditary nonpolyposis colon cancer (HNPCC) cases, a relatively common genetic disease that accounts for about 5% of all colorectal cancers. Most HNPCC individuals inherit a mutation in one allele of either MLH1 or MSH2 (Liu *et al.*, 1996, Muller *et al.*, 2002). These tumours show extensive microsatellite instability because microsatellites (short repetitive sequences such as $[GT]_{30}$) are prone to mismatch formation during replication. In addition to HNPCC, microsatellite heterogeneity and

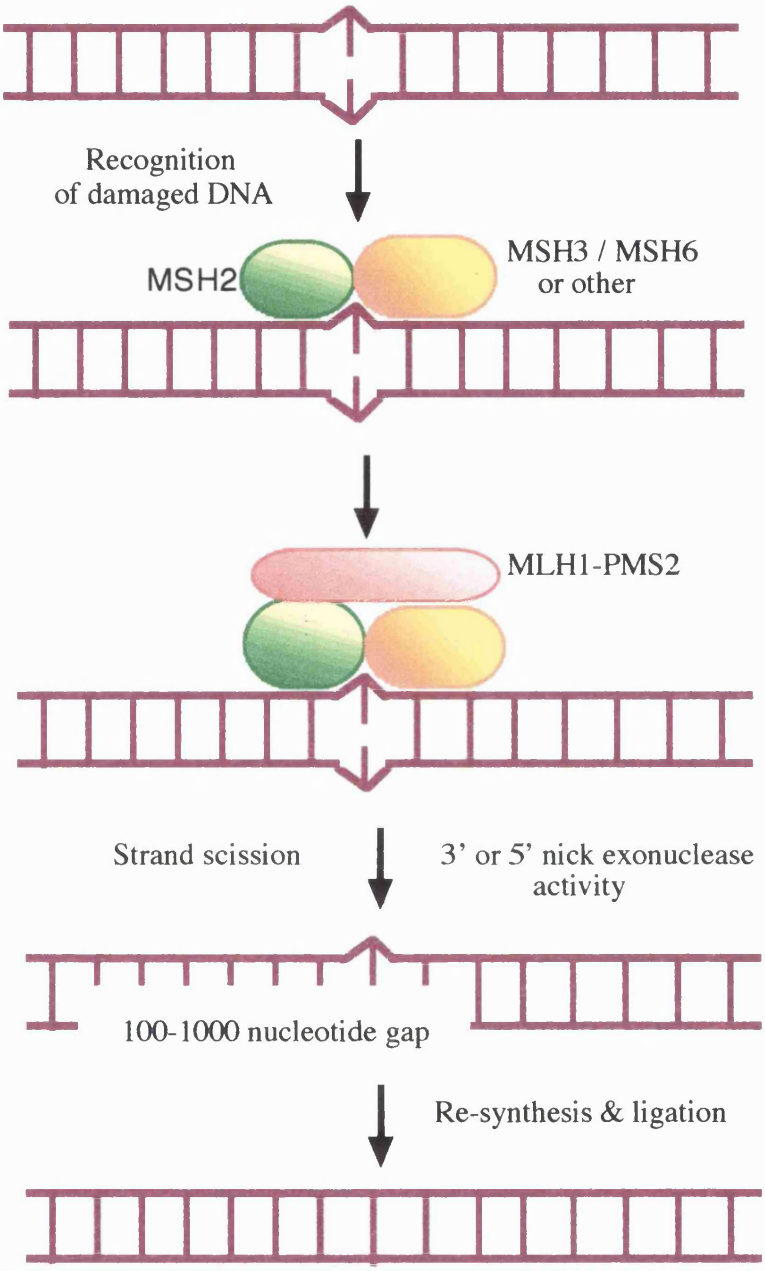


Figure 1.8. Mechanism of MMR.

Either a MSH2-MSH6 or MSH2-MSH3 complex recognises and binds to damaged DNA. Then, MLH1 and PMS2 bind to this complex and make a 3' or 5' nick followed by exonuclese activity, resulting in a 100-1000 nucleotide gap in the newly synthesised strand, which is then filled in by resynthesis and ligation.

mismatch repair defects have been detected in a number of sporadic tumours (Hansen and Kelley, 2000, Hoeijmakers, 2001).

Cells defective in MMR pathway are resistance to the drugs cisplatin and carboplatin, the alkylating agent busulfan, methylating agents procarbazine and temozolomide, the antimetabolite 6-thioguanine, and the topoisomerase II inhibitors etoposide and doxorubicin (Fink *et al.*, 1998). In a number of sporadic cancers hypermethylation of hMLH1 gene promoter occurs at high frequency resulting in loss of MLH1 expression. Cisplatin resistant ovarian tumour cell lines derived from A2780 cells lack MLH1 expression due to methylation of hMLH1 gene promoter. Treatment of these cells with the demethylating agent 2'-deoxy-5-acadytidine (DAC) restores MLH1 expression and sensitises these cells to cisplatin *in vitro* (Strathdee *et al.*, 1999). Similarly, DAC treatment can also sensitise drug resistant ovarian and colon xenografts to cisplatin, carboplatin, temozolomide and epirubicin *in vivo* (Plumb *et al.*, 2000).

It has been shown that MMR proteins recognise DNA adducts and stimulates signalling cascades for apoptosis. Loss of this system results in impaired damage recognition and apoptosis. For example in the absence of MMR, cisplatin induced intrastrand cross-links are bypassed during replication resulting a cisplatin resistant phenotype (Durant *et al.*, 1999). Inhibition of replication by the treatment with DNA polymerase inhibitor aphidicolin increases the sensitivity of MMR-deficient cells to cisplatin (Moreland *et al.*, 1999). These studies have suggested that MMR is involved in producing a signal during replication that triggers cell death and in the absence of

MMR DNA lesions are bypassed leading to cellular resistance. Therefore, in addition to repair of DNA mismatches, MMR also contributes to genome stability by stimulating DNA damage induced apoptosis.

1.3.3. Base Excision Repair (BER)

BER has evolved primarily to protect cells from deleterious effects of endogenous DNA damage including that resulting from reactive oxygen species, methylation, deamination, hydroxylation and other endogenous metabolites that modify DNA base structure. This repair pathway is also important in the repair of some of the DNA base damage produced by ionising radiation and alkylating agents. The mechanism of BER is outlined in figure 1.9. Firstly, a structure specific DNA glycosylase flips the altered base out of the DNA helix. Different DNA glycosylases remove different types of damaged bases and the specificity of the repair pathway is determined by the type of glycosylase involved. Once the base is removed, strand incision at the resulting apurinic/apyrimidinic site (AP-site) is initiated by an APE1 endonuclease. Then DNA polymerase β performs a one nucleotide gap filling reaction and removes the 5'-terminal baseless sugar via its lyase activity. This is followed by sealing of the remaining nick by the XRCC1-ligase III complex restoring the damage. The XRCC1 scaffold protein has separate binding sites for DNA polymerase β and ligase III and may be instrumental in protein exchange (Lindahl *et al.*, 1997, Hansen and Kellary, 2000 and Hoeijmakers, 2001). This represents the primary pathway of BER, which involves one nucleotide repair gap and is termed 'short patch repair'. BER can also involve in a repair patch larger than one nucleotide. In this so called 'long patch'

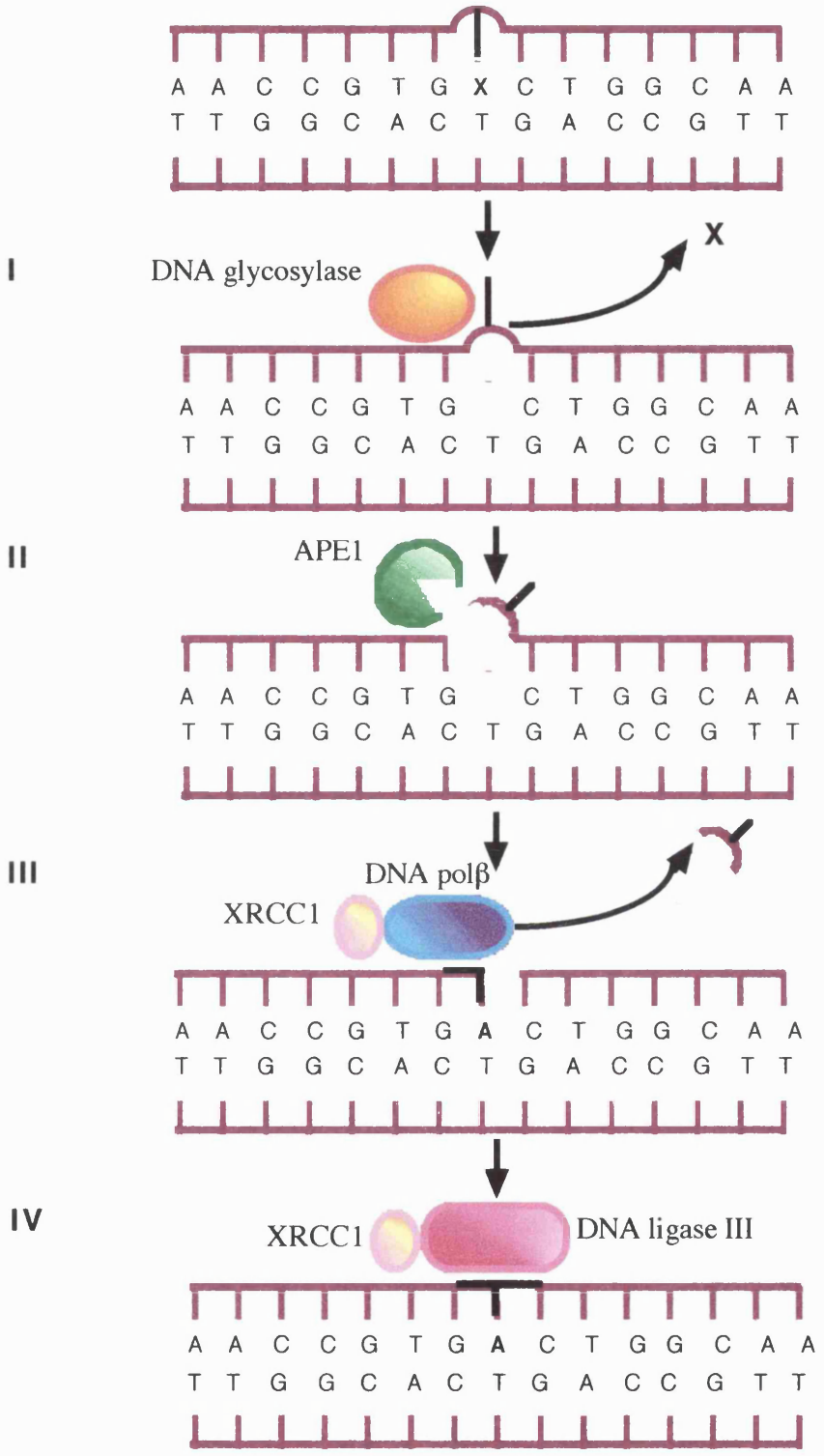


Figure 1.9. Mechanism for base excision repair (BER).
1. Altered base residue is released by DNA glycosylase. II. Abasic site is cleaved by APE1 endonuclease. III. DNA polβ fills in one nucleotide gap and exices the base free residue. IV. XRCC1-ligase III complex then complete the reaction by sealing the remainng nick.

pathway, approximately 2-10 nucleotides are excised and replaced by the combined actions of the DNA polymerase δ, ε or β, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C) and the endonuclease that cleave 'flap' structures, FEN1. The long patch pathway seems to predominate when repair is initiated at oxidised or reduced AP sites generated by X-rays or chemical agents, whereas the short patch repair occurs when regular AP sites are generated (Hansen and Kelley, 2000).

In humans, there are methylpurine-, uracil-, thymine- and 8-oxoguanine DNA glycosylases with narrow substrate ranges and one major AP endonuclease (Sancar, 1996). 3-methyladenine DNA glycosylase (MPG/AAG) is involved in the repair of some DNA damage induced by alkylating agents. This enzyme is most active on 3-methyladenine, a major lesion resulting from alkylating agents. MPG also functions to cleave the major product of all alkylated DNA, N7-methylguanine (Friedberg *et al.*, 1995). Studies in this laboratory have shown that *S.cerevisiae* mutants lacking MPG are hypersensitive to nitrogen mustard, HN2 indicating the importance of BER in the repair of HN2 induced DNA damage (McHugh *et al.*, 1999). Additionally, mouse cells with a null mutation in MPG gene display hypersensitivity to several alkylating agents including mitomycin C, but surprisingly, not to HN2 (Allan *et al.*, 1998). These results suggest that the relative contribution of MPG in the repair of HN2-induced lesions differ in these two organisms.

The uracil DNA glycosylase (UDG) is specifically involved in the removal of uracil containing DNA. This enzyme is well conserved from bacteria to man emphasizing the

essential nature of this type of function in preventing mutations arising from deaminated cytosine residues in DNA (Seeberg *et al.*, 1995). Cytosine residues of DNA can be methylated at C5 position and upon deamination, thymine is generated in the context of a G:T mispair. Thymine DNA glycosylase (TDG) is involved in the correction to G:T mispair to G:C (Hansen and Kelley, 2000). Oxidative stress from normal metabolism or ionising radiation causes oxidative damage to both purines and pyrimidines. 7,8-dihydro-8-oxoguanine (8-oxoG), which result from oxidation of guanine opposite cytocine in DNA are removed by 8-oxoguanine DNA glycosylase (Ogg1). This abundant lesion is mutagenic because it may result in GC to TA transversions (Hansen and Kelley, 2000).

1.3.4. Nucleotide excision repair (NER)

NER is a DNA repair pathway which can act with varying efficiency on a wide variety of adducts in DNA and is most effective on bulky or helix distorting lesions (Sancar, 1996, de Laat *et al.*, 1999, Hansen and Kellary, 2000 and Hoeijmakers, 2001). Among DNA repair pathways, nucleotide excision repair plays a major role in the removal of DNA adducts produced by DNA alkylating agents. In humans, the major function of NER is the removal of UV-induced lesions, cyclobutane pyrimidine dimers and (6-4) photoproducts caused by exposure of cells to sunlight. This elaborate repair system removes DNA damage in the form of 24-32 nucleotides long oligomers by incising the damaged strand on both sides of the lesion, followed by DNA synthesis, using a complementary strand as the template, and ligation. It is the most extensively studied and most complicated of the excision repair processes involving the products of some

30 genes. Two modes of NER can be distinguished with partly distinct substrate specificity; repair of lesions over the entire genome termed global genome repair (GG-NER), and repair of transcription blocking lesions present in transcribed DNA strands termed transcription coupled (TCR) (Sancar, 1996, de Laat *et al.*, 1999).

1.3.4.1. NER deficiency syndromes

Deficits within the NER genes can lead to inherited disorders like Xeroderma Pigmentosum (XP), Cockayne's Syndrome (CS), and Trichothiodistrophy (TTD) (Lehmann, 1995, de Boer and Hoeijmakers, 2000). Defects in one of the seven XP complementation groups genes (XPA-XPG) leads to a > 1000-fold increase in skin cancers, primarily after exposure to sunlight or UV irradiation, and neurological abnormalities (Lindahl *et al.*, 1997). In contrast, CS and TTD are NER disorders that show no evidence of increased cancer risk, but these disorders are associated with congenital neurological degeneration and skeletal abnormalities and demonstrate some overlapping symptoms with XP. CS include features of premature aging, which may be related to the increased trigger for apoptosis induced by transcriptional arrest from endogenous lesions in combination with the TCR defect (de Boer and Hoeijmakers, 2000). TTD shares many symptoms with CS, but with additional hallmarks of brittle hair, nails and scaly skin. Mutations in XPB and XPD, subunits of transcription factor TFIIH, which have dual functions in NER and transcription, can lead to defects in both repair and transcription (de Boer and Hoeijmakers, 2000).

1.3.4.2. History of NER

The NER pathway was first extensively studied in prokaryotes, mainly *E. coli* and later in eukaryotes. Research leading to the discovery of the NER mechanism was initiated in *E.coli* in 1960's. Studies in UV-sensitive yeast mutants lead to the discovery of RAD (radiation sensitive) loci. Over 30 RAD genes have been identified and these genes have been useful in understanding NER in mammalian cells. The mechanism of NER in eukaryotes is very similar to that of prokaryotes, regarding the biochemical strategy used, but very different in the nature and the number of proteins involved (Petit and Sancar, 1999).

Two mammalian cell systems have been extensively investigated in the process of isolation of genes involved in NER. One consists of cell lines derived from the three NER defective human hereditary diseases, XP, CS, and TTD. The second consist of series of rodent cell lines selected in the laboratory for defective NER (Friedberg *et al.*, 1995).

Initial attempts to isolate human NER deficient genes involved studies with XP cell lines. Cells with XP are assigned to nine different complementation groups according to phenotypic improvement in DNA repair following cell fusion studies. Attempts to characterize the genes responsible for the DNA repair deficit in XP cells by transfection of foreign DNA has been unsuccessful because human cells are generally poor recipients of DNA.

Among the mutant rodent cell lines used for studies on NER, the majority originate from Chinese Hamsters, although a number of murine cell lines have also been isolated. Most of these mutants were generated by exposure of exponentially growing cell population to UV radiation and allowing suitable periods for fixation of damage-induced mutations. The largest and most completely characterized collection of UV sensitive rodent mutant cell lines has been derived from the Chinese Hamster Ovary (CHO) cell line AA8 (Thompson *et al.*, 1980, Busch *et al.*, 1980). Many of these UV-sensitive mutant CHO cell lines were defective in NER, and therefore they were used to identify human NER genes. Transfection of these mutants with human genomic DNA has resulted in the identification and cloning of series of a complementing genes termed ERCC (for Excision Repair Cross Complementing), denoting the fact that rodent cells were corrected for mutant phenoypes by human DNA (Friedberg *et al.*, 1995).

The above studies resulted in the molecular cloning of genes termed ERCC1, ERCC2, ERCC3, ERCC4, ERCC5 and ERCC6, named for the RCG1 (Rodent Complimenting Group 1) through RCG6 (Lehmann *et al.*, 1994). In all cases human genes isolated have turned out to be involved in NER in human cells. Apart from ERCC1, all other ERCC genes have been proven to be involved in NER defective diseases XP, CS and TTD. Therefore these genes were given the designation XP-, CS- or TTD-, depending on the complementing group (see table 1.1).

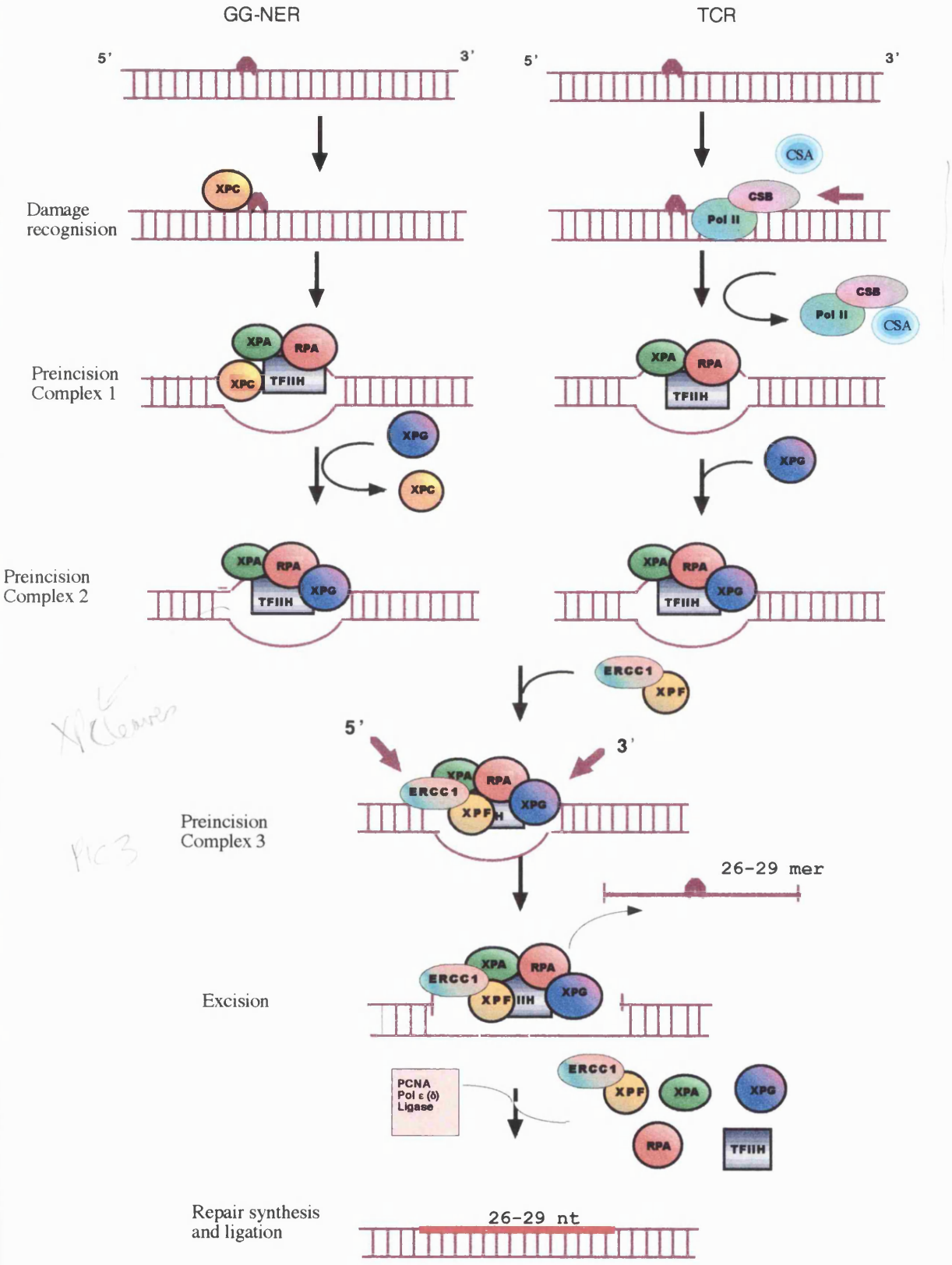
Rodent complementation group	Human complementation group	Human Gene	*S. cerevisiae* gene
RCG-1		ERCC1	*RAD10*
RCG-2	XP-D / CS-XPD / TTD-XPD	XPD	*RAD3*
RCG-3	XP-B / CS-XPB / TTD-XPB	XPB	*RAD25 (SSl2)*
RCG-4	XP-F	XPF	*RAD1*
RCG-5	XP-G / CS-XPG	XPG	*RAD2*
RCG-6	CS-B	CSB	*RAD26*
	XP-A	XPA	*RAD14*
	XP-C	XPC	*RAD4*
	CS-A		*RAD28*
	XP-E		

Table 1.1. Genes involved in NER pathway and their complementary groups.

1.3.4.3. Mechanism of NER

NER is a multi-step process and the mechanism is outlined in figure 1.10. Firstly, NER proteins recognize the damage and an open structure is formed in the DNA around the lesion. In GG-NER, for many DNA lesions, there is increasing evidence that the XPC-hHR23B complex plays an important role early in the damage recognition steps (Wood, 1999, Volker *et al.*, 2001). XPC-hHR23B binds tightly to a distorted region and alters the DNA structure so that other components of repair apparatus can enter (Sugasawa *et al.*, 1998). If the structure is already sufficiently distorted, XPC is not needed (Mu *et al.*, 1996, 1997, Mu and Sancar 1997). Several studies have shown that other factors such as XPA and replication protein A (RPA) assist in the recognition

Figure 1.10. The mechanism of global (GG-NER) and transcription coupled (TCR) NER pathway. In GG-NER, XPC-hHR23B complex plays a role early in the damage recognition step and other repair factors are recruited. The first high specific damage recognition complex is formed with combination of XPC-hHR23B, XPA, RPA and TFIIH proteins, termed PIC1. In TCR, damage is detected by the elongating RNA polymerase II. CSA and CSB are involved in the processing of stalled RNA polymerase II, and other repair factors; TFIIH, XPA and RPA are recruited. DNA is then unwound by the helicase activity of XPB and XPD subunits of TFIIH. In GG-NER, XPG binds to PIC1 to form PIC2, and XPC-hHR23B leaves. The rest of the pathway is identical in GG-NER and TCR. Binding of XPF-ERCC1 to PIC2 leads to the formation of PIC3. XPG carry out the 3' incision followed by 5' incision by XPF-ERCC1 resulting the release of a 24-32 nucleotide fragment. Following release of the excised oligomer, repair synthesis is carried out by PCNA and DNA polymerase δ and ε and finally DNA is ligated by DNA ligase.


GG-NER
TCR
5'
3'
Damage recognision
XPC
CSA
CSB
Pol II
Preincision Complex 1
XPA
RPA
TFIIH
XPG
Preincision Complex 2
ERCC1
XPF
Preincision Complex 3
26-29 mer
Excision
PCNA
Pol ε (δ)
Ligase
Repair synthesis and ligation
26-29 nt

process (Jones and Wood 1993, Wood, 1999, Kusumoto *et al.*, 2001). However, a recent study using immunofluorescent labeling to investigate assembly of NER proteins in UV-irradiated human cells have shown that XPA is not required for damage recognition (Volker *et al.*, 2001).

In TCR, damage is detected by the elongating RNA polymerase II complex that attracts NER complex when it encounters a lesion (Donahue *et al.*, 1994). Because the stalled RNA polymerase II hinders the accessibility of NER proteins, it has to dissociate from the lesion for repair to occur. Cells defective in CSA and CSB genes are specifically defective in TCR. CSA protein is a member of the WD-40 repeats family of proteins that are implicated in chromatin remodeling, and CSB is a member of the SW1/SNF family of DNA-dependent ATPases also implicated in chromatin remodeling and the removal of bound proteins from DNA (Tornaletti and Hanawalt, 1999). Interactions between CSB and RNA polymerase II has been reported and it has been suggested that CSA and CSB proteins are involved in the processing of stalled RNA polymerase II complex (Tantin, 1998). CSA protein is rapidly translocated to the nuclear matrix in a CSB dependent manner following treatment of human fibroblasts with UV, cisplatin or hydrogen peroxide indicating a role of CSB and CSA proteins in TCR of DNA lesions induced by these agents (Kamiuchi *et al.*, 2002). However, the exact role of CSA and CSB in TCR still remains to be elucidated.

Recent studies have shown that in GG-NER, the first high specific damage recognition complex is formed with the combination of four repair factors, XPA, RPA, XPC and

TFIIH (containing XPB/ERCC3, XPD/ERCC2, p62, p44 and p34). This complex is termed pre-incision complex 1 (PC1). TFIIH creates a 10-20 nucleotide opened DNA complex around the lesion by virtue of its helicases XPB and XPD, and this step requires ATP (Wakasugi and Sancar, 1998). XPB is a 3' to 5' helicase, whereas XPD acts with opposite polarity (Sung *et al.*, 1993, Ma *et al.*, 1994).

Binding of 3' endonuclease XPG to PC1 greatly increases the stability of the DNA protein complex and leads to formation of pre-incision complex 2 (PC2), which is of very high affinity and specificity (Evans *et al.*, 1997a,b, Mu and Wakasugi, 1997, Wakasugi *et al.*, 1997). XPC leaves PC1 upon entry of XPG. Hence XPC is absent in PC2 (Wakasugi and Sancar, 1998). This explains why XPC is not needed for transcription coupled repair (Veneme *et al.*, 1991). XPC is therefore known as a molecular matchmaker in damage recognition.

The XPF-ERCC1 complex cannot associate with PC1 and that the nuclease function of XPG is not required for the formation of PC2. Finally, binding of XPF-ERCC1 to PC2 leads to formation of pre-incision complex 3 (PC3), which does not have much greater specificity than PC2, but is essential intermediate on the pathway to dual incision. Thus, it is apparent that XPG, in addition to its 3' nuclease function, is involved in recruiting the XPF-ERCC1 5' nuclease to the excinuclease complex (Wakasugi and Sancar, 1998). Recently it has been shown that recruitment of XPF-ERCC1 also depend on XPA protein (Volker *et al.*, 2001).

Following PC3 formation, XPG and the XPF-ERCC1 complex, which have junction cutting nuclease activity carry out the dual incision releasing a fragment of 24-32 nucleotide length. 3' incision by XPG occurs first and is followed by 5' incision by XPF-ERCC1 within a fraction of a second (Evans *et al.*, 1997a,b, O'Donovan *et al.*, 1994, Sijbers *et al.*, 1996a, McCutchen-Maloney *et al.*, 1999, Mu *et al.*, 1996). The 3' incision is at the 6th ± 3 phosphodiester bond 3' to the damage and the 5' incision occurs at the 20 ± 5th phosphodiester bond (Petit and Sancar, 1999). Incision by XPG requires the presence of XPA and it is likely that incision by XPG is activated by XPA in cooperation with RPA (Volker *et al.*, 2001).

Following the release of the excised oligomer some, as yet unknown components of the nuclease remain bound to the post-incision gap and protect the single stranded region from non-specific degradation. Then, repair synthesis is carried out by PCNA and DNA polymerase δ and ε. Finally DNA is ligated by DNA ligase (de Laat *et al.*, 1999, Petit and Sancar, 1999, Shivji *et al.*, 1995).

1.3.5. Double strand break repair pathways.

DSBs can arise as intermediates during several cellular processes such as meiotic recombination, V(D)J recombination in B- and T-lymphocytes in mammals, or by exposure to ionizing radiation or free radical producing agents. Furthermore, repair of certain types of lesions, or replication past single strand breaks or gaps may also result in the generation of DSBs. If not repaired DSBs can cause gene deletion, chromosome loss or other chromosomal aberrations that might ultimately produce cancers.

DSBs are repaired by means to two main mechanisms: homologous recombination (HR) and non-homologous end-joining (NHEJ). Both mechanisms operate in all eukaryotes, but their relative contributions differ between higher and lower eukaryotes. In *S.cerevisiae*, HR is the major mechanism of repair of DSBs. In this organism, the contribution of NHEJ is minor and can only usually be detected when HR is defective (Pastink and Lohman, 1999). Until recently, it has been speculated that mammalian cells may appear to favour NHEJ pathway for DSB repair because all DSB-repair mutants analysed have been found to be defective in NHEJ (Weaver, 1995). However, recent identification of number of mammalian genes involved in HR have shown that this pathway also play an important role in the repair of DSBs and the maintenance of genomic stability in mammalian cells (Thacker, 1999). The repair pathway utilised to resolve DSBs may depend on the stage of the cell cycle. Studies in vertebrate cells have shown that NHEJ pathway plays a dominant role in repairing γ-radiation-induced DSBs during G_1-early S phase, while HR pathway is used in late S-G_2 phase (Takata *et al.*, 1998). In addition to these two main mechanisms, DSBs can also be repaired by a single strand annealing (SSA) pathway (Pastink and Lohman, 1999).

1.3.5.1. Homologous recombination.

Homologous recombination (HR) makes use of the sequence identity between sister chromatids on the same chromosome or the undamaged homologous chromosome to repair the damaged DNA.

Many genes and proteins involved in HR have been identified, mainly by yeast genetics, using mutants that are hypersensitive to ionizing radiation (IR) (Thompson and Schild, 1999). In *S.cerevisiae*, IR sensitivity is associated with mutations in the *RAD52* epistasis group, which consists of *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, XRS2,* and *MRE11* (originally called RAD58) (Thompson and Schild, 1999). Within the *RAD52* epistasis group, defects in *RAD51, RAD52* and *RAD54* genes confer the most IR sensitivity phenotype and severe defects in recombination and DSB repair, suggesting that these genes encode key factors in HR (Pastink and Lohman, 1999). The yeast Rad51 protein has structural homology to the well-studied bacterial RecA recombination protein, and this similarity has been shown to extend to function. Both are involved in the formation of underwound nucleoprotein filaments on duplex DNA and the promotion of homologous pairing and strand exchange reactions (Thompson and Schild, 1999). Rad52 can bind single stranded DNA and interacts with single stranded DNA binding protein RPA. Rad52 can also interact with Rad51 and it has been suggested that it might facilitate loading of Rad51 onto DNA (Thacker, 1999). Rad54 is structurally related to a family of DNA helicases and has also been shown to interact with Rad51. It has been suggested that these Rad proteins may form a complex or 'recombinase' that operates in homology search and early events of strand invasion (Thacker, 1999).

HR is conserved from bacteria to mammals, but biochemical details of mammalian HR appear to differ from those of yeast. Based on sequence similarity, human and mouse homologs of the *RAD51*, *RAD52* and *RAD54* genes have been isolated (Thompson and

Schild, 1999). Human Rad51 protein is 68% identical to yeast Rad51 and shows a low but significant similarity to the RecA protein (Shinohara *et al.*, 1993, Yoshimura *et al.*, 1993). Human and mouse homologues of yeast Rad52 and Rad54 are less conserved and show only 30% and 50% identity respectively (Muris *et al.*, 1994, Kanaar *et al.*, 1996).

The cloning of human DNA sequences that functionally complement mammalian mutant cell lines that are hypersensitive to ionising radiation has also lead to the identification of genes involved in HR (Jeggo, 1998). A large number of mutant mammalian cell lines with sensitivity to X-rays have been isolated (Collins, 1993). These mutants, derived from rodents, have been assigned to approximately nine genetic complementing groups. In a number of these cell lines, it has been found on further testing that the major sensitivity is to DNA damaging agents other than X-rays (Caldecott and Jeggo, 1991). Therefore it is misleading to classify these mutants as primarily radiation sensitive. In several of these mutants, defective genes have been isolated and characterised and are termed X-ray Repair Cross Complementing (XRCC) genes (Collins, 1993). These genes have been turned out to be involved in a number of different repair pathways. Among XRCC genes, recent studied have confirmed that XRCC2 and XRCC3 play a major role in homologous recombination (Liu *et al.*, 1998).

The XRCC2 and XRCC3 genes encode members of an emerging family of Rad-51 related proteins (Liu *et al.*, 1998, Tambini *et al.*, 1997). These two genes were

identified in independent somatic cell hybrid studies (Jones *et al.*, 1995, Tebbs *et al.*, 1995), on the basis of their ability to complement mitomycin C (MMC) sensitivity of mutants irs1 (Jones *et al.*, 1987) and irs1SF (Fuller *et al.*, 1988), respectively. Importantly, irs1 and irs1SF are phenotypically similar and are sensitive to diverse DNA damaging agents, including ionizing radiation (~2-fold), UV radiation (2-3-fold), ethyl methanesulfonate (EMS, 2-10-fold) and MMC (60-100-fold). The extreme sensitivity to many DNA cross-linking agents, including cisplatin and nitrogen mustard is a hallmark of irs1 and irs1SF among the XRCC mutants (Caldecott *et al.*, 1991). Interestingly, although they are sensitive to ionising radiation, irs1 and irs1SF cells show no defects in the repair of radiation induced DSBs (Cheong *et al.*, 1992, Fuller *et al.*, 1988, Jones *et al.*, 1990, Thacker *et al.*, 1990). However, recent studies have shown that both the XRCC2 (Johnson *et al.*, 1999) and XRCC3 (Pierce *et al.*, 1999) genes are involved in the repair of I-SceI endonuclease induced DNA double strand breaks by homologous recombination. Another common feature of these mutants is their highly elevated frequency of chromosomal aberrations, both spontaneous and mutagen induced (Fuller *et al.*, 1988, Tebbs *et al.*, 1995, Liu *et al.*, 1998).

Although human Rad51 is quite similar to *S. cerevisiae* Rad51, XRCC2 and XRCC3 show only 20% identity to human and yeast Rad51, suggesting that they are not orthologs but paralogs of yeast Rad51 (proteins that have evolved new functions arising from duplication of an ancestral gene) (Tatusov *et al.*, 1997). Three additional members of the mammalian Rad51 paralog family have been identified by searching

databases for Rad51-like sequences: *RAD51B*, *RAD51C* and *RAD51D* (Thacker, 1999, Thompson, 1999). The reason for the larger Rad51 family in higher eukaryotes is unclear, but it is likely that they act as accessory factors, to promote filament formation and exchange mediated by Rad51. Although, the yeast *RAD51* gene is not essential for yeast viability, deletion of homologs in mice is lethal, suggesting the importance of Rad51 in mammals (Lim and Hasty, 1996, Tsuzuki *et al.*, 1996).

Models for the repair of DSBs by HR in yeast (Figure 1.11) (Hoeijmakers 2001, Haber, 2000b) suggests that, DSBs are enzymatically resected in the 5' to 3' direction first by exonuclease activity of the Rad50/Mre11/Xrs2 complex. Then, the resulting 3' overhanging ends invade an unbroken double stranded homologue to form a heteroduplex, and synthesis on this template reconstitutes the damaged strand. Rad51, Rad52 and Rad54 may play a role in this process. Separation of the joint product from this reaction requires the activity of enzymes cutting and rejoining the newly synthesised DNA strands. Depending upon which strands are cut and rejoined, this reaction may result in crossing over of DNA strands. Since HR utilise extensive regions of homology in another DNA duplex, no loss of genetic information usually results.

1.3.5.2. Non-homologous end joining

In contrast to HR, non-homologous end joining (NHEJ) requires very little or no homology. As shown in figure 1.12, this mechanism repairs DSBs by direct rejoining of broken molecules and can be precise or error prone (Zdzienicka, 1999). The crucial

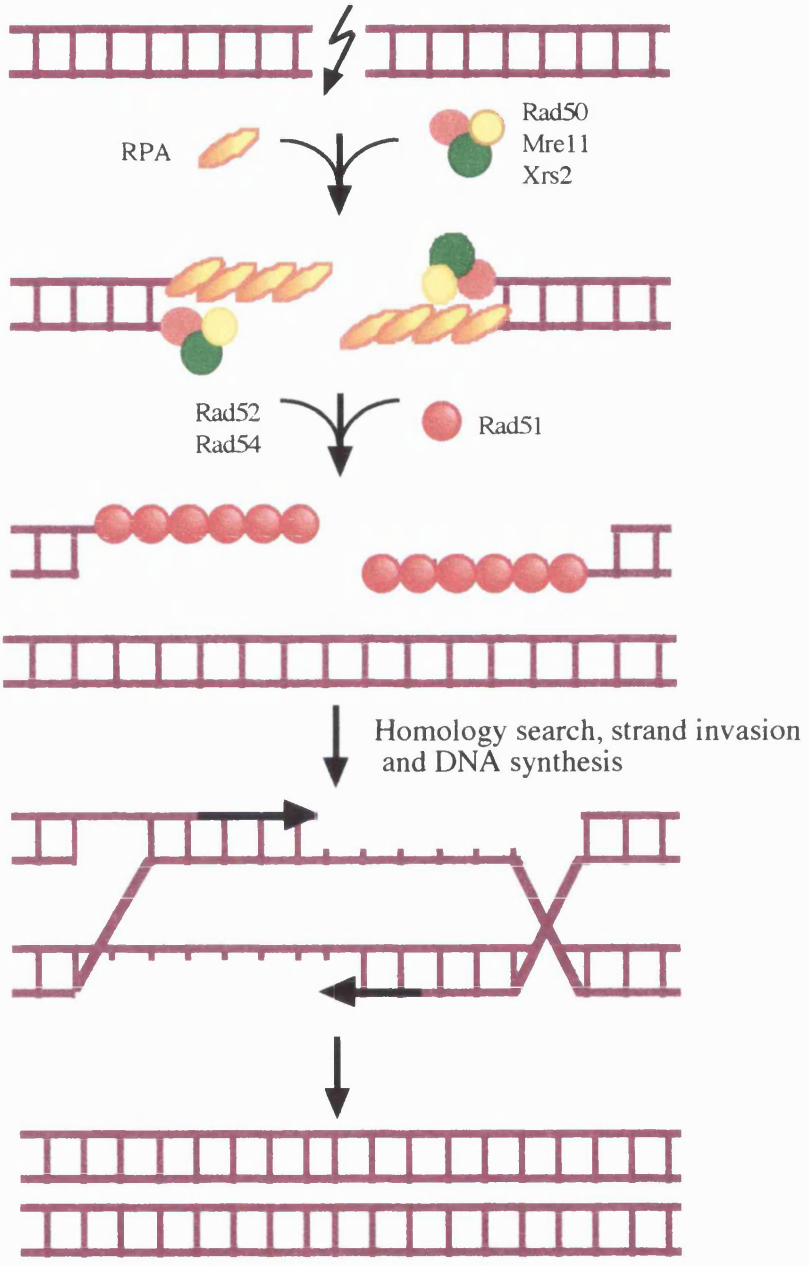


Figure 1.11. The mechanism of homolgous recombination. To facilitate strand invasion, 3' ends are exposed by 5'-3' exonuclease activity of the RAD50/MRE11/Xrs2 complex. RPA promote assembly of Rad51 nucleoprotein filament and other RAD51 related proteins may stimulate this process. Rad51 has the ability to exchange the single strand with a homologous double stranded DNA molecule and Rad54 and Rad52 may play a role in this process. Following identification of homologous sequence, broken ends are repaired by DNA synthesis using homologous double stranded DNA as the template. Finally the so-called Holiday-junctions are resolved by resolvases (reviewed in Hoeijmakers 2001, Haber 2000b).

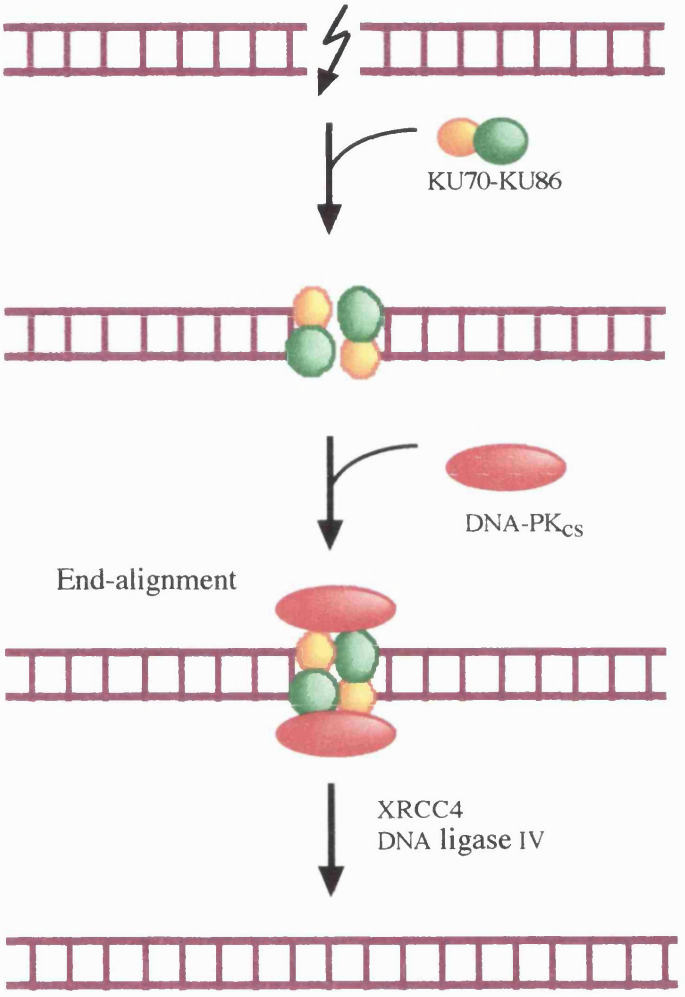


Figure 1.12. Mechanism of the Non-Homologous End Joining pathway. This process joins the ends of a double strand break without use of a template. Ku 70 and Ku86 heterodimer binds to DNA ends of the double strand break and recruits DNA-PKcs. This process leads to the activation of serine/threonine activity of DNA-PKcs which phospharylate target proteins involved in resolving the DSB. Finally repair is completed by ligation of DNA ends by DNA ligase IV (XRCC4) (Zdzienicka, 1999, Jeggo,1998 Hoiejmakers 2001).

protein, which plays the central role in this pathway is the DNA protein kinase (DNA-PK). It is a heterotrimeric enzyme composed of a large catalytic subunit (460 kDa, DNA-PKc) and a regulatory component consisting of Ku 86 (86 kDa) and Ku 70 (70 kDa) proteins. Ku 86 and Ku 70 proteins form a heterodimer and binds to DNA ends of the DSBs, Ku protein recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and activates its serine/threonine kinase activity, which phosphorylates target proteins involved in resolving the DSB (Jeggo, 1998, Zdzienicka, 1999).

Mammalian mutants in four X-ray complementation groups (XRCC4-7), show high sensitivity to IR and are defective in the repair of IR induced DSBs (Jeggo, 1998). These mutants are defective in genes involved in NHEJ pathway. The XRCC4 gene encodes a protein of 38 kDa which interacts with ligase IV. Ku80 and Ku70 subunits are encoded by XRCC5 and XRCC6 genes, respectively, and DNA-PKc is encoded by the XRCC7 gene (Jeggo, 1998, Zdzienicka, 1999, Muller *et al.*, 1999). Ku protein (consist of Ku86 and Ku70) was originally identified as an antigen present in the sera of certain autoimmune patients. It is a highly abundant nuclear protein in human cells and has a characteristic property of binding to double stranded DNA ends. It has a DNA dependent ATPase and 3'-5' helicase activity. Ku mutants, XRCC5 and XRCC6 lack both DNA end-binding and DNA PK activities. These mutants are defective in ionising radiation induced DSB repair, and are unable to perform V(D)J recombination (Zdzienicka, 1999). V(D)J recombination occurs in mammals in developing B and T cells. This process is initiated by the generation of blunt DNA DSB between variable (V), diversity (D) and joining (J) gene segments. Severe combined immunodeficiency

(*scid*) mice which carry a mutation in the XRCC7 gene are defective in V(D)J recombination and also show impaired repair of IR induced DSBs, indicating a link between V(D)J recombintaion and repair of DSBs induced by IR (Araki *et al.*, 1997). Similarly, mice deficient in Ku70 and Ku80 are also impaired in V(D)J recombintaion and hypersensitive to IR (Nussenzweng *et al.*, 1997, Gu *et al.*, 1997).

DNA-PKcs protein is a member of the phosphatidylinositol kinase (PIK-3) family of proteins (Muller *et al.*, 1999). *In vitro* a large number of protein substrates of DNA-PKcs have been identified, most of which are DNA binding proteins, including transcription factors, DNA-PKcs and Ku themselves.

1.3.5.3. Single Strand Annealing (SSA).

The SSA is a sub-pathway of homologous recombination and depends on the presence of direct repeats on both sides of the break (Pastink and Lohman, 1999). The length of these repeats can be as small as 29 bp (Sugawara *et al.*, 2000). As shown in figure 1.13 the first step is a 5'-3' degradation of both ends of the break by a specific exonuclease. This allows the exposure of regions of homology, which permits the formation of joint molecules. The non-homologous ends are then removed and DNA synthesised to fill in gaps. Finally, repair is completed by ligation of the ends. Inevitably, SSA has the potential to create deletions between repetitive sequences.

The RAD52 gene, which plays a major role in homologous recombination, is also involved in the SSA pathway. Recent studies have shown that human and yeast Rad52

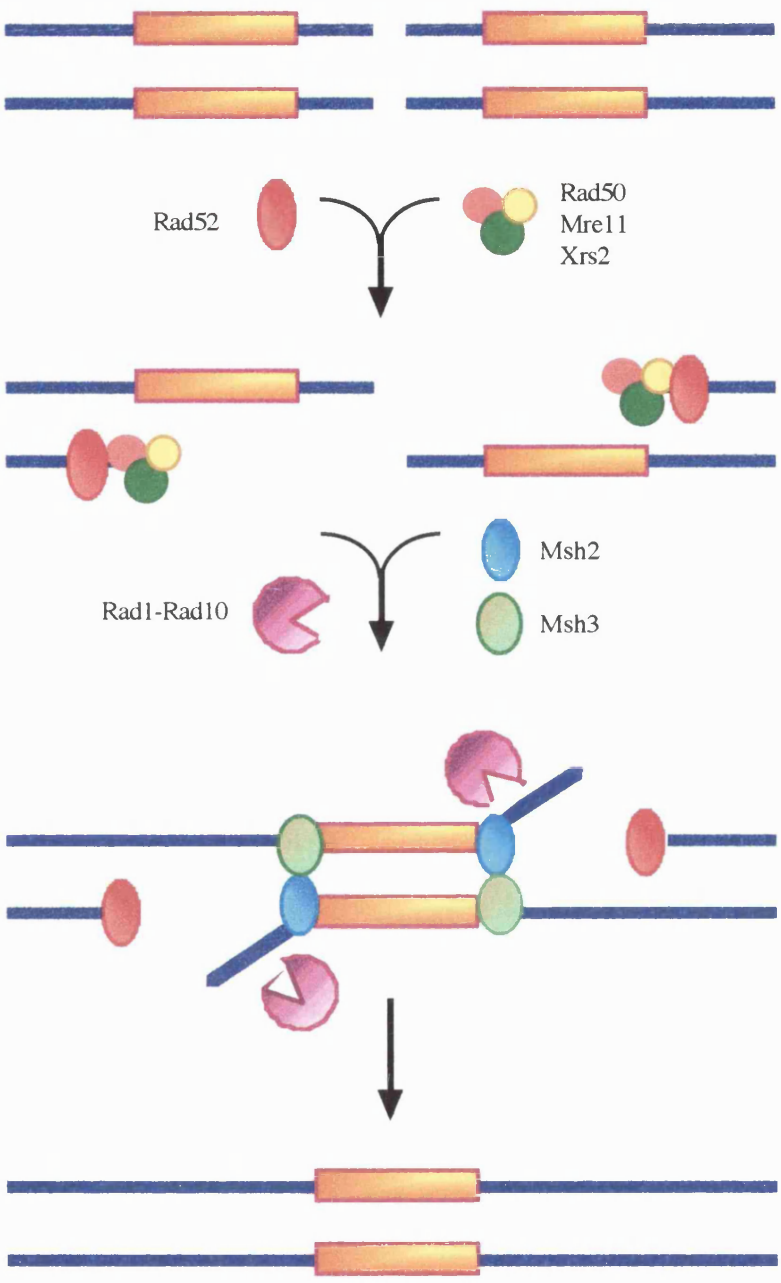


Figure 1.13. Single strand annealing model. When a DSB is created within one of the two repeats, one strand on either side of the DSB is resected in the 5'-to-3' direction by Rad50/Mre11/Xrs2 complex, leaving a 3' tail. Rad52 binds to partially resected DSBs and protect them from nuclease attack. When complementary strands on opposite side are exposed, they can anneal forming a branched intermediate. The single strand tails are then removed by Rad1-Rad10 endonuclease together with Msh2 and Msh3 proteins. The gaps are then filled in and the remaining nicks are ligated.

proteins bind specifically to DSBs and partially resected DSBs to protect them from nuclease attack and to facillitate end to end interactions (Van Dyck *et al.*, 1999, Kim *et al.*, 2000, Parsons *et al.*, 2000). Multiple rings of human Rad52 bind resected DNA termini, directly promote annealing of complementary sequences and remains bound to the heteroduplex DNA intermediate, probably to prevent further attempts of annealing (Van Dyck *et al.*, 2001).

SSA also requires the Rad50, MRE11, Xrs2 protein complex in *S. cerevisiae* and functionally analogous proteins in humans: Rad50, Mre11, and Nbs1 (Haber, 2000a, Karran, 2000). It is likely that this complex is involved in the resection of DNA ends. Rad59 also play a role in SSA probably by participating in strand annealing and stabilising annealed intermediates. The role of Rad59 has a larger impact when the length of the homology sequences are shorter (Sugasawa *et al.*, 2000). However, SSA pathway is independent of the *RAD51*, *RAD54*, *RAD55*, and *RAD57* genes involved in HR (Ivanov *et al.*, 1996).

In *S.cerevisiae*, RAD1 and RAD10 genes, which are homologs of human XPF and ERCC1 genes respectively, are involved in the removal of 3'-ended regions of non-homologous DNA from SSA intermediates (Fishman-Lobell *et al.*, 1992, Davis *et al.*, 1995). These genes belong to RAD3 epistasis group and are also involved in 5' incision in the NER mechanism. As discussed earlier, XPF and ERCC1 are also involved in NER. Because of the structural homology of mammalian XPF and ERCC1 to RAD1 and RAD10 genes, it has been postulated that XPF and ERCC1 may also

play a role in recombination (Weeda *et al.*, 1997). In agreement with this suggestion, recently it has been shown that ERCC1 is required for the removal of long non-homologous tails from the 3'-ends of invading DNA strands during targeted homologous recombination (Adair *et al.*, 2000) suggesting a similar role of XPF and ERCC1 in receombintion in mammalian cells analogous to that of RAD1 and RAD10 in yeast.

Interestingly, the products of *S.cerevisiae* mismatch repair genes MSH2 and MSH3 also play a role in SSA (Sugawara *et al.*, 1997). These proteins along with Rad1 and Rad10 are involved in removing non-homologous 3'-ended DNA tails from annealed intermediates. However, the requirement of these proteins decrease dramatically as the annealed regions increase from 205 bp. It is believed that these proteins play a role in stabilising short annealed heteroduplexes.

1.3.4. Repair of DNA interstrand cross-links

For most bifunctional agents, the critical cytotoxic lesion is the DNA ICL. Since these adducts covalently link complementary DNA strands, they distort DNA structure and inhibit DNA replication and transcription. Repair of ICLs is an important mechanism of acquired clinical resistance to cross-linking agents. For example, resistance in B-cell chronic lymphocytic leukemia (B-CLL) (Bramson *et al.*, 1995) and breast cancer cell lines (Batist *et al.*, 1989) are associated with enhanced repair of nitrogen mustard induced ICLs. Recent studies in this laboratory have demonstrated a direct relation

between repair of ICLs and resistance to cross-linking agents in patients with multiple myeloma (Spanswick *et al.*, 2002).

Repair of DNA ICLs has been studied extensively in *Eschericia coli*. Over twenty years ago, studies by Cole and co-workers have demonstrated the involvement of both NER and recombination pathways in the repair of these lesions in *E. coli* and proposed a model of ICL repair (figure 1.14) (Cole *et al.*, 1973, 1975, 1976). Biochemical and genetic work carried out by this group using psoralen ICLs demonstrated that products of *uvr*A, uvrB, *uvr*C, *uvr*D, *rec*A and *pol*A genes are required for repair of these lesions. Cole proposed that ICL repair is initiated by uvr(A)BC mediated excision on one side of the cross-link to unhook and release the lesion in the form of an oligonuceotide still attached to the complementary strand by the cross-linking agent. Then the excision patch is replaced by a recombination event whereby information is exchanged between an intact duplex and the damaged duplex. A second excision then occurs to remove the still cross-linked complementary strand. Subsequent investigations by other groups have provided additional insights into the model proposed by Cole. Using psoralen ICLs, Sancar's group (Van Houten *et al.*, 1986) demonstrated that the initial incisions made at the site of the lesion are located 9 nucleotides to the 5' side and three nucleotides to the 3' side. The same group also showed that (A)BC excinuclease will cleave a triple stranded cross-linked substrate which mimics the recombination intermediate in Cole's model (Cheng *et al.*, 1988 & 1991). Studies by Sladek *et al.*, (1989) showed that a substrate with a dual incision on either side of the cross-link did not stimulate strand exchange by RecA, but if the nick

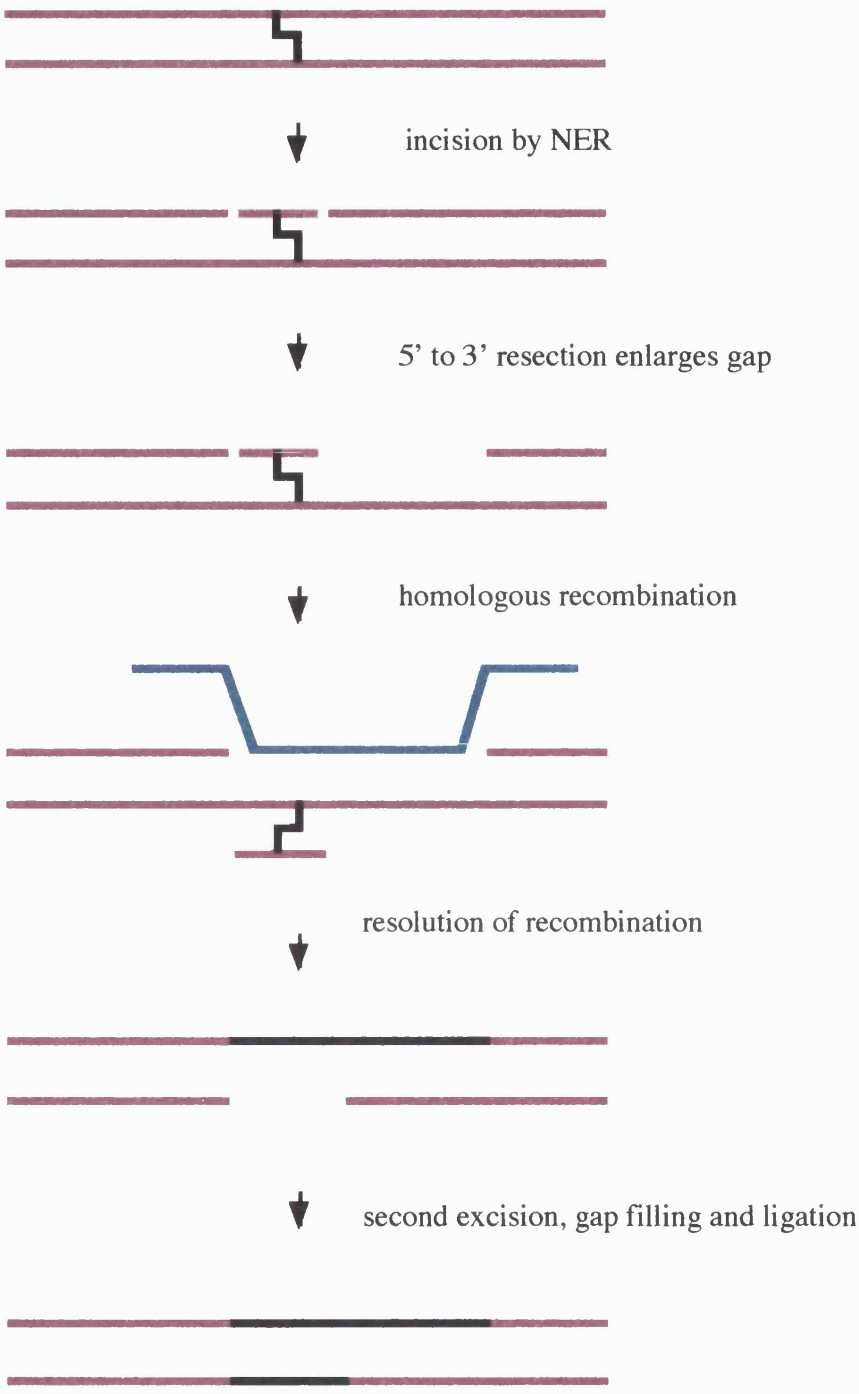


Figure 1.14. Cole's model for the repair of ICLs in bacteria. ICL is incised by NER apparatus, and the resulting gap is enlarged by 5' to 3' exonuclease activity. The lost genetic infomation is replaced by homologous recombination. A second incision event completely remove the ICL and the gap is filled by DNA synthesis and ligation.

is processed into a gap by 5'-exonuclease activity of DNA polymerse I, RecA-mediated strand exchange is stimulated, suggesting that an exonucleolytic step is required prior to recombination. Taken together, these findings strongly support the recombination mode of ICL repair suggested by Cole *et al.*, (1973, 1975, 1976). Although, the NER-recombination pathway appears to be the primary mechanism of ICL repair in *E.coli*, recent evidence has suggested a recombination independent pathway for ICL repair in which the gap created by uvr(A)BC excinuclease is repaired by translesion bypass by DNA polymerase II in order to circumvent a deficiency in recombination or a lack of homologous donor sequence (Berardini *et al.*, 1997, 1999).

In the yeast *S. cerevisiae*, ICL repair has not yet been characterised in as much detail as repair in *E.coli*. Significant amount of genes involved in ICL repair has been discovered. In this organism, members of the RAD3 epistasis group, which are deficient in NER, and members of the RAD52 epistasis group deficient in homologous recombination are highly sensitive to cross-link damage (Jachymczyk *et al.*, 1981, McHugh *et al.*, 2000). It has been shown that repair of psoralen-induced cross-links induced DSBs, and rejoining of these breaks depended on homologous recombination (Jachymczyk *et al.*, 1981, Magana-schwencke *et al.*, 1982). Production of these DSBs depended on the NER genes *RAD2* and *RAD3*. Recent studies in this laboratory have also demonstrated that DSBs are induced during processing of nitrogen mustard-induced ICLs, but in contrast to psoralen cross-links, formation of DSBs resulting from the processing of nitrogen mustard-induced cross-links does not depend on NER (McHugh *et al.*, 2000). In exponentially growing cells, repair of these DSBs depends

on homologous recombination pathway but NHEJ pathway is not involved. *S. cerevisiae* mutants defective in homologous recombination genes *RAD51*, *RAD52*, *RAD54* and *MRE11* show hypersensitivity to nitrogen mustard and defective in the repair of nitrogen mustard induced DSBs. *RAD58* defective mutant only showed marginal sensitivity and DSB repair defect (McHugh *et al.*, 2000).

As in the case in *E. Coli*, recombination independent mechanism may also play a role in eukaryotes. The yeast *pso1* mutant is characterised by hypersensitivity to psoralen induced ICLs (Henriques *et al.*, 1980), and genetic analysis has demonstrated allelism between the *pso1-1* mutant and *rev3-1* mutant (Cassier-Chauvat *et al.*, 1988). The REV3 gene encodes the catalytic subunit of the yeast translesion DNA polymerase ζ (Morrison *et al.*, 1989, Nelson *et al.*, 1996), and studies by McHugh *et al.*, (2000) have recently shown that REV3 is important for the processing of nitrogen mustard induced-ICLs in non-replicating cells, further substantiating a lesion bypassed-based recombination independent mechanism in ICL repair in yeast.

In mammals, the mechanism of ICL repair is largely unknown. The majority of mammalian NER mutants exhibit only mild sensitivity to ICL inducing agents, although mutants defective in either ERCC1 or XPF do exhibit extreme sensitivity (Andersson *et al.*, 1996, Hoy *et al.*, 1985). The reason for the extreme sensitivity of ERCC1 and XPF mutants to ICL inducing agents compared to cells defective in other NER components, is not well understood, but it has been suggested that it could be due to the dual involvement of ERCC1 and XPF in NER and recombination (Thompson

1996, Weeda *et al.*, 1997). Recently, evidence of recombinational repair of ICLs has emerged through the characterisation of two hamster mutants, irs1 and irs1SF, both of which show an extreme sensitivity to ICL inducing agents (Fuller *et al.*, 1988, Jones *et al*, 1987). Both the XRCC2 and XRCC3 genes, which complement the repair deficiency of irs1 and irs1SF mutants, respectively, show structural similarity to the hRAD51 family (Liu *et al.*, 1998). Studies by Johnson *et al.*, (1999) and Pierce *et al.*, (1999) have demonstrated that both cell lines are defective in homologous recombination. In addition, studies by Li *et al.*, (1999) have shown that the presence of an ICL in a plasmid substrate stimulate repair synthesis in mammalian cell extracts and this repair synthesis is also observed in an undamaged plasmid co-incubated in the same extract suggesting that ICL repair can involve recombinational repair synthesis.

A recent study by Wang *et al.*, (2001) employing site-specific psoralen cross-links, have demonstrated the presence of a recombination-independent, error-prone pathway for ICL repair in mammalian cells. Using an *in vivo* reactivation assay, this group demonstrated that a single psoralen cross-link placed between the promoter and the coding region of a plasmid reporter gene was removed in repair proficient cells in the absence of an undamaged homologous sequence. This repair process was dependent on the whole NER system but was independent of homologous recombination. These observations provide clues for the involvement of not only NER and recombination pathways but also recombination independent error prone pathways in the repair of ICLs in mammalian cells as in yeast and bacteria.

Recently it has been shown that the MMR protein hMutSβ play a role in the repair of psoralen-induced ICLs (Zhang *et al.*, 2002). HMutSβ complex can specifically bind to psoralen ICLs and this binding stimulated by PCNA. This protein is also required for the incision of psoralen ICLs in combination with XPF and ERCC1, but hMutSα is not required (Zhang *et al.*, 2002). These findings indicate a novel role of MMR proteins in the repair of ICLs in mammalian cells.

In addition to XPF, ERCC1, XRCC2 and XRCC3 mutants, Fanconi anemia (FA) cell lines are also hypersensitive to interstrand cross-linking agents (Grompe and D'Andrea, 2001). FA is a recessive genetic disorder characterised by congenital abnormalities, bone marrow failure and increased incidence of malignancy. So far eight FA complementation groups have been defined and six genes have been maped and cloned; FANCA, FANCC, FANCD2, FANCE, FANCF and FANCG (Grompe and D'Andrea, 2001). The exact functions of FANC genes are currently unclear. However, it has been shown that chromatin associated protein extracts from normal human cells produce incisions on either side of a psoralen ICL on both sides of DNA and in FA cells these incisions are markedly reduced (Kumarasen *et al.*, 1995, 2000, McMahon *et al.*, 2001). Therefore it is most likely that FANC gene products may play an important role in the repair of ICLs which still remains tot be discovered.

1.4. Objectives of this study

Previous studies of mammalian ICL repair have mainly focused the attention on psoralen cross-links. The repair pathways and the mechanism of removal of ICLs induced by widely used chemotherapeutic agents such as the nitrogen mustards, cisplatin, and novel sequence specific minor groove binders, still remains to be elucidated. Using a panel of CHO cell lines defective in genes involved in NER, HR and NHEJ pathways this study aimed to understand the repair of ICLs induced by these drugs. Using mono-functional analogues of these agents repair of bifunctional and monofunctional DNA adducts were compared. In addition, the cytotxicity of the novel sequence specific minor groove binding agent SJG-136 was compared with that of the conventional major groove binding agents and the repair pathways involved in the removal of both monofunctional and bifunctional lesions induced by this drug was analysed.

CHAPTER 2: MATERIALS AND METHODS

2.1. Cell Culture

2.1.1. Materials

- Tissue culture plastics (Gibco Life Technologies, UK).
- F12-Ham Hepes medium (Sigma, Pool, U.K.).
- 200 mM L-glutamine (Sigma).
- Foetal calf serum (FCS) (Gibco Life Technologies, U.K.).
- Versine : 1mM Disodium EDTA, 0.5% phenol red.
- Trypsin (0.05%) (Gibco Life Technologies).
- Dimethyl Sulfoxide (DMSO) (Sigma).
- Freezing vials (Sigma).

2.1.2. Cell culture conditions

All cell lines were grown in T80 cm^2 tissue culture flasks in F12-Ham Hepes medium (Sigma) supplemented with 2 mM glutamine and 10% foetal calf serum which had been heat inactivated by a thirty minute treatment at 56°C. Cell cultures were grown at 37°C in a humidified incubator with 5% CO_2.

2.1.3. Passaging cells

Cells were passaged by trypsinisation when semi-confluent. Monolayers were washed in 5 ml / 75 cm^2 growth area in versine, then incubated with 1:1 ratio of versine and

trypsin until the cells had become detached (about five minutes). Cells were then centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and cells were re-suspended in fresh media. The cell suspension was diluted 1:10 in fresh medium and seeded into new flasks.

2.1.4. Cryo-Preservation

Cells were frozen in complete medium containing 10% DMSO in 1ml aliquots containing 1 x 10^6 cells in 2 ml freezing vials. Cells were frozen by slowly lowering the temperature to -20°C over 2 hours, storing at -80°C overnight and then into liquid nitrogen (-196°C). Thawing of cells was carried out as quickly as possible in a 37°C water bath and the cells transferred straight into fresh medium pre-warmed at 37°C. Every three months cells in culture were discarded and frozen stocks thawed to insure that cells are not passaged for too long.

2.1.5. Growth curves

5 ml of 1 x 10^4 cells / ml cell suspension was added into each of five T25 cm^2 tissue culture flasks and placed in the incubator. One flask was trypsinised by adding 2.5 ml versine and 2.5 ml trypsin at 24 h intervals and the cell density was determined using a haemocytometer. A graph of the cell density against the time was plotted and the doubling time of the cell line was calculated by reading off the exponential portion of the growth curve and using the following equation:

$$\text{Doubling time} = 1 / r$$

$$r = \frac{3.32 (\log N_2 - \log N_1)}{(T_2 - T_1)}$$

Where, T_1 = the time when exponential growth starts

T_2 = the time when exponential growth stops

N_1 = the cell density at time T_1

N_2 = the cell density at time T_2

As an example, the growth curve for AA8 cells is shown in figure 2.1. The doubling times of each cell line are shown in table 2.1.

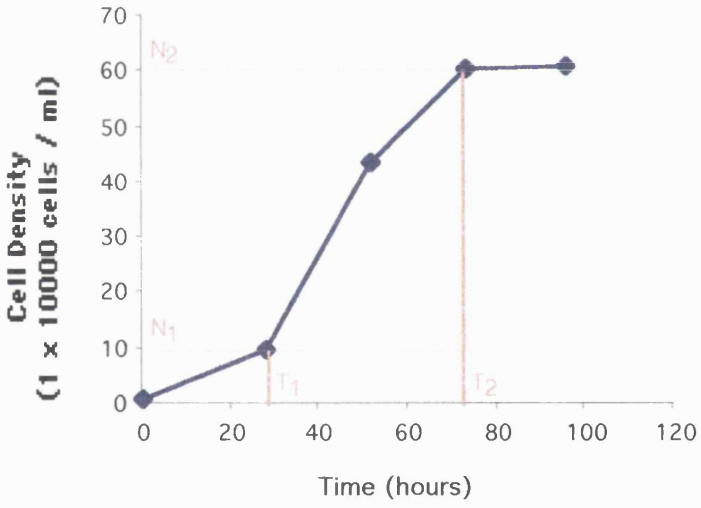


Figure 2.1. Growth curve of AA8 parent cell line. AA8 cells were plated in T25 cm^2 flasks and the cell density was measured at 24 h intervals as explained in section 2.1.5. A graph of cell density against the time was then plotted.

Cell line	Doubling time (hours)
AA8	17.3
UV23	17.9
UV42	16.2
UV47	18.2
UV61	17.6
UV96	18.9
UV135	16.9
CHO-K1	17.9
V79	18.1
Irs1	16.7
Irs1SF	16.9
Xrs5	17.3

Table 2.1 Doubling times of the cell lines used in this study.

2.2. Measurement of growth inhibition

Growth inhibition was assessed using the Sulforhodamine B (SRB) assay (*Rubinstein et al.*, 1990; Skehen, *et al.*, 1990). SRB is a dye which binds basic amino acids in cells which can be used to determine the amount of cellular protein.

2.2.1. Materials

- 0.4% SRB (w / v) in 1% acetic acid.
- 10 mM Tris base.
- 10% Trichloroacetic acid (TCA).
- 1 % Acetic acid.
- 96-well flat bottom tissue culture plates (Gibco Life Technologies, UK).
- Cisplatin, 3.3 mM solution, stored at room temperature (David Bull Laboratories, Faulding Pharmaceuticals PLC, Queensway, Royal Leamington Spa, Warwickshire).

- Mechlorethamine (HN2) (Sigma), stored at -20°C. 10 mM stock solution was made in sterile water immediately before use.
- 2-dimethylaminoethylchloride hydrochloride 99% (HN1) (Sigma), stored at room temperature, 100 mM stock solution was made in sterile water immediately before use.
- Melphalan (Sigma), stored at room temperature. 1 mM stock solution was made in absolute ethanol with 0.5% (v/v) concentrated HCL. This stock solution was stored at -20°C.
- Mono-melphalan, 10 mM stock solution in methanol, stored at -20°C (obtained from Dr. M. J. Tilby, University of Newcastle Upon Tyne).
- SJG-136, 10 mM stock solution in methanol (obtained from Dr. S. J. Gregson, CRC Gene Targeted Drug Design Research Group, University of Nottingham), stored at -20°C.
- MMY, 10 mM stock solution in methanol (obtained from Dr. S. J. Gregson, CRC Gene Targeted Drug Design Research Group, University of Nottingham), stored at -20°C.

2.2.2. Handling of cytotoxic drugs

All the cytotoxic drugs used were handled with extreme care. Gloves, lab coat and safety goggles were worn at all times. All the drugs were weighed in a fume hood. All the equipment and medium contaminated with drugs were decontaminated by soaking in 0.5 M NaOH solution prior to disposal.

2.2.3. Cell Plating

Stock cultures were trypsinised and cells were seeded into the wells of 96-well flat bottomed microtitre plates in a volume of 100 µl and left to adhere overnight. The cells were plated at a density (3 x 10^3 cells/well), which ensures that the untreated control cells are still in exponential growth at the end of the assay.

2.2.4. Drug treatment

Test drugs were prepared at their final concentration in culture medium without FCS immediately before use. Medium in wells was removed and 100 µl of drug-media mixture was added per well. Six replicates were used for each drug concentration. Plates were incubated for 1 hour at 37°C. After exposure, the media was replaced with 200 µl of fresh complete media and the plates were incubated for three days in a humidified incubator at 37°C.

2.2.5. SRB staining

Medium in wells was removed and 100 µl of ice cold 10% TCA was added per well to fix the cells. The plates were incubated at 4°C for twenty minutes and then washed four times in tap water. Cells were stained with 100 µl per well of 0.4% SRB (w/v) in 1% acetic acid for twenty minutes at room temperature. Unbound dye was removed by five washes in 1% acetic acid, and plates were dried overnight at room temperature. The dye was solubilised by the addition of 100 µl of 10 mM tris base per well. Plates were left at room temperature for twenty minutes and the optical density at 540 nm was determined using a Titretech 420 microtitre plate reader equipped with Titresoft II

software (Flow laboratories). A picture of a stained plate is shown in figure 2.2 as an example. The fraction of control A540 (surviving fraction) was calculated from the following equation;

Fraction of control A540 = OD of treated cells / OD of untreated control

The mean surviving fraction for each drug concentration with standard deviations were calculated. Dose response curves were plotted and the IC_{50} values were determined from curves, i.e., the drug concentration needed to produce 50% inhibition of cell growth. When comparing parental and mutant cell lines, the relative sensitivity was determined by the following equation:

Relative sensitivity = IC50 value in parent cell line / IC50 value in the mutant cell line

At least three independent experiments were carried out for each drug.

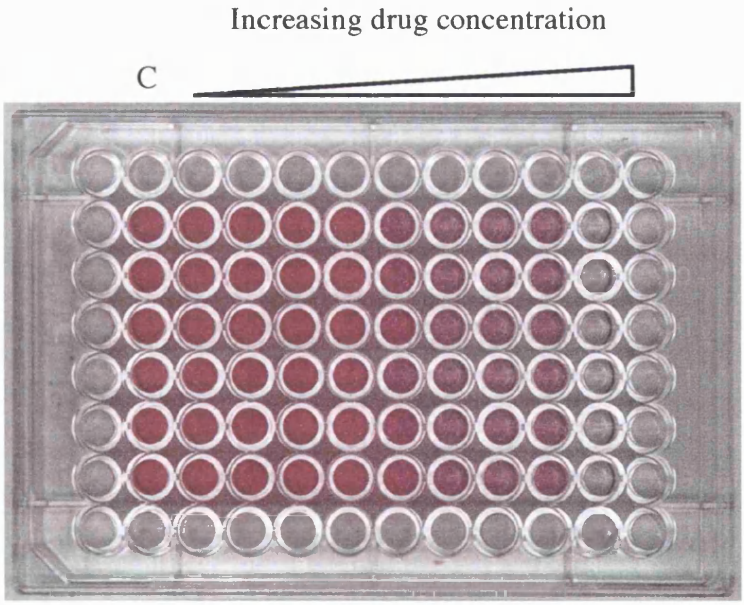


Figure 2.2. Picture of a SRB assay plate. AA8 cells were treated with increasing concentrations of cisplatin for 1 h, washed and incubated in drug free medium for 3 days. Then the cell survival was determined by staining with SRB as described above. C = control untreated wells.

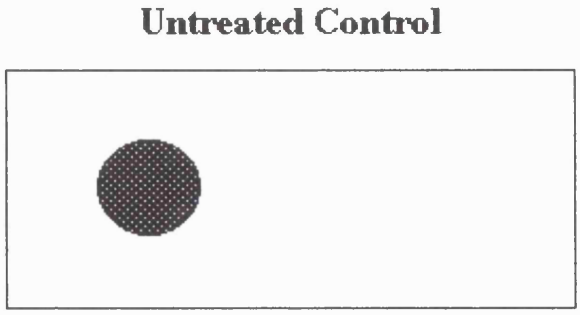
2.3. Measurement of DNA ICLs using the single-cell gel electrophoresis (Comet) assay

2.3.1. Background of the assay

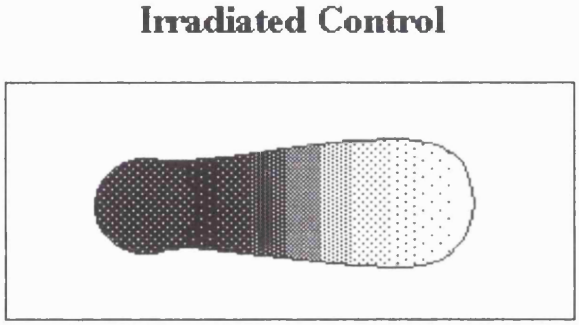
The single-cell gel electrophoresis or comet assay was originally develop by Ostling and Johanson (1984) as a method which allows visualisation of DNA damage in individual cells. Subsequently it was also suggested that this technique could be adapted to measure DNA ICLs (Tice *et al.*, 1992).

In order to detect DNA ICLs cells are irradiated immediately prior to analysis to deliver a fixed level of random DNA strand breakage (Spanswick *et al.*, 1999). The DNA is then denatured under alkaline conditions and subjected to electrophoresis. During electrophoresis any relaxed or broken DNA fragments migrate further than supercoiled undamaged DNA. Following staining the DNA resembles a comet with a brightly fluorescent head and a tail. The length and the intensity of the tail is determined by the level of strand breakage produced within the cells (figure 2.3 a and b). The tail moment (TM) is used as a measure of damage and is defined as the product of the percentage of DNA in the comet tail, and the distance between the means of the head and tail distributions, based on the definition by Olive *et al.*, (1990) (figure 2.3c). The presence of DNA ICLs will retard the migration of the irradiated DNA during electrophoresis giving a reduced tail moment compared to the non-cross-linked irradiated control. Removal of ICLs can be assessed as an increase is tail moment with time following a drug free incubation period.

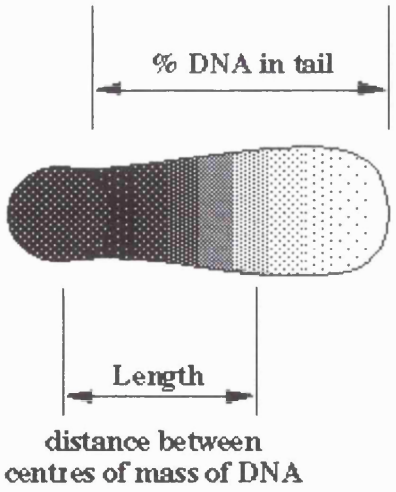
a



b



c



Tail Moment (μm) = % DNA in tail x Length

Figure 2.3. Diagramatic representation of typical results from the comet assay. a) Untreated control. b) Irradiated control. c) Calculation of tail moment.

2.3.2. Materials

- Single frosted glass microscope slides (Sigma).
- 24 x 40 mm cover slips (Sigma).
- Agarose type I-A (Low melting point) (Sigma).
- Agarose type VII (low gelling temperature) (Sigma).
- Lysis buffer : 100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris-HCl, pH to 10.5 with NaOH. Stored at 4°C. Immediately before use 1% Triton X-100 (Sigma) was added.
- Alkali buffer : 50 mM NaOH, 1mM Na_2EDTA, pH 12.5. Stored at 4°C.
- Nutralisation buffer : 0.5 M Tris-HCL, pH to 7.5 with NaOH. Stored at 4°C.
- PBS, pH 7.4. Store at 4°C.
- Flat bed electrophoresis tank. 30 x 25 cm gel tank from Flowgen Instruments, Lichfield, UK.
- Propidium iodide (Sigma). 2.5 μg/ml solution prepared immediately prior to use.
- Nikon inverted microscope DIAPHOT model MTD - Epi-Fluorescence microscope equipped with high pressure mercury light source using a 580 nm dichromic mirror, 510-560 nm excitation filter and 590 nm barrier filter for propidium iodide staining.
- Images were visualised, captured and analysed using Kinetic imaging komet software (Kinetic imaging, Liverpool, UK).

2.3.3. Pre-coating microscope slides

1% Type-IA agarose solution was made and kept at 40°C in a water bath. 1 ml of this solution was pipetted onto the centre of a microscope slide and a glass cover slip was placed on top. Once the agarose solidified, the cover slip was removed and slides were left to dry overnight at room temperature.

2.3.4. Preparation of cells and drug treatment

The cell line to be investigated was harvested and plated at 3.5 x 10^4 cells / well / in 2 ml of medium in 6 well plates, and incubated overnight at 37°C in a humidified incubator with 5% CO_2. The required concentration of the drug was made in FCS-free medium immediately prior to the treatment. Medium was removed from wells and 1 ml of drug containing medium was added into appropriate wells. Control wells received FCS free medium alone. Cells were incubated with the drug for 1 hour at 37°C. Following incubation, drug containing medium was removed, cells were washed with 2 ml of PBS and wells were replaced with 2 ml of fresh complete medium. Plates were returned to the incubator under standard conditions for the required post-treatment time.

2.3.5. Cell harvesting and irradiation

All subsequent steps were performed on ice and samples were protected from light wherever possible, to minimise light induced DNA damage or cellular DNA repair. After the appropriate post incubation interval cells were harvested by trypsinising and re-suspended in complete medium maintained at 4°C. Cells were counted and diluted

at a density of 2.5 x 10^4 cells/ml. All drug treated samples, plus one control, were subjected to 12.5 Gy X-irradiation on ice. A second control remained un-irradiated.

2.3.6. Slide preparation

1% molten Type-VII Agarose solution was prepared and placed in a water bath maintained at 40°C. A 24-well plate was placed on ice. 0.5 ml of cell sample was placed in a well, to which 1 ml of molten agarose solution was added. After mixing, 1 ml of this solution was placed onto a pre-coated slide maintained at 4°C. A cover slip was place on top, and then removed once the gel solidified. Duplicate slides were prepared for each sample. Slides were placed in a tray on ice.

2.3.7. Lysis and electrophoresis

Ice cold lysis buffer containing 1% Triton X-100 was added into the tray containing slides, ensuring that all slides were sufficiently covered, and incubated in the dark for 1 hour. The lysis buffer was removed carefully so as not to disturb gels. Ice cold ddH_2O was added to completely cover the slides and incubated for 15 minutes. This was repeated a further 3 times. Slides were carefully transferred to a flat-bed electrophoresis tank and covered with 2 L of ice cold alkali buffer. After incubating for 45 minutes in the dark, electrophoresis was performed at 18 V (0.6 V/cm), 250 mA for 25 minutes.

2.3.8. Neutralisation and staining

Once electrophoresis was completed, slides were removed from the tank and placed on a horizontal rack. Slides were neutralised by flooding each slide with neutralisation buffer and leaving for 10 minutes. Slides were then washed with phosphate buffered saline and left to dry overnight. At this stage, dried slides could be stored in slide boxes until required. Prior to staining, slides were re-hydrated by flooding with ddH_2O and leaving for 30 minutes. Slides were then stained by flooding twice with 1 ml of propidium iodide solution and leaving in the dark for 30 minutes. Following staining, slides were washed three times with ddH_2O and left to dry in the dark overnight. Stained slides were stored in slide boxes until required for analysis.

2.3.9. Visualisation

A few drops of ddH_2O was placed on each slide, covered with a cover-slip and analysed using a Nikon DIAPHOT TDM inverted epi-fluorescent micnroscope (high pressure mercury-vapour light source, a 580 nm dichromic mirror, 510-560 nm excitation filter and 590 nm barrier filter) at 20 x magnification. Fifty cells were analysed per slide (100 per dose taking into account duplicates) using Komet Assay Software (kinetic Imaging, Liverpool).

2.3.10. Comet Analysis

The Comet Assay software captures comet images and measures the intensity of the comet. The software calculates the proportion of DNA in the head and the tail from areas under each respective line. It determines the centres of the mass of comet head

and comet tail, and measures the distance (d) between the centres of the mass (as shown in figure 2.3). This enables Tail Moment, which represent the most sensitive measure of the extent of comet tail to be calculated as followed.

$$\text{Tail Moment (TM)} = \%\ \text{DNA in Tail} \times d$$

The degree of DNA inter-strand cross-linking present in a drug treated sample was determined by comparing the tail moment of irradiated drug treated sample with irradiated untreated sample and un-irradiated untreated sample. The level of inter-strand cross-linking is proportional to the decrease in the tail moment in the irradiated drug treated sample compared to the irradiated untreated control. The decrease in tail moment is calculated by the following formula :

$$\%\ \text{Decrease in Tail Moment} = \{ 1 - (TMdi - TMcu) / (TMci - TMcu) \} * 100$$

where; TMdi = Mean tail moment of drug treated, irradiated sample.

TMci = Mean tail moment of irradiated control sample.

TMcu = Mean tail moment of un-irradiated control sample.

2.4. Measurement of cisplatin 1,2 intra-strand cross-links using competitive ELISA

2.4.1. Background and general features of the assay

Cisplatin intrastrand adduct levels were determined using the competitive Enzyme Linked Immunosorbant Assay (ELISA) method developed by Tilby *et al.*, (1991). This assay is based upon a monoclonal antibody, ICR4, obtained from rats immunised with

cisplatin (Tilby *et al.*, 1991). ICR4 specifically recognises cisplatin intrastrand cross-links. The principle of this assay is summarised in figure 2.4. ELISA plates are firstly coated with a standard amount of platinated DNA. A solution of sample being assayed is incubated with a small amount of antibody, to allow binding of ICR4 antibody to cisplatin intrastrand adducts in the sample. This mixture is then put in an ELISA well that is coated with the platinated DNA. The unbound antibody in the assay sample-antibody mixture will bind to the platinated DNA coated in the ELISA well. The amount of antibody that binds to the coated platinated DNA is inversely related to the amount of cisplatin intra-strand adducts present in the assay sample.

In order to maximise the sensitivity of this assay, it is necessary to use the lowest antibody concentration possible. Therefore, it is important to use a very sensitive detection system. In this assay, detection of monoclonal antibody bound to the well was originally done by use of a ß-galactosidase conjugated anti-rat reagent (Tilby *et al.*, 1991). This has now been superseded by biotinylated goat anti-rat antibody and a ß-galactosidase-streptavidin conjugate. The amount of ß-galactosidase enzyme is then quantitated by use of methylumbelliferyl ß-galactoside (MUG) substrate. Cleavage of MUG by β-galactosidase yields the fluorescent molecule 4-methylumbelliferone (7-hydroxy-4-methylcoumarin, 4MU). 4-MU is fluorescent above pH 8. When excited by 365 nm light, 4MU emits light at 460 nm.

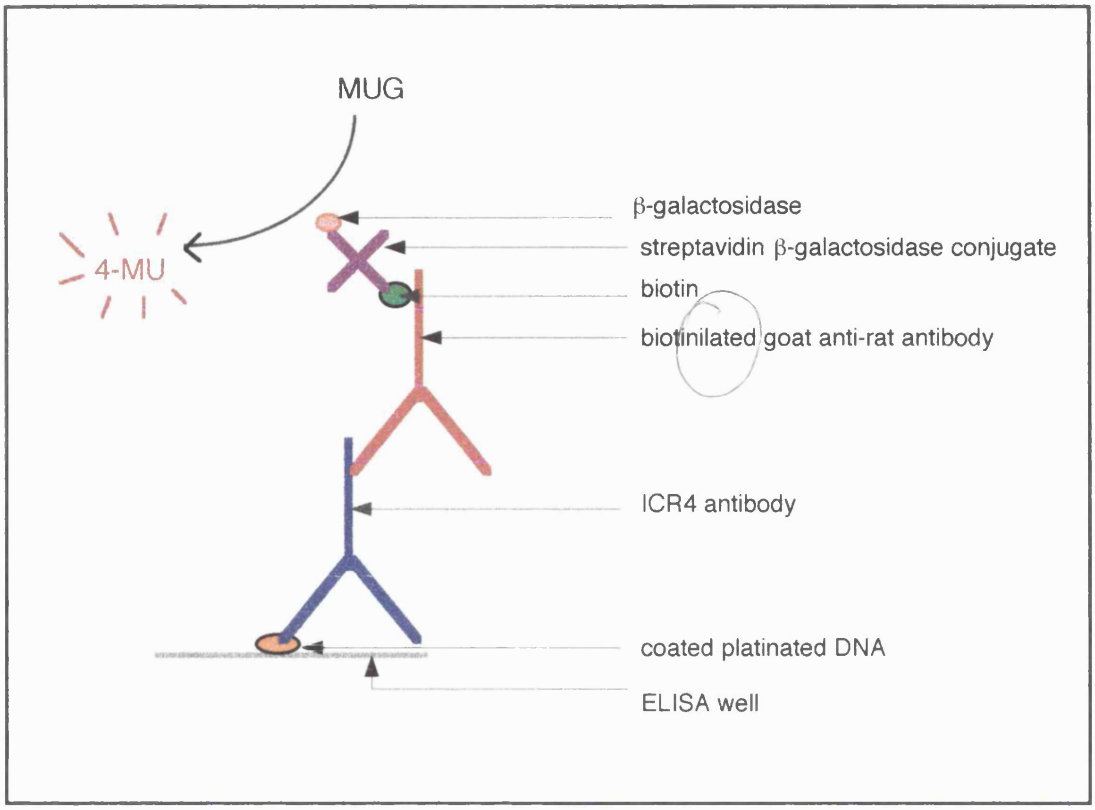


Figure 2.4. Summary of the ELISA.

The standard assay involves the serial dilution of each sample to be assayed. Each dilution is then treated as a separate sample and the mean fluorescence intensity for each is plotted against the dilution factor. A curve can then be fitted to the set of points to calculate the dilution of sample that gives 50% inhibition of the assay signal (i.e. 50% inhibition of antibody binding). Using a standard curve with known amount of platinated DNA, the number of adducts which leads to 50% inhibition of the assay signal can then be calculated. This value can then be used to calculate the number of adducts in the assay sample. If the DNA concentration of the assay sample is known, the number of adducts per μg of DNA can be calculated.

The assay plates are prepared as replicate pairs. For each pair the layout of the plates gives 4 wells for each antigen dilution, 4 wells with no monoclonal antibody and 20 wells with no competitor. The wells with no monoclonal antibody give the background signal comprising non-specific binding of reagents to the plates and innate fluorescence of the plastic of the plates.

The assay has been described in detail (Tilby *et al.*, 1987; Tilby *et al.*, 1991). Recently, changes have been made to the procedure to increase the sensitivity of this assay. The revised procedures include a more efficient coating method, and the antibody detection method. The unpublished revised protocol was obtained from Dr. M. J. Tilby.

2.4.2. Materials and reagents.

- PBS: 10 mM Na/K phosphates, 140 mM NaCl (KCl is optional), 0.02% sodium azide.

- PBST: PBS + 0.1% v/v Tween 20.

- High-salt coating buffer: 1 M NaCl, 50 mM sodium phosphate, 0.02% sodium azide, pH 7.0.

- DB buffer: 50 mM NaCl, 50 mM sodium phosphate, pH 7.0.

- 20% Tween: DB + 20% v/v Tween 20 [measure Tween 20 with a syringe].

- Phenol red solution: 2 mg/ml in PBS + 0.02% sodium azide.

- 1 M $MgCl_2$ solution (for supplementing buffers).

- ICR4 antibody: was provided by Dr. M. J. Tilby, University of Newcastle upon Tyne.

- Platinated DNA for coating ELISA plates: Denatured calf thymus DNA that has been reacted with cisplatin to give an adduct level of 35.2 μM/g DNA (lot 331) was provided by Dr. M. J. Tilby, University of New castle upon Tyne. It is stored at -80 °C.

- Platinated DNA for standard curve: Native calf thymus DNA (lot. 333) reacted with cisplatin was provided by M. J. Tilby, University of Newcastle upon Tyne. The platinum adduct concentration = 996 pmol/ml. DNA concentration = 490 μg/ml. It is stored at -80°C.

- Biotinilated goat anti-rat reagent:

  Goat-anti-rat antibody from Sigma. This is diluted at 4 μl per 10 ml buffer as followed:

PBS	9 ml
10% BSA soln.	1 ml
20% Tween soln.	100 μl
biotinylated anti-rat	4 μl

  5 ml of this reagent was required for each assay plate.

- ß-galactosidase-streptavidin conjugate:

  This is purchased from Boehringer. The stock (1 mg) was dissolved in 1 ml of PBS.

  This reagent is then diluted x 10,000 fold as follows:

PBS	18 ml
10% BSA soln.	2 ml
20% Tween soln.	100 µl
1 M $MgCl_2$ soln.	200 µl
ß-gal-SA conj.	2 µl

10 ml of this reagent was required for each assay plate.

- Substrate solution:

  4-methyl-umbelliferyl ß-D-galactoside (MUG) was purchased from Sigma. An 80 µg/ml solution was made in PBS containing 10 mM $MgCl_2$ and azide. MUG was initially dissolved with a drop of PBS, and then rest of the buffer added. The solution was gently warmed until it became clear, and then filtered through a wattmann filter paper. The substrate solution was stored at -20 °C until required.

- 96-well ELISA plates: High-bind 96 well flat bottom ELISA plates and plate sealers were purchased from Greiner.

2.4.3. Preparation of cells and drug treatment

Exponentially growing cells in T80 cm^2 flasks were treated with the required dose of cisplatin for 1 h, and then incubated in drug free medium for the desired post incubation time. Cells were then washed two times with PBS, harvested and genomic DNA was isolated as described below.

2.4.4. Isolation of genomic DNA from cells

Genomic DNA was isolated from cells using a QIAmp DNA Mini Kit purchased from Qiagen Ltd (UK). The isolation procedure was carried out as described in the QIAmp DNA Mini Kit handbook.

2.4.5. DNA digestion

Genomic DNA was digested with Hind III (Promega) at 37 °C overnight, ethanol precipitated and re-suspended in 100 µl of H_20. The DNA concentration was then measured using a fluorimeter as described in section 2.5.

2.4.6. Coating and blocking ELISA plates

Assay plates were prepared the day before the assay. Platinated DNA stock (lot 331) was dilluted 2000-fold in coating buffer and 50 µl of this solution was added into each well of the assay plate. Wells were checked to insure that the solution completely covered the bottom. The assay plate was covered with a plate sealer and left in a 37 °C incubator overnight. ELISA wells were then emptied and blocked with BSA solution (1% w/v in PBS) by adding 150 µl/well and incubating at room temperature for at least 30 minutes.

2.4.7. Preparation of standard and assay samples

Platinated DNA standard stock solution (lot 333) containing 996 pmol/ml of cisplatin adducts, was diluted 1250-fold in DB buffer to make a solution containing 797 fmol/ml of adducts. 260 µl of this solution was added to a 1.5 ml eppendorf tube. An initial dilution of each assay sample was also made in a total volume of 260 µl and placed in 1.5

ml eppendorf tubes. The caps of the eppendorfs were sealed with parafilm and the standard and assay samples were placed in boiling water for 5 minutes to increase the immunoreactivity. The samples were then placed in ice, spun briefly and stored on ice until required.

2.4.8. Preparation of the antibody solution

A solution containing the following reagents was made.

PBS	16.8 ml
10% BSA	0.4 ml
4.5 M NaCl	0.4 ml
20% Tween	0.2 ml
Phenol red solution	0.2 ml

1.8 ml of this solution was placed in a bijou bottle and 0.2 ml of PBS was added to make the control solution (solution A) without the antibody. Using a freshly thawed aliquot of ICR4 antibody, a 6000-fold dilution was made in PBS. 2 ml of this diluted antibody solution was added to the rest of the mixture prepared above to make the antibody solution (solution B).

2.4.9. Preparation of serial dilutions of the standard and the assay samples

96 well tissue culture plates (dilution plate) were used for preparation of serial dilutions. One row of a 96 well flat bottom plate was allocated for each assay sample or standard. 120 µl of DB buffer was added to each of wells 3 - 11 of the plate. 260 µl of the pre-diluted, pre-heated assay sample or standard was added to well 2. Using a multi-channel

pipette to synchronously dilute several samples, 120 µl was transferred from well 2 to well 3 and mixed. This was repeated for well 3 to 4, then 4 to 6 skipping 5. Then 6 to 7, 7 to 8, 8 to 9, 9 to 10 and well 11 was left as it is.

55 µl of solution in each well of the dilution plate was transferred to a 96 well U-bottom plate (mixing plate) using a multi-channel pipette. 55 µl of solution A was then added to wells D5 and D11 designated for as the no antibody control. 55 µl of solution B was added to the other wells. 120 µl of water was added to the outer wells of the plate, the plate was covered with a plate sealer and incubated at 37 °C for 30 min.

2.4.10. Detection procedure

ELISA plates containing blocking buffer (from step 1) were washed two times with PBST buffer taking care to avoid the wells drying out. 50 µl from each row of wells in the mixing plate was transferred to the equivalent rows in each of the two replicate ELISA assay plates. Plates were covered with plate sealers and incubated for 1 h at 37 °C.

Plates were washed 5 times with PBST, and 50 µl of biotinylated anti-rat solution was added to each of the wells. Plates were covered with plate sealers and incubated at 37 °C for 30 minutes. Plates were then washed 3 times with PBST and 50 µl of streptavidin-β-galactosidase reagent was added to each of the wells. Plates were covered with plate sealers and incubated at 37°C for 30 minutes.

Plates were washed 7 times with PBST, and 50 µl of the substrate solution was added to each of the wells including the outer wells. Plates were covered with plate sealers and placed in dark for 2 - 4 hours. To measure the flourescence of each well, plates were read at 360 nm emission and 465 nm excitation. The mean background reading was subtracted from all the readings and the percentage inhibition of maximum fluorescence was calculated for each serial dilution of the assay samples and the standard.

2.5. Measurement of DNA concentration - Fluorescent DNA assay

2.5.1. Materials

- 20 x SSC stock solution: 175.3 g sodium chloride, 88.2 g of sodium citrate in 800 ml of H_20, pH adjusted to 7.0 with sodium hydroxide and the volume was adjusted to 1 L with H_2O.
- Salmon testes DNA solution for standard (Sigma): 100 x stock at 1 mg /ml in SSC.
- Hoechst Dye (Sigma): 100 x stock at 4 mg in 50 ml SSC.
- Flourimeter: Perkin-Elmer LS-2B filter flourimeter.

2.5.2. Method

DNA concentrations were measured flourimetrically as described by Kowalski et al., (1979). In summary, a 20 x SSC solution was diluted to make a 1 x solution. Salmon testis DNA and Hoechst dye solutions were diluted to obtain 1 x solutions with SSC. Tubes containing the reagents in table 2.2 were made for the standard curve. Duplicate tubes were made for each unknown DNA samples with the following reagents; 1190 µl

SSC, 1000 µl Hoechst dye and 10 µl test sample. Tubes were covered and left for 10 minutes. The fluorescence value of standard and test samples were read at 360 nm excitation and 450 nm emission using a Perkin-Elmer LS-2B Filter Fluorimeter which automatically read the DNA concentrations of the test samples using the readings of the standard curve.

Tube	µg DNA / tube	Volume of DNA (µl)	Volume of SSC (ml)	Volume of Hoechst (ml)
R0	0	0	2	1.0
R10	10	1000	1.0	1.0
Rinse	0	0	2	1.0
0a	0	0	2	1.0
0b	0	0	2	1.0
0.25a	0.25	25	1.975	1.0
0.25b	0.25	25	1.975	1.0
1.0a	1.0	100	1.900	1.0
1.0b	1.0	100	1.900	1.0
5.0a	5.0	500	1.500	1.0
5.0b	5.0	500	1.500	1.0
10.0a	10	1000	1.0	1.0
10.0b	10	1000	1.0	1.0
Rinse	0	0	2	1

Table 2.2. Preparation of DNA samples for fluorescent DNA assay

2.6. NBP assay

The reactivity of an alkylating agent is often defined as its time dependent alkylation of 4-(4'-nitrobenzyl) pyridine (NBP) in the presence of competing nucleophiles such as buffer and solvent molecules (Spears, 1980). The reactivity of NBP molecules toward alkylating agents results from the relatively strong nucleophilicity of the anionoid electron pair of the pyridine ring nitrogen. NBP assay as described by Spears (1981) was employed to compare the alkylation potential of HN2 and HN1.

2.6.1. Materials

- NBP solution (2% w/v in acetone) (Sigma).
- Ethyl acetate.
- 200 mM tris pH 7.
- 0.25 M NaOH.
- Glass screw cap tubes.

2.6.2. Method

1 ml of H_2O, 200 µl of 200 mM tris pH 7, and 1 ml of NBP were mixed (in that order) in a glass screw cap tube. 100 µl of test sample was then added to this mixture, mixed well, the tubes were closed tightly and boiled in a heating block at 110 °C for 30 minutes. Tubes were chilled on ice for 10 minutes and then allowed to warm to room temperature. 1 ml of acetone and 2.5 ml of ethyl acetate were added sequentially and mixed. To develop the blue colour which indicates the alkylation potential, 1 ml of

NaOH was added. 1 ml of this mixture was transferred into a glass cuvette and the absorbance was read at 540 nm.

2.7. Pulse-field gel electrophoresis (PFGE)

2.7.1. Background of the assay

The advent of pulse-field gel electrophoresis (PFGE) as a method to examine larger molecular weight DNA has led to the application of this technique for measuring DSBs in mammalian cells (Schwartz and Cantor, 1984). The theory behind PFGE is that by alternating the direction of the electric field at regular intervals, DNA is forced to reorient in the new field direction, and smaller DNA pieces will reorient faster than larger ones because of the physical resistance of the agarose matrix. In contour-clamped homogeneous field electrophoresis (CHEF) (Chu *et al.*, 1986), the direction of the electric field changes electronically to reorient the DNA by changing the polarity of an electrode array. The result is that far superior separation of large molecular weight DNA according to size can be achieved. This method has many technical advantages over other methods used to measure DNA DSBs, including good reproducibility and large sample handling capacity. To perform the assay, about 10^6 cells are embedded in low gelling temperature agarose in plugs which are then immersed in a lysing solution generally containing the detergent SDS, and proteinase K, and often incubated at high temperatures (50 °C) for several hours. The plugs are then sealed in the wells of a pre-formed gel, and subjected to PFGE for various times. The method of embeddding intact cells in agarose prior to lysis considerably reduces

the background level of strand breaks, thus increasing the sensitivity of the method. In practice, the amount of DNA that migrates out of the well and into the lane is often used as a sensitive indicator of DSBs in mammalian cells.

2.7.2 Materials

- CHEF Genomic Plug Kit (Bio-Rad).
- Pulse field certified agarose (Bio-Rad).
- 10 x Tris-Borate-EDTA (TBE) Buffer.
- Biometra Rotaphor Type V.

2.7.3. Method

Cells growing in monolayer in T80 cm^2 flasks were treated with test agent for one hour, washed with 10 ml of PBS and incubated with fresh medium for required repair time. Cells were trypsinised, 3 x 10^6 cells were harvested and CHEF plugs were prepared using the Bio-Rad mammalian CHEF Genomic Plug Kit as instructed by the manufacturer. CHEF was performed with a 0.7% gel (pulse field certified, Bio-Rad) in 0.25 x TBE buffer using a Biometra Rotaphor Type V apparatus as instructed in the manual. Electrophoresis was run for 120 hours at 14°C with the following parameters: Interval 5000-1000 seconds log, angle 110-100° linear, voltage 50-45 V linear. On completion, gels were stained with 2 μg/ml ethidium bromide for 1 hour and de-stained overnight with ddH_2O. For analysis, gels were placed on a UV illuminator and photographed. Quantitative data was obtained by measuring the absolute integrated OD of each lane using Gel Pro Analyser (Media Cybergenetics) and calculating the percentage of DNA released from the DNA plug.

CHAPTER 3: REPAIR OF HN2-INDUCED ICLs IN MAMMALIAN CELLS

3.1. INTRODUCTION

DNA interstrand cross-linking agents such as the nitrogen mustards, mitomycin C, cisplatin and psoralen are widely used in cancer chemotherapy and phototherapy, and are thought to exert their cytotoxic effects by preventing efficient DNA replication and transcription. Since ICLs affect both strands of DNA, repair of these lesions presents a special problem. As explained in chapter 1, in *E. coli* and *S. cerevisiae*, the repair of ICLs depends on both NER and homologous recombination (Cole *et al.*, 1973 & 1975, Jachymczyk *et al.*, 1981, Magana-Schwencke *et al.*, 1982, Van Houten *et al.*, 1986, McHugh *et al.*, 2000). In *S. cerevisiae* the repair of psoralen photoinduced DNA ICLs involves a double-strand break intermediate, resulting partially from NER incisions and these DSBs are repaired by homologous recombination (Davies *et al.*, 1995, Jachymczyk *et al.*, 1981, Megana-Schwencke *et al.*, 1982, Malcova *et al.*, 1996). In contrast, recent studies from this laboratory have demonstrated the occurrence of NER-independent DSBs in yeast following treatment with nitrogen mustard (McHugh *et al.*, 2000). In common with psoralen-induced cross-links these DSB intermediates are repaired by homologous recombination (McHugh *et al.*, 2000).

In mammalian systems ICL repair is poorly understood. However, the isolation and characterisation of mutant cell lines with extreme sensitivities to cross-linking agents

can provide mechanistic clues. For example, CHO cells with mutations in the XPF and ERCC1 genes involved in the NER pathway, and mutations in XRCC2 and XRCC3 involved in homologous recombination pathway are extremely sensitive to cross-linking agents suggesting a role of these genes in ICL repair (Anderson *et al.*, 1996, Damia *et al.*, 1996, Hoy *et al.*, 1985, Fuller Painter, 1988, Jones *et al.*, 1987).

In this chapter, the roles of NER, HR and NHEJ pathways in the repair of HN2-induced ICLs in mammalian cells are determined using CHO mutant cell lines defective in these repair pathways. The sensitivity of the mutant cell lines to HN2 was compared with that of its monofunctional analogue HN1, employing the SRB assay. The sensitivity data was then compared the ability of the mutant cell lines to unhook HN2-induced ICLs using the modified single cell gel electrophoresis or comet assay. Results presented here confirm the hypersensitivity of ERCC1, XPF, XRCC2 and XRCC3 mutant CHO cells to HN2, and show a correlation between this hypersensitivity and a defect in the "unhooking" of ICLs in the ERCC1 and XPF defective cells, but not in the homologous recombination mutants XRCC2 and XRCC3. Using pulsed field gel electrophoresis (PFGE), induction of DSBs following treatment of CHO cells with HN2 is shown, and the importance of XRCC2 and XRCC3 in the repair of these DSBs is illustrated.

3.2. RESULTS

3.2.1. Nitrogen mustard sensitivity of NER and recombination defective cell lines.

To confirm the roles of NER and recombination in the repair of ICLs in mammalian cells the sensitivity to the bifunctional alkylating agent nitrogen mustard (HN2) of CHO cell lines defective in these pathways was determined. The results are consistent with those obtained by other workers (Anderson *et al.*, 1996, Caldecott *et al.*, 1991, Damia *et al.*, 1996, Hoy *et al.*, 1985) using a variety of cross-linking agents, implying that these observations are of general significance to ICL repair.

Amongst the mutant cell lines defective in NER (Figure 3.1A), UV47 and UV96, defective in XPF and ERCC1, respectively, were highly sensitive (> 15-fold) to HN2 compared to their isogenic parent cell line AA8. In contrast, UV135, defective in XPG responsible for the 3' incision in NER, was only slightly sensitive (< 2-fold). Similarly, the UV23 and UV42 cell lines bearing mutations in the XPB and XPD helicases, respectively, were also only slightly sensitive to HN2. Surprisingly, the UV61 CSB mutant cell line, defective in the transcription coupling of NER, was slightly resistant to HN2 compared to its isogenic parent.

To differentiate the repair mechanisms involved in the removal of ICLs to that of mono-adducts, sensitivities of NER mutants to a monofunctional analogue of HN2, HN1 was also investigated. HN1 is a mono-functional alkylating agent which can form N-alkylpurine mono-adducts but not ICLs. When IC50 values of HN2 and HN1 were compared in the AA8 parent cell line, HN1 was 225 fold less cytotoxic than HN2

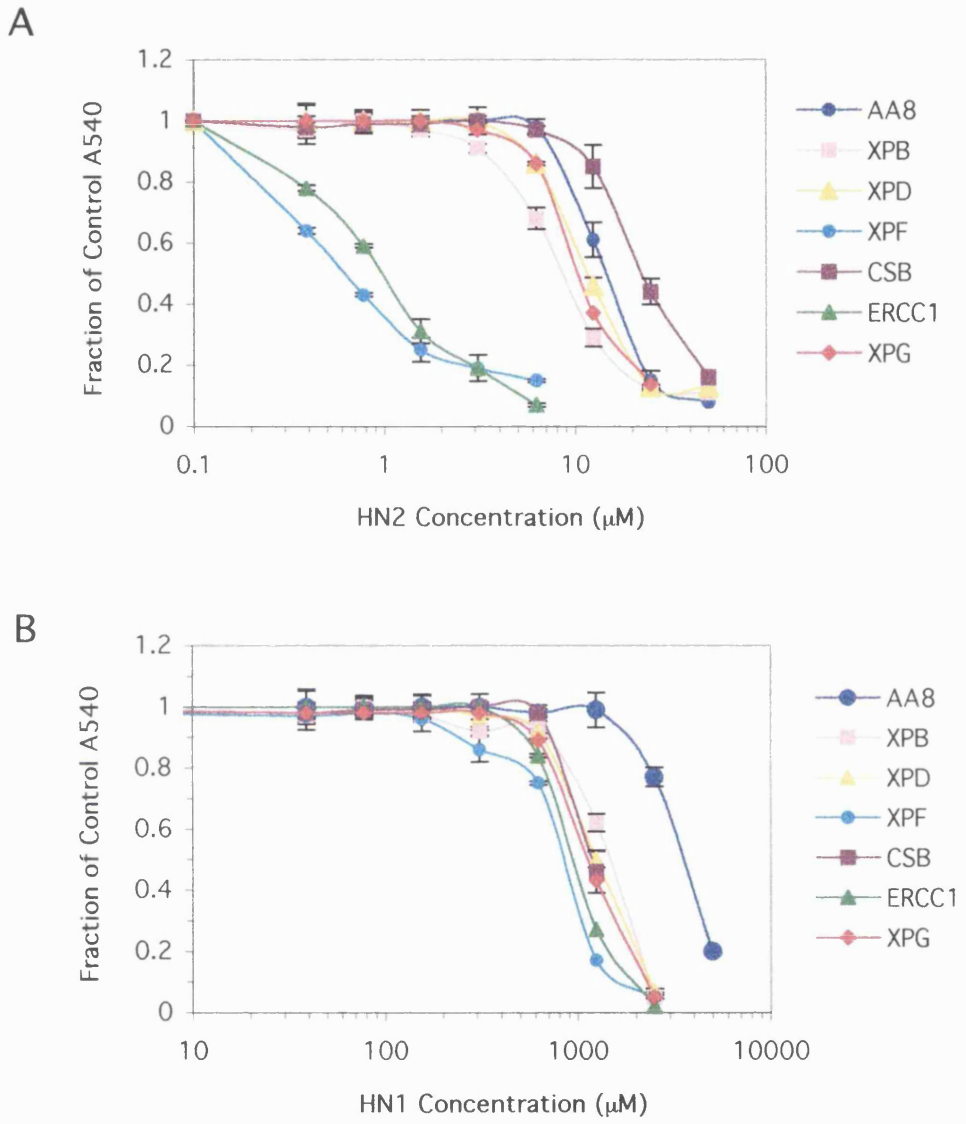


Figure 3.1. Cytotoxicity of HN2 (A) and HN1 (B) in AA8 wild type cells and in NER mutants; XPB (UV23), XPD (UV42), XPF (UV47), CSB (UV61), ERCC1 (UV96) and XPG (UV135). Exponentially growing cells were treated with increasing concentrations of the drug for 1 h, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition was calculated for each dose as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

(figure 3.1B) indicating that the cytotoxicity of HN2 is mainly due to the induction of bifunctional lesions. At high doses of HN1, all the mutants tested, including the XPF and ERCC1 mutants demonstrated 2.4 - 4 fold higher sensitivity than the parent cell line (figure 3.1B). These results suggest that the extreme sensitivity of the XPF and ERCC1 mutants to HN2 is due to an inability to repair ICLs.

Figure 3.2 shows the sensitivities of the XRCC mutants to HN2. The HR defective irs1 (XRCC2 mutant) and irs1SF (XRCC3 mutant) lines derived from V79 and AA8, respectively, were extremely sensitive to HN2 (12-fold and 26-fold, respectively). In contrast, the NHEJ defective cell line xrs5, bearing a mutation in the XRCC5 gene product, was only slightly sensitive to HN2 (1.5-fold) compared to its parent cell line CHO-K1.

Surprisingly, HR and NHEJ mutants also showed a similar pattern of sensitivity to HN1 as for HN2, when treated with high doses (figure 3.3). XRCC2 and XRCC3 mutants were 11 and 15-fold more sensitive to HN1 than the parent cell line respectively. The NHEJ mutant XRCC5 was only 2.5 fold more sensitive than the parent cell line. These results imply that XRCC2 and XRCC3 factors are not only important in the processing of lesions induced by HN2, but also required for the repair of DNA damage induced by high doses of HN1.

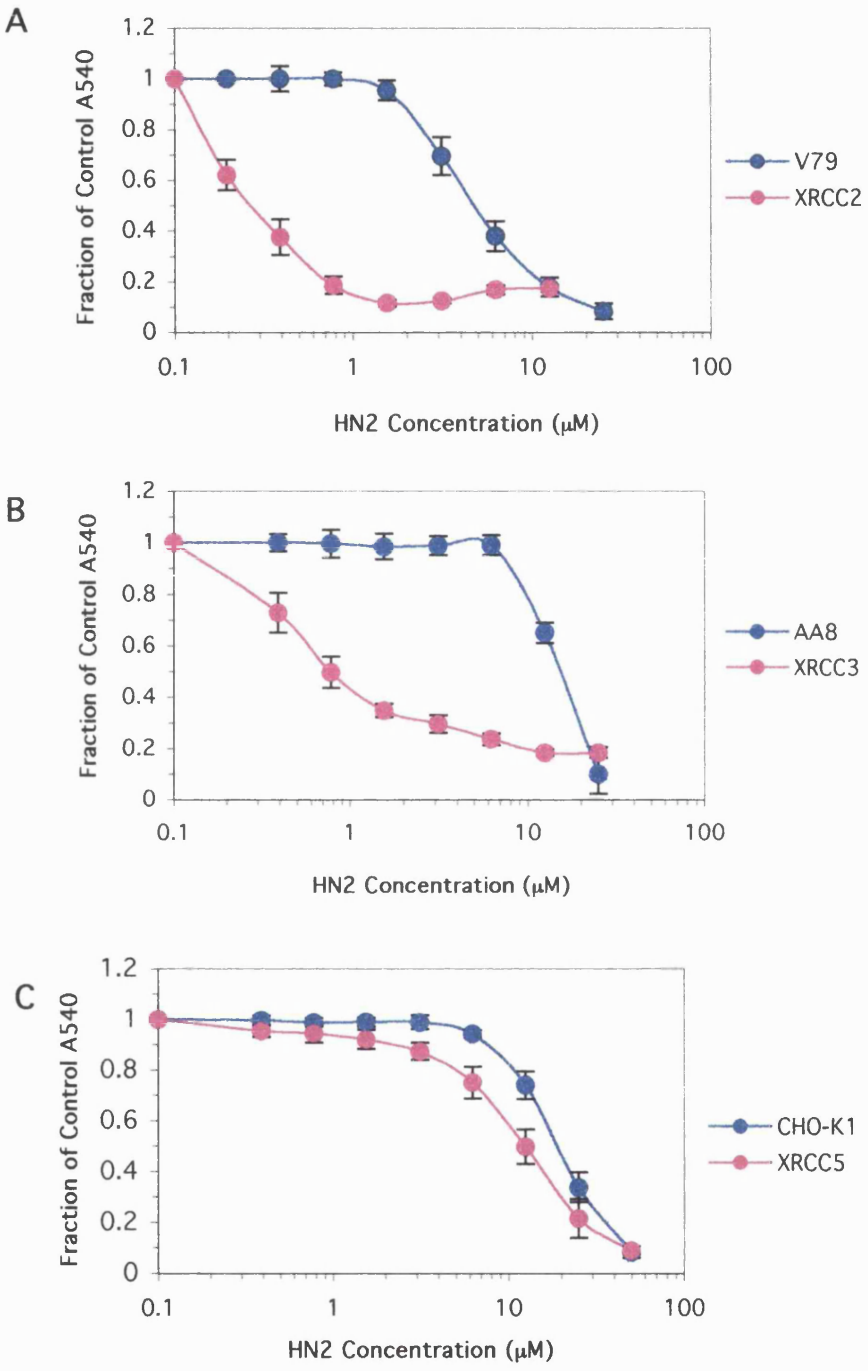


Figure 3.2. Cytotoxicity of HN2 in homologous recombination mutants, A. XRCC2 (irs1) and the wild type cell line V79, B. XRCC3 (irs1SF) and wild type AA8 cell line, and C. non-homologous end-joining mutant, XRCC5 (xrs5) and the wild type cell line CHO-K1. Cells were treated with increasing concentrations of HN2 for 1 hour, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition for each dose was calculated as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

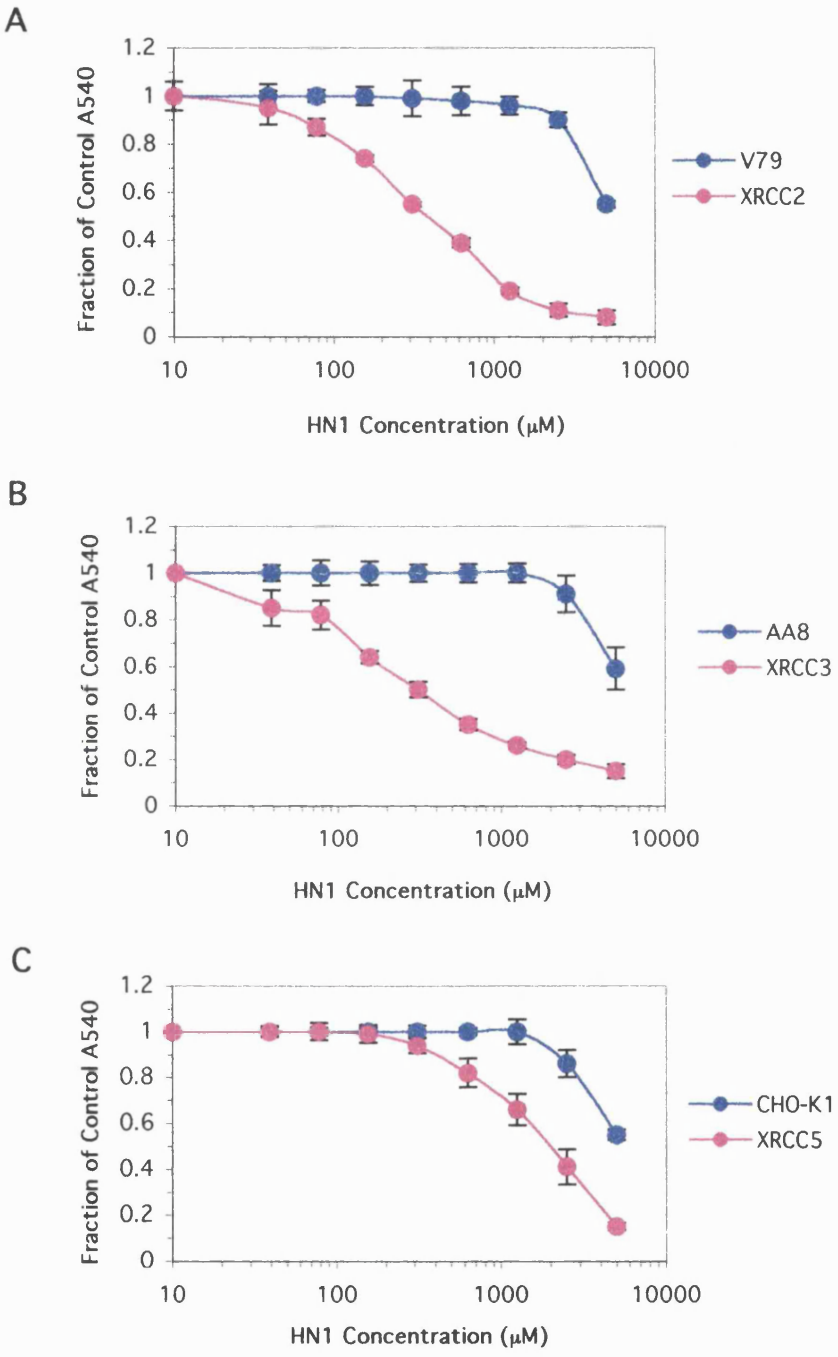


Figure 3.3. Cytotoxicity of HN1 in homologous recombination mutants, A. XRCC2 (irs1) and the wild type cell line V79, B. XRCC3 (irs1SF) and wild type AA8 cell line, and C. non-homologous end-joining mutant, XRCC5 (xrs5) and the wild type cell line CHO-K1. Cells were treated with increasing concentrations of HN1 for 1 hour, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition for each dose was calculated as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

3.2.2. The unhooking of ICLs in CHO repair defective cell lines.

To investigate whether the extreme sensitivities of the XPF, ERCC1, XRCC2 and XRCC3 mutants are due to a defect in the incision step of cross-link repair, the efficiency of "unhooking" of HN2-induced cross-links in these mutants, and the less sensitive XPB, XPD, XPG and XRCC5 mutants were compared with their isogenic parents. Cross-linking was measured at the single cell level using a modified version of the Comet assay as explained in Materials and Methods (section 2.3). This assay allows the initial cross-link incisions on one strand, releasing the covalent linkage of the two strands to be followed. Prior to cell lysis, samples received a dose of X-rays to induce random DNA single strand breakage. The presence of ICLs retards the migration of the irradiated DNA during electrophoresis resulting in a reduced tail moment compared to the unirradiated control. Examples of typical comet images are sown in figure 3.4. In control non-drug treated unirradiated cells no DNA damage was detected and the high molecular supercoiled DNA remained intact as shown in figure 3.4A. Following irradiation of cells with 12.5 Gy to introduce a fixed level of random single strand breaks, the resulting shorter fragments of DNA migrated from the bulk of the DNA to produce the typical comet images (figure 3.4B). Following treatment of cells with HN2, drug induced single strand breaks were not detected in unirradiated cells (figure 3.4C) and these cells showed similar profiles to unirradiated controls (figure 3.4A). When the drug treated cells were irradiated (figure 3.4D) comet tails were visible but with a reduced length and intensity compared to the irradiated controls. The decrease in tail moment compared to untreated irradiated controls was

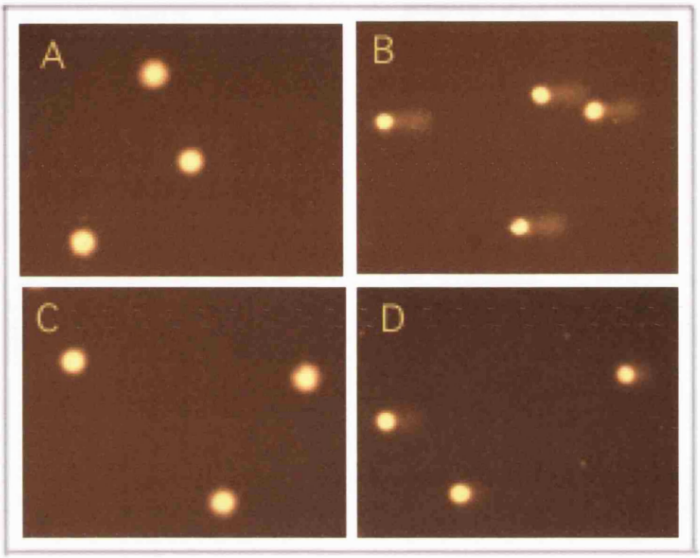


Figure 3.4. Typical Comet images of AA8 cells treated with HN2. Following irradiation (12.5 Gy) of untreated AA8 cells distinct comets were observed (B) compared to the untreated control (A). When cells were treated with 16 μM HN2 for 1 h, single strand breaks were not observed in unirradiated cells (C). Following irradiation of HN2 treated cells comet tails were visible but with reduced length and intensity due to the presence of DNA ICLs.

used to quantitate the level of DNA ICLs as explained in Materials and Methods (section 2.3.10).

Figure 3.5 shows the decrease in tail moment compared to the irradiated control due to the presence of cross-links induced following 1 h treatment with increasing concentrations of HN2. The ability of cells to unhook the cross-link can therefore be observed as an increase in tail moment following a repair period in drug-free medium.

Figure 3.6A illustrates the unhooking efficiency of XPF, ERCC1, XPB and XPG mutants compared to that of their parent AA8. The XPB and XPG mutants showed unhooking kinetics indistinguishable from AA8. These cells were all able to unhook approximately 65% of the cross-links by 24 h and more than 85% by 48 h. In contrast, and in agreement with their extreme sensitivity, the XPF and ERCC1 mutants were highly defective in the unhooking of HN2 cross-links. Less than 15% of the cross-links were unhooked after 48 h in drug free medium.

The unhooking kinetics of the recombination mutants is shown in Figure 3.6B and 3.6C. Although the XRCC2 and XRCC3 mutants are highly sensitive to HN2, they displayed similar unhooking kinetics to their isogenic parents indicating a lack of correlation between their hypersensitivity and the ability to unhook cross-links. Attempts to measure unhooking kinetics in the xrs5 line were unsuccessful because a high level of background DNA strand breaks was observed which obscured the results of this assay.

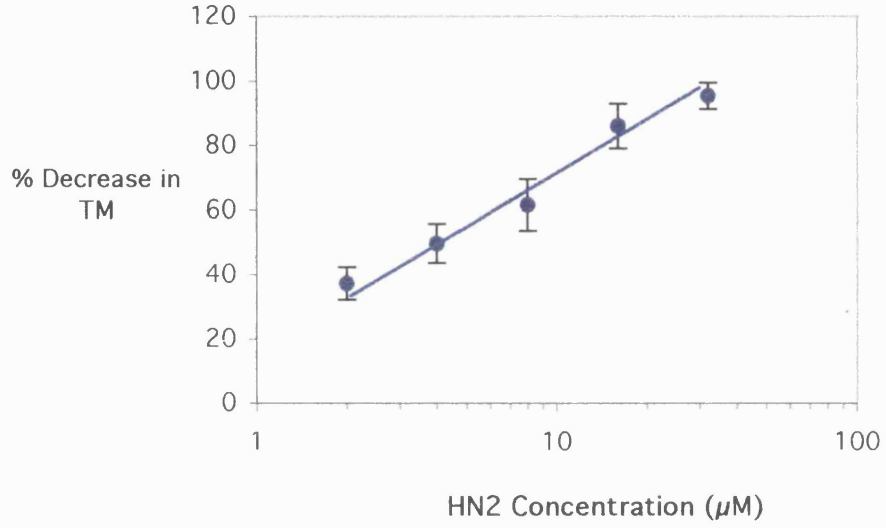


Figure 3.5. DNA interstrand cross-linking measured as the decrease in TM with increasing concentration of HN2. AA8 cells were treated with increasing doses of HN2 for 1 h and analysed immediately using the comet assay. The percentage decrease in tail moment compared to untreated samples was then calculated as described in Materials and Methods. Results shown above are the mean of three independent experiments, and error bars show the standard error of the mean.

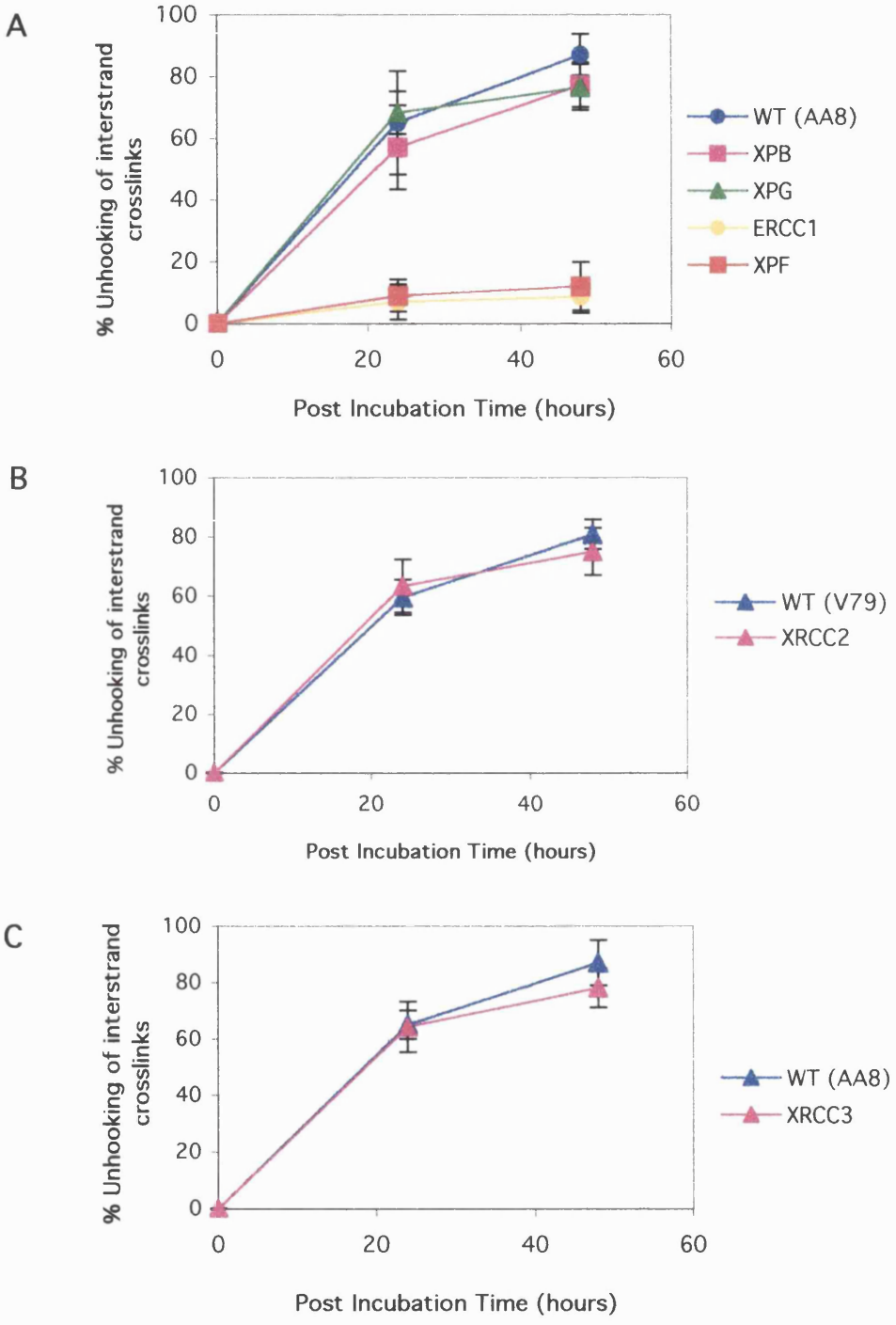


Figure 3.6. The efficiency of unhooking of interstrand cross-links following 1 h treatment with 16 μM HN2. A. NER mutants: XPB (UV23), XPG (UV135), ERCC1 (UV96) and the AA8 wild type cell line. B. Homologous recombination mutant XRCC2 (irs1) and wild type cell line V79. C. XRCC3 mutant (irs1SF) and wild type cell line AA8. Results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

3.2.3. Evidence for the occurrence of DSBs following treatment with HN2.

It has been clearly demonstrated that in *S. cerevisiae* the repair of ICLs, including those induced by HN2, involves the formation of DSBs (Jachymczyk *et al.*, 1981, Magana-Schwencke *et al.*, 1982, Malcova *et al.*, 1996, McHugh *et al.*, 2000). However the formation of DSB intermediates during the repair of ICLs in mammalian systems has not been systematically explored. To address this, exponentially growing and non-dividing cell cultures were treated with a range of doses of HN2 from 0 to 32 μM for 1 h and DSB formation assessed using PFGE. Non-dividing cells were obtained by allowing cells to reach to confluence and culturing them in medium containing 0.5% serum.

As shown in figure 3.7A, DSBs were formed following a 1 h treatment with HN2 in a dose dependent manner. In exponentially growing cells, release of low molecular weight DNA from the high molecular weight DNA plugs as a result of DSBs was evident at doses as low as 4 μM. DSB induction in non-dividing cells was significantly less efficient compared to that of diving cells, as has been previously shown for *S. cerevisiae* (McHugh *et al.*, 2000). Semi-quantitative analysis of the percentage of DNA released into the gel from the plugs (Figure 3.7B) showed that following 16 μM HN2 treatment for 1 h, approximately 80% of DNA is released from dividing cell plugs compared to only 10% from non-dividing cell plugs.

In order to rule out that DSBs were the result of a DNA degrading activity of HN2, genomic DNA embedded in agarose plugs was treated with the same doses of HN2 (0-

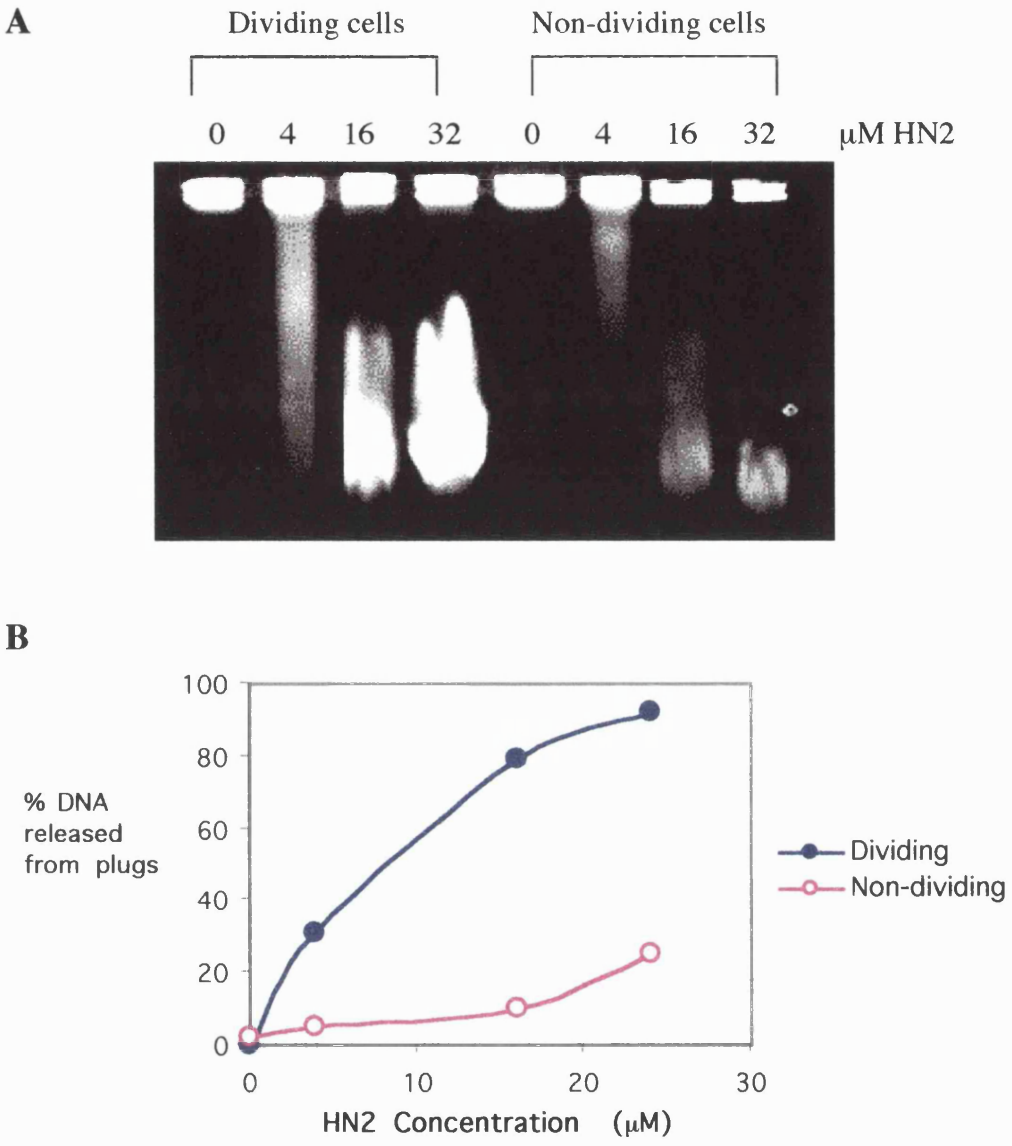


Figure 3.7. A. Induction of DSBs in dividing and non-dividing parental AA8 cells determined by PFGE. Cells were treated with 0, 4, 16 and 32 μM HN2 for 1 h, embedded in agarose plugs as described in Materials and Methods and run on PFGE gels. Release of DNA from plugs indicates the presence of DNA double-strand breaks. B. Quantitative analysis of the percentage of the DNA released from plugs following HN2 treatment.

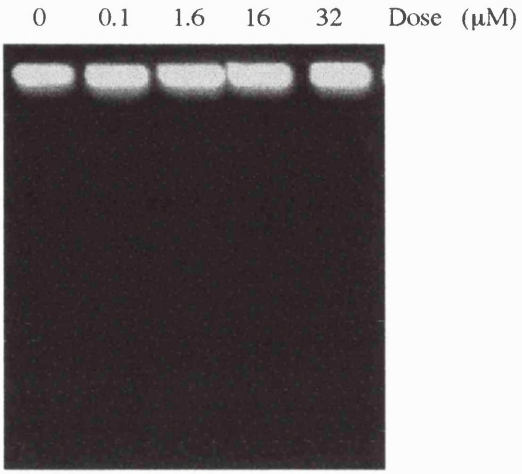


Figure 3.8. Analysis of DSBs following treatment of genomic DNA with increasing doses of HN2. PFGE gel plugs containing AA8 cells were prepared and cells were lysed as described in Materials and Methods. Following cell lysis, plugs were treated with increasing doses of HN2 for 1 h, washed 3 times and analysed immediately by PFGE.

32 μM) and analysed by PFGE. No DSBs were induced even at much higher effective doses than when cellular DNA was exposed to HN2 (figure 3.8), indicating that these breaks are a result of cellular activities during the processing of HN2-induced DNA damage. To investigate whether these DSBs arise due to the processing of ICLs and are not due to the processing of mono-adducts, induction of DSBs by HN2 and HN1 were compared. Comparison of the alkylation potential of HN2 and HN1 using the NBP assay (Spears *et al.*, 1981) described in Materials and Methods (section 2.6) indicated that at equimolar doses both agents alkylate DNA to a similar extent (figure 3.9). As shown in figure 3.10, in exponentially growing cells no DSBs were observed following treatment with a dose of 16 μM HN1, equivalent to that which produces a high level of DSBs with HN2 (figure 3.7). Even at a 100 μM dose, HN1 failed to induce DSBs. However, at a dose of 3600 μM, which is the IC50 dose of HN1 and equivalent to 16 μM HN2, DSBs were observed. This suggests that in dividing cells, DSBs are generated in the presence of ICLs induced by HN2, but not by a similar level of mono-adducts induced by HN1. However, in the presence of very high levels of mono-alkylation (when cells were treated with 3600 μM HN1) DSBs can be induced.

3.2.4. Homologous recombination repairs DSBs resulting from HN2 exposure in mammalian cells.

To examine the pathways involved in the repair of DSBs resulting from HN2 exposure, DSB repair was followed in both homologous recombination and NHEJ defective cell lines. Exponentially growing cells were treated with 16 μM HN2 for 1 h and subsequently incubated in fresh medium for 0, 4, 8, and 24 h to allow repair to

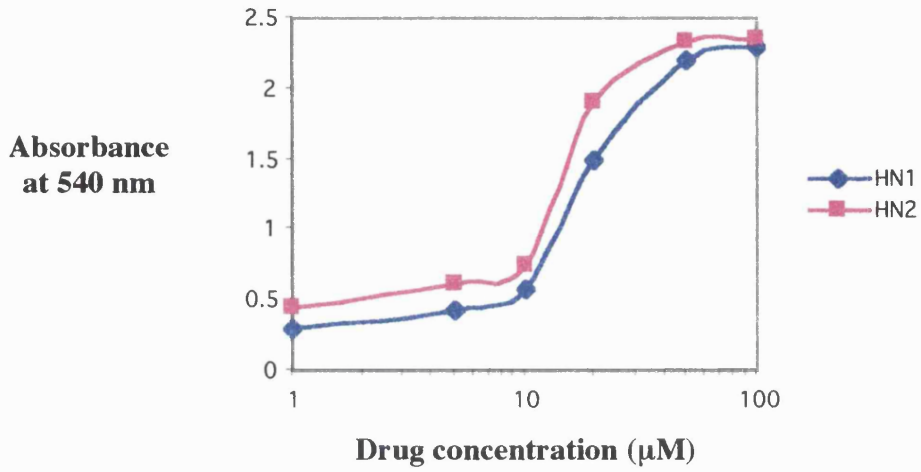


Figure 3.9. Alkylation potential of HN1 compared to that of HN2, determined using the NBP assay as described in Materials and Methods.

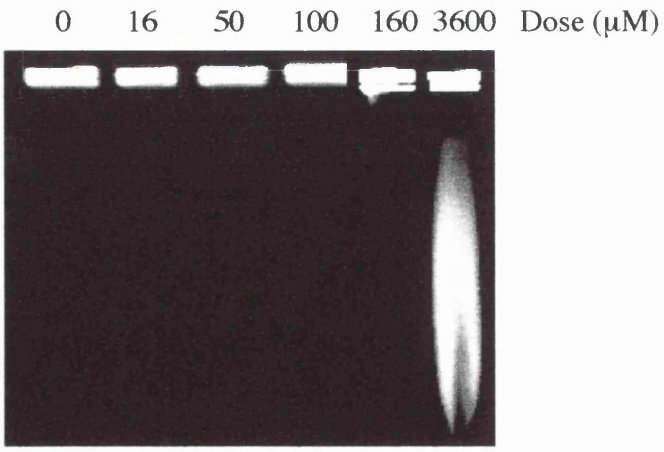


Figure 3.10. Induction of DSBs following treatment of AA8 cells with increasing doses of HN1. Exponentially growing AA8 cells were treated with HN1 for 1 hour and DSB were analysed immediately using PFGE.

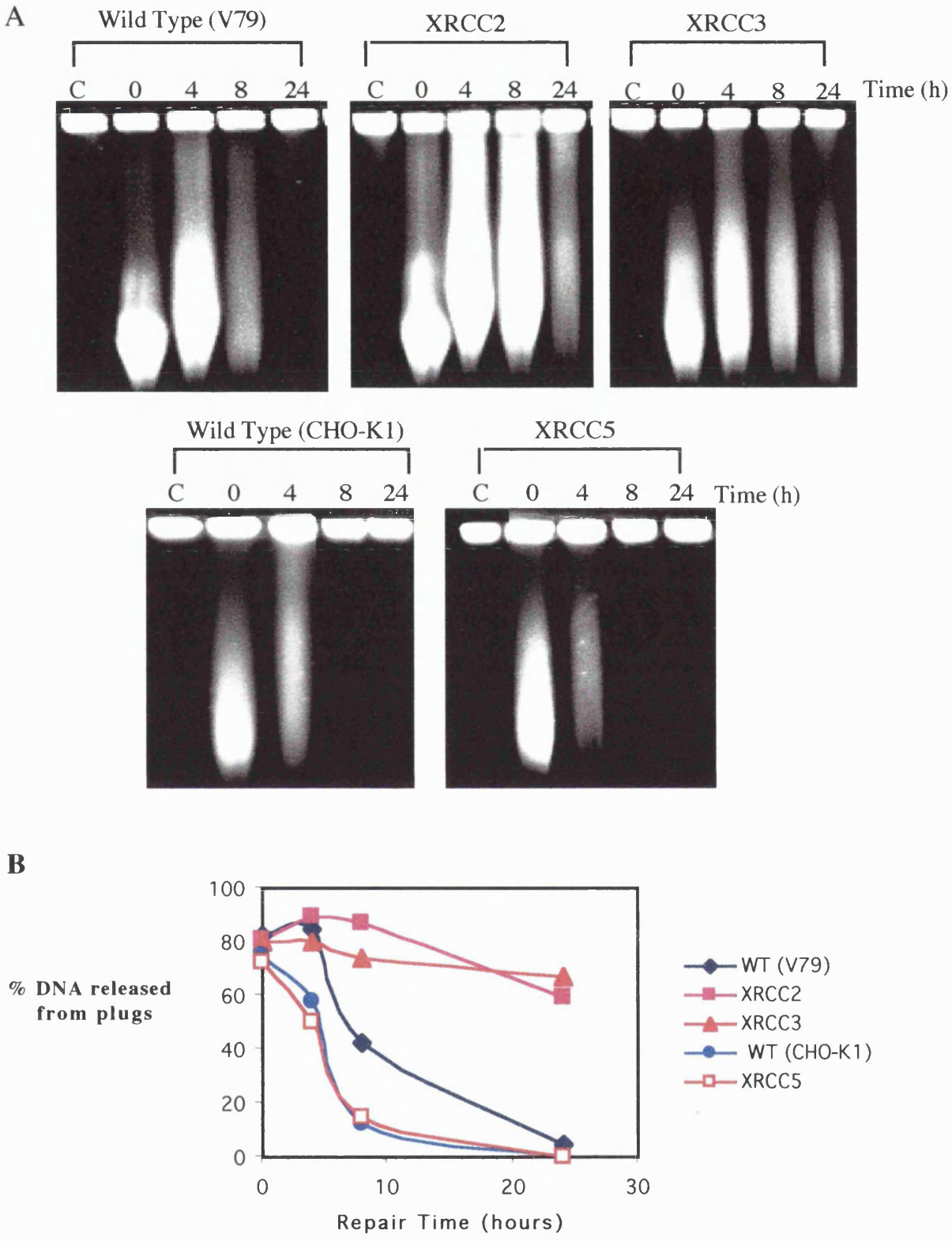
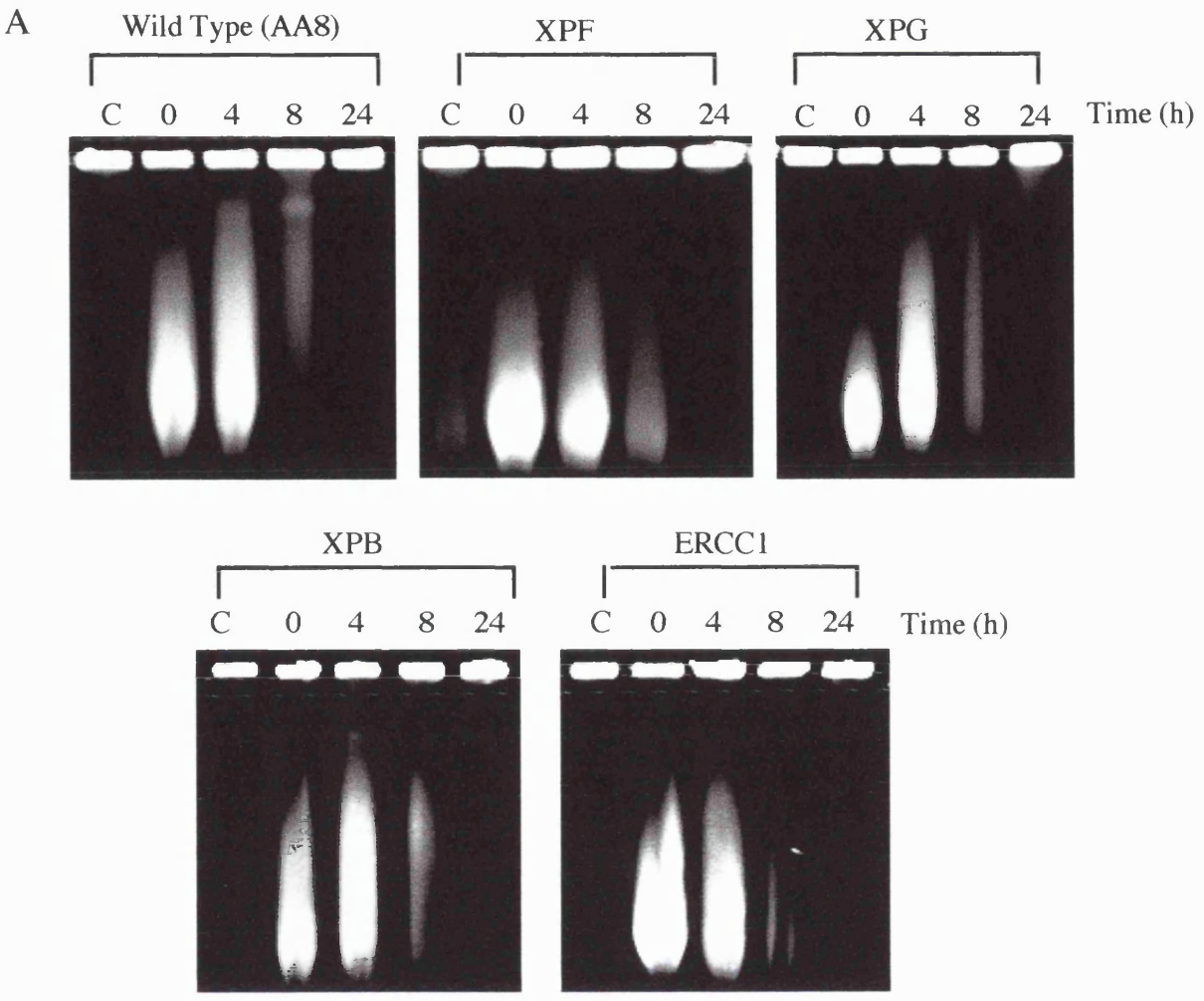


Figure 3.11. A. Induction and repair of DSBs in the V79 wild type cell line and XRCC mutants XRCC2 (irs1), XRCC3 (irs1SF), CHO-K1 parent cell line and XRCC5 (xrs5). Exponentially growing cells were treated with 16 μM HN2 for 1 h and subsequently allowed to repair in fresh medium for 0, 4, 8, and 24 h. Control cells (C) were non-drug treated. Samples were then analysed by PFGE. B. Quantitative analysis of the percentage of DNA released from plugs for the gels shown in A. Results ahown are from a single representative experiment.

occur. Then, the level of DSBs was analysed using PFGE. Immediately following 1 h drug exposure (at 0 h time point), high level of DSBs were observed in all the cell lines (figure 3.11A). These DSBs disappeared with time in the parent cell lines. Semi-quantitative analysis of the gels (figure 3.11B) indicated that within 24 h complete DSB repair had occurred for the parent cell lines. However, the HR mutants XRCC2 and XRCC3 were severely impaired in the repair of these DSBs (figure 3.11). In the XRCC2 and XRCC3 mutants, more than 60% of DNA was still released from the plugs after 24 h, indicating that little repair of DSBs had occurred. These results imply a direct relationship between the extreme sensitivities of XRCC2 and XRCC3 mutants to HN2 and inability to repair DSBs resulting from HN2 exposure. The XRCC5 mutant, defective in the NHEJ pathway, showed DSB repair kinetics indistinguishable from its isogenic parent cell line CHO-K1 (figure 3.11). Consistent with sensitivity data these results suggest that NHEJ is not involved in the repair of DSBs resulting from HN2 exposure. Interestingly, the CHO-K1 parent cell line, consistently repaired DSBs more rapidly than the AA8 and V79 parent cell lines (Figures 3.11).

3.2.5. The NER-independent origin of DSBs resulting from HN2.

It is clear from figure 3.1, and from other studies (Bessho *et al.*, 1997, Anderson *et al.*, 1996, Damia *et al.*, 1996, Hoy *et al.*, 1985), that XPF and ERCC1 are involved in the processing of ICLs. Therefore to investigate whether NER activities are required for the induction of ICL-associated DSBs, the formation of DSBs in NER mutant cells was investigated. Surprisingly, as shown in figures 3.12, all mutants tested, including XPF and ERCC1, induced DSBs indistinguishably from their AA8 parent. These



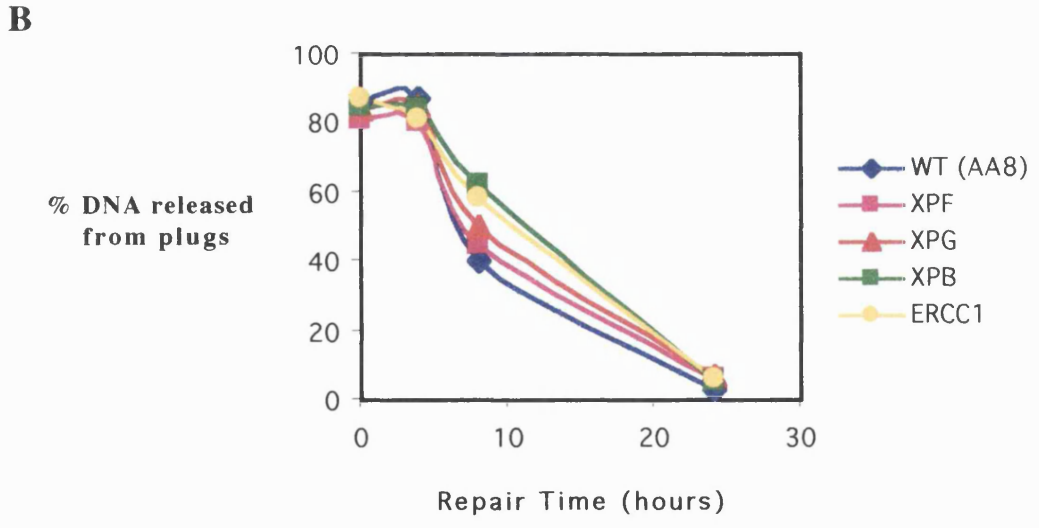


Figure 3.12. A. Induction and repair of DSBs in the AA8 wild type cell line and NER mutants XPF (UV47), XPG (UV135), XPB (UV23), ERCC1 (UV96). Exponentially growing cells were treated with 16 μM HN2 for 1 h and subsequently allowed to repair in fresh medium for 0, 4, 8, and 24 h. Control cells (C) were non-drug treated. Samples were then analysed by PFGE. B. Quantitative analysis of the percentage of DNA released from plugs for the gels shown in A. Results shown are from a single representative experiment.

results indicate that the DSBs are not a result of incision activities associated with NER. It is also evident from figure 3.12 that the repair of DSBs in ERCC1 and XPF is normal. This suggests that the extreme sensitivity of these mutants is entirely the result of a defect in the unhooking step of ICL repair.

3.3. DISCUSSION

The precise mechanism by which mammalian cells eliminate ICLs remains largely unknown, but it is thought that, as in yeast and *E. coli,* both NER and recombination are involved. The results presented here provide evidence for XPF and ERCC1 acting in the incision step, and XRCC2 and XRCC3 in a DSB-repair recombination step, during the elimination of HN2 induced ICLs, but that ERCC1 and XPF are not involved in the recombination arm of repair as has previously been suggested (Sargent *et al.*, 1997, Thompson, 1996, Weeda *et al.*, 1997).

In agreement with previous reports (Anderson *et al.*, 1996, Damia *et al.*, 1996, Hoy *et al.*, 1985) results presented here confirm that CHO cells defective in XPF and ERCC1 are highly sensitive to HN2, but that the mutants XPB, XPD and XPG are only slightly sensitive. The high sensitivity of XPF and ERCC1 mutants is due to an inability to repair ICLs, since these mutants were not highly sensitive to HN1, which do not induced ICLs. Consistent with their extreme sensitivities, XPF and ERCC1 cells were defective in the unhooking of HN2-induced ICLs. In addition to ICLs HN2 induces much more abundant, but less toxic, mono-adducts. The slight sensitivity of XPB,

XPD and XPG to HN2 seems likely to be due to their involvement in the elimination of these lesions since they are clearly proficient in the initiation of cross-link excision.

All NER mutants tested demonstrated a similar level of sensitivity to HN1, suggesting that the whole NER system is required for the elimination of mono-adducts. Indeed, a requirement for these components in the excision of mono-adducts produced by the closely related nitrogen mustard, melphalan has been reported (Grant *et al.*, 1998). As expected, the XRCC mutants, including the highly sensitive XRCC2 and XRCC3 lines, had normal unhooking kinetics. Therefore, of the components examined, XPF and ERCC1 are the only mammalian NER factors required to produce the incisions that release an ICL.

Bessho *et al.*, (1997) have reported that during the repair of psoralen-induced ICLs by CHO cell extracts the NER system excises a 22- to 28-nucleotide oligomer from the 5' side of the cross-link. If such an activity is responsible for removal of cross-links *in vivo* then the XPB, XPD and XPG mutants would be expected to show hypersensitivity to cross-linking agents and an inability to unhook cross-links. Results presented do not favour such an incision activity in the repair of ICLs. The same workers recently demonstrated that the gap generated by removal of this oligomer is filled by a futile DNA synthesis reaction (Mu *et al.*, 2000). In 90% of the cases, this repair patch terminates at a nick adjacent to the cross-link, and in 10% of the cases ligation occurs. In both cases the cross-link remains. They also demonstrated that in the presence of RPA, the XPF-ERCC1 heterodimer could act as a 3' to 5' exonuclease

on cross-linked DNA with high processivity. This exonucleolytic digestion either terminates immediately past the cross-link or can continue to the 5' terminal of the linear duplex substrates completely removing one strand (Mu *et al.*, 2000). This is consistent with our results, since it does not require other factors involved in NER apart from XPF and ERCC1, and it results in the unhooking of ICLs in intact cells.

Previous studies have clearly demonstrated that DSBs are generated at both psoralen and HN2-induced ICLs in dividing yeast cells (Jachymczyk *et al.*, 1981, Magana-Schwencke *et al.*, 1982, McHugh *et al.*, 2000). Results presented here now provide evidence for the dose-dependent induction of DSBs following HN2 treatment of mammalian cells. DSBs were not induced following treatment of cells with equimolar dose of the mono-functional agent HN1, indicating that ICLs are responsible for the induction of DSBs. The ICL-associated DSBs were repaired rapidly with the majority disappearing by 8 h and complete repair at 24 h. These DSBs arise as a result of cellular activity, and are not due to DNA degradation by HN2 since DSBs were not observed when genomic DNA was treated with HN2. DSB induction has also been observed following treatment of cells with other clinically used nitrogen mustard derivatives such as melphalan (discussed in Chapter 5). Therefore DSBs are probably a common intermediate following treatment of dividing cells with the nitrogen mustard family of cross-linking anticancer drugs.

Results obtained with yeast indicate that a necessary role for recombination might be restricted to S phase, since non-dividing haploid yeast cells do not lose significant

viability even when homologous recombination is completely disabled (in *rad52* disruptants) (McHugh *et al.*, 2000). In these quiescent cells the activity of polymerase ζ (REV3 gene) appears to be important for processing ICL intermediates. Therefore it can be suggested that DSBs are not an obligate intermediate in the repair of ICLs, but are nevertheless inevitably generated during the *in vivo* replication of cross-linked DNA.

A possible origin for these DSBs is via incision by NER components, or as a result of the initiation of recombination. However, the normal induction of DSBs in all the repair defective mutants examined make either of these possibilities unlikely. Furthermore, the maximum yield of DSBs was detected immediately after drug treatment when very little ICL repair would have occurred. Our observation that the induction of DSBs following HN2 treatment is more efficient in dividing cells implies that a likely origin of these breaks is the processing of arrested replication forks. This pathway is currently poorly understood in eukaryotes (Cox *et al.*, 2000, Flores-Rosaz and Kolodner, 2000), although these events are well described for *E. coli* (Cox *et al.*, 2000). Finally, there are reports of a novel human chromatin-associated DNA endonuclease complex, which is involved in the repair of DNA ICLs (Lambert *et al.*, 1988, 1992, 1997 and 1999). Although the contribution of such an activity to the repair of ICLs in whole cells is not known, it is conceivable that these factors may play a part in the origin of the DSBs observed following HN2 treatment.

HR is the predominant mechanism by which ICL associated DSBs are repaired in *S. cerevisiae*. Both the *RAD52* and *RAD54* genes are essential for this, as is the activity of Mre11 (McHugh *et al.*, 2000). In this organism a *rad51* disruptant displayed only a partial defect in ICL-associated DSB repair, and the contribution of NHEJ is very minor and can only be detected when *RAD52* is disrupted (McHugh *et al.*, 2000). In mammalian cells, both the NHEJ and homologous recombination pathways play a role in the repair of DSBs. The contribution of the former is well established (reviewed in Pastink *et al.*, 1999), but the importance of the latter is just emerging and it appears that the pathway employed depends on the nature of the DSB substrate. DSBs resulting from ionising radiation treatment are primarily repaired by NHEJ (Pastink and Lohman, 1999), whereas those resulting from I-*Sce*I endonuclease cleavage are repaired by XRCC2 and XRCC3-dependent homologous recombination (Johnson *et al.*, 1999, Pierce *et al.*, 1999).

The identification of mammalian cell lines sensitive to ionising radiation has facilitated the isolation of genes involved in mammalian DSB repair. Out of nine XRCC complementation groups the cell lines in the XRCC4-7 groups are defective in a DNA ligase IV-interacting protein, Ku86, Ku70 and DNA-Pk_{cat} respectively, all components of the NHEJ apparatus (Pastink and Lohman, 1999, Rathmell and Chu, 1998). These are all highly sensitive to ionising radiation and are all defective in the repair of ionising radiation induced-DSBs (Jeggo *et al.*, 1983, Pastink and Lohman., 1999 and Rathmell and Chu, 1998). In agreement with previous reports (Fuller and Painter, 1988, Jones *et al.*, 1987), the results presented in this study confirm that the XRCC2

and XRCC3 mutants are highly sensitive to HN2, but that the NHEJ mutant XRCC5 is only slightly sensitive. Consistent with these observations, the XRCC2 and XRCC3 mutants showed impaired repair of DSBs resulting from HN2 exposure, whereas the XRCC5 mutant repaired these DSBs efficiently. When XRCC mutants were treated with high doses of HN1, these mutants exhibited a similar pattern of sensitivity to that of HN2. XRCC2 and XRCC3 mutants were highly sensitive to HN1, and the XRCC5 mutant was only slightly sensitive. This high sensitivity of XRCC2 and XRCC3 mutants to HN1 was surprising, since previous studies have described XRCC2 and XRCC3 mutants as being highly sensitive to bifunctional cross-linking agents but not to monofunctional agents (Caldecott *et al.*, 1991). However, since DSBs are induced at high doses of HN1, the sensitivities of XRCC2 and XRCC3 mutants could be due to inability to repair these lesions.

The XRCC2 and XRCC3 mutants did not show a complete defect in the repair of HN2 induced DSBs, suggesting that there is redundancy in the repair of different types of DSBs. In this respect, the roles played by XRCC2 and XRCC3 in homologous recombination are currently only partly understood. Both XRCC2 and XRCC3 show limited homology to human Rad51 (Liu *et al.*, 1998). They are both involved in maintaining chromosome stability during cell division (Cui *et al.*, 1999) and it has been shown that mammalian Rad51 induces the formation of sub-nuclear foci in response to DNA damage by ionising radiation, UV irradiation, methylmethanesulphate and cisplatin (Bishop *et al.*, 1998, Haaf *et al.*, 1995). Interestingly, in the absence of XRCC3, Rad51 foci are not formed suggesting XRCC3

plays a role in stabilisation of Rad51 and these foci during DNA repair (Bishop *et al.*, 1998), and two hybrid studies have also shown that Rad51 protein and XRCC3 protein interact with one another directly (Liu et al., 1998). Collectively these findings suggest that XRCC2 and XRCC3 may act as accessory factors for Rad51 in homologous recombination.

The presence of several Rad51-like genes in mammals (reviewed in Thacker *et al.*, 1999) suggests the presence of sub-pathways of recombination which involve different sets of protein factors. Human and mouse Rad52 show only 30% identity with the *S. cerevisiae* factor, and studies in mouse and chicken cells have shown that the phenotype of RAD52 deficient vertebrate cells clearly differs from that of *S. cerevisiae* (Thompson, 1999). Human and mouse Rad54 are about 50% identical to *S. cerevisiae* Rad54 and their function is conserved in mammalian cells (Thompson, 1999). In comparison to XRCC2 and XRCC3 mutants, *rad54*$^{-/-}$ mouse cells show only a small degree of sensitivity to MMC and no UV sensitivity (Essers *et al.*, 1997). This again suggests the existence of sub-pathways of mammalian homologous recombination, and different proteins may contribute differentially in the repair of various types of DNA lesions including cross-links.

Results presented in this chapter showed that XPF and ERCC1 are required for the unhooking of HN2 induced ICLs, but other NER factors are not required. Data also showed that DSBs are induced in mammalian cells following treatment with HN2 in a NER independent mechanism, and that homologous recombination, but not the NHEJ pathway resolves these DSBs.

CHAPTER 4: REPAIR OF INTRA- AND INTERSTRAND CROSS-LINKS INDUCED BY CISPLATIN

4.1. INTRODUCTION

In the previous Chapter the repair of ICLs induced by the bifunctional alkylating agent HN2 was discussed. This chapter focuses on the repair of DNA lesions induced by the bifunctional platinum agent, cisplatin. As described in Chapter 1, cisplatin forms a variety of DNA adducts, including DNA mono-adducts, inter- and intra-strand cross-links and DNA protein cross-links. About 90% of the cisplatin adducts are 1,2-intra-strand cross-links, and ICLs and mono-adducts occur only at very low frequency (1-2%) (Fichtinger-Schepman *et al.*, 1985). Although cisplatin has been used in cancer chemotherapy for many years, the major cytotoxic lesion induced by this drug is still not clear. Different studies have put forward the 1,2 intra-strand cross-links (Chu *et al.*, 1994) and ICLs (Zwelling *et al.*, 1979) as candidates for the major cytotoxic lesion.

Since cisplatin induces various types DNA adducts, *in vivo* analysis of repair of individual types of cisplatin lesions has been difficult. Repair of individual cisplatin DNA adducts has been studied *in vitro* using oligonucleotides containing site specifically modified cisplatin adduct, and cell extracts or purified repair proteins (Hansson *et al.*, 1989, Evans *et al.*, 1997a & 1997b, Zamble *et al.*, 1996). These studies have demonstrated that three major cisplatin adducts, 1,2-d(GpG), 1,2-d(ApG), and 1,3d(GpXpG) intra-strand cross-links are incised by NER (Zamble *et al.*, 1996).

Previous studies have shown that CHO cell lines defective in the XPF and ERCC1 components of the NER machinery are highly sensitive to cisplatin (Hoy *et al.*, 1985, Damia *et al.*, 1996). These studies have suggested that the extreme sensitivity of XPF and ERCC1 mutants are because the XPF and ERCC1 proteins play a role in recombination in addition to NER.

This study aimed to investigate whether the difference in sensitivity of NER mutants to cisplatin is due to a difference in their ability to repair cisplatin induced ICLs or intra-strand cross-links. To confirm previously reported data, the sensitivity of mutants to cisplatin was studied using the SRB cytotoxicity assay. Induction and repair of cisplatin-induced ICLs in parent and NER mutants was analysed employing the modified comet assay. It has been suggested that as a result of arrest of replication forks at the sites of cisplatin-DNA lesions, DSBs may arise as secondary DNA damage, and these lesions are subsequently repaired by the recombination pathway (Zdraveski *et al.*, 2000). In agreement with this suggestion, XRCC2 and XRCC3 mutant cell lines defective in the homologous recombination pathway are highly sensitive to cisplatin (Caldecott and Jeggo, 1991). However, to date, the occurrence of DSBs following cisplatin treatment has not been experimentally proven. Therefore, induction of DSB following cisplatin-induced DNA damage was investigated using PFGE. To confirm previously reported data, the cytotoxicity of cisplatin in the XRCC2 and XRCC3 homologous recombination mutants together with the XRCC5 mutant defective in NHEJ pathway was measured and their role in the processing of DSB was investigated.

4.2. RESULTS

4.2.1. Cisplatin sensitivity of NER and recombination defective cell lines

The cytotoxicity of cisplatin in NER and recombination mutants was compared with their isogenic parent cell lines using the SRB assay. Exponentially growing cells were treated with varying concentrations of cisplatin for 1 hour, and incubated in drug-free medium for three days before SRB staining. The fraction of growth inhibition was then calculated for each drug concentration as described in the Materials and Methods.

As shown in figure 4.1, the UV47 and UV96 cell lines, defective in XPF and ERCC1 respectively, showed extreme sensitivity to cisplatin compared to their isogenic parent cell line AA8. When IC50 values were compared, XPF and ERCC1 mutants were 40 and 37 fold more sensitive to cisplatin than the parent cell line respectively, indicating the importance of these two factors in the repair of cisplatin induced DNA damage. In contrast, the XPB and XPD helicase mutants were only 1.4 and 1.3 fold more sensitive than the parent cell line, respectively. This suggests a very minor role of these proteins in the repair of cisplatin induced DNA damage. The XPG mutant showed a higher sensitivity (3.1-fold) than the parent cell line indicating a possible role for this protein in the repair of cisplatin adducts. Interestingly, the transcriptional coupling repair mutant CSB, showed 5.2 fold higher sensitivity than the parent cell line. This suggests that repair of cisplatin adducts in transcriptionally active regions of the genome significantly contribute to the cytotoxicity of cisplatin.

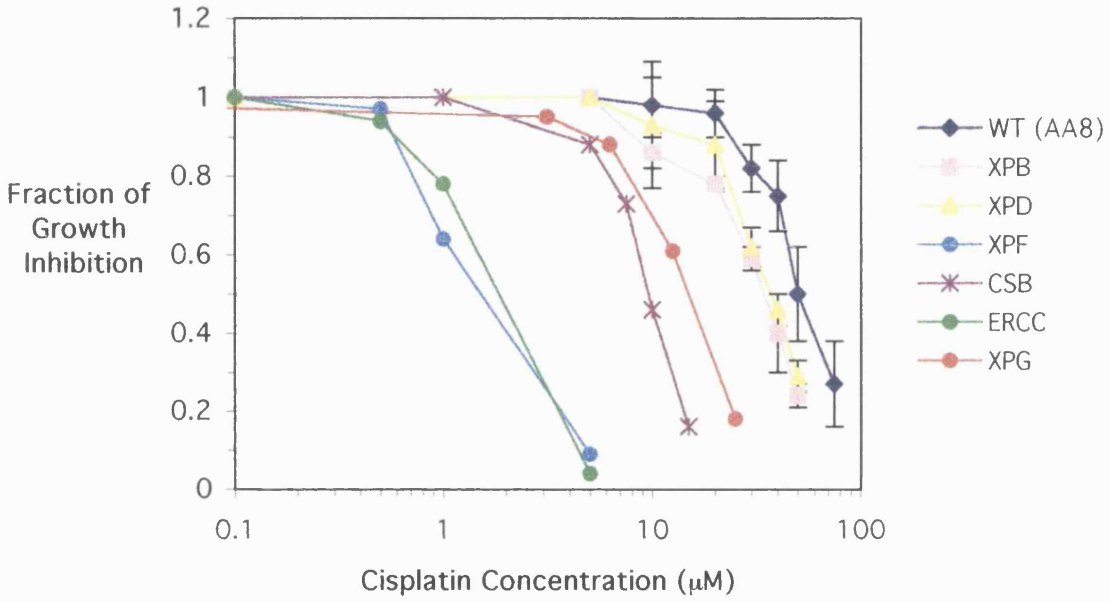


Figure 4.1. Cytotoxicity of cisplatin in AA8 wild type cells and in NER mutants; XPB (UV23), XPD (UV42), XPF (UV47), CSB (UV61), ERCC1 (UV96) and XPG (UV135). Exponentially growing cells were treated with increasing concentrations of cisplatin for 1 h, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition was calculated for each dose as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

It has been suggested that recombination also plays an important role in the repair of cisplatin DNA lesions. To investigate this, cisplatin cytotoxicity was studied in the HR mutants irs1 and irs1SF, defective in the XRCC2 and XRCC3 genes, respectively, and in a NHEJ mutant xrs5, defective in the XRCC5 gene. The sensitivity of these mutants to cisplatin was compared with their isogenic parent cell lines. Irs1 and irs1SF cell lines are derived from V79 and AA8 cell lines, respectively, and the xrs5 cell line is derived from CHO-K1. The cytotoxicity of cisplatin in these mutants together with their parent cell lines is shown in figure 4.2. Confirming previously published data (Caldecott and Jeggo, 1991), the XRCC2 and XRCC3 mutants showed extreme sensitivity to cisplatin, being 50 and 38 fold more sensitive compared to their parent cell lines. These results suggest that XRCC2 and XRCC3 HR factors are extremely important in the repair of cisplatin DNA damage in addition to XPF and ERCC1 proteins discussed above. In contrast, the NHEJ mutant xrs5 showed only a slight increase in sensitivity (1.7 fold) compared to the parent cell line, suggesting the involvement of the NHEJ pathway in the repair of cisplatin DNA damage is very minor.

4.2.2 Induction and repair of cisplatin induced ICLs

ICLs are believed to be the major cytotoxic lesion induced by many bifunctional DNA damaging agents. However, in the case of cisplatin, whether ICL is the major cytotoxic lesion still remains to be determined. Positive (Zwelling *et al.*, 1979), as well as negative (Fichtinger-Schepman *et al.*, 1995) results have been reported in this regard. These lesions comprise only a minor adduct (~1%) formed by cisplatin (Fichtinger-

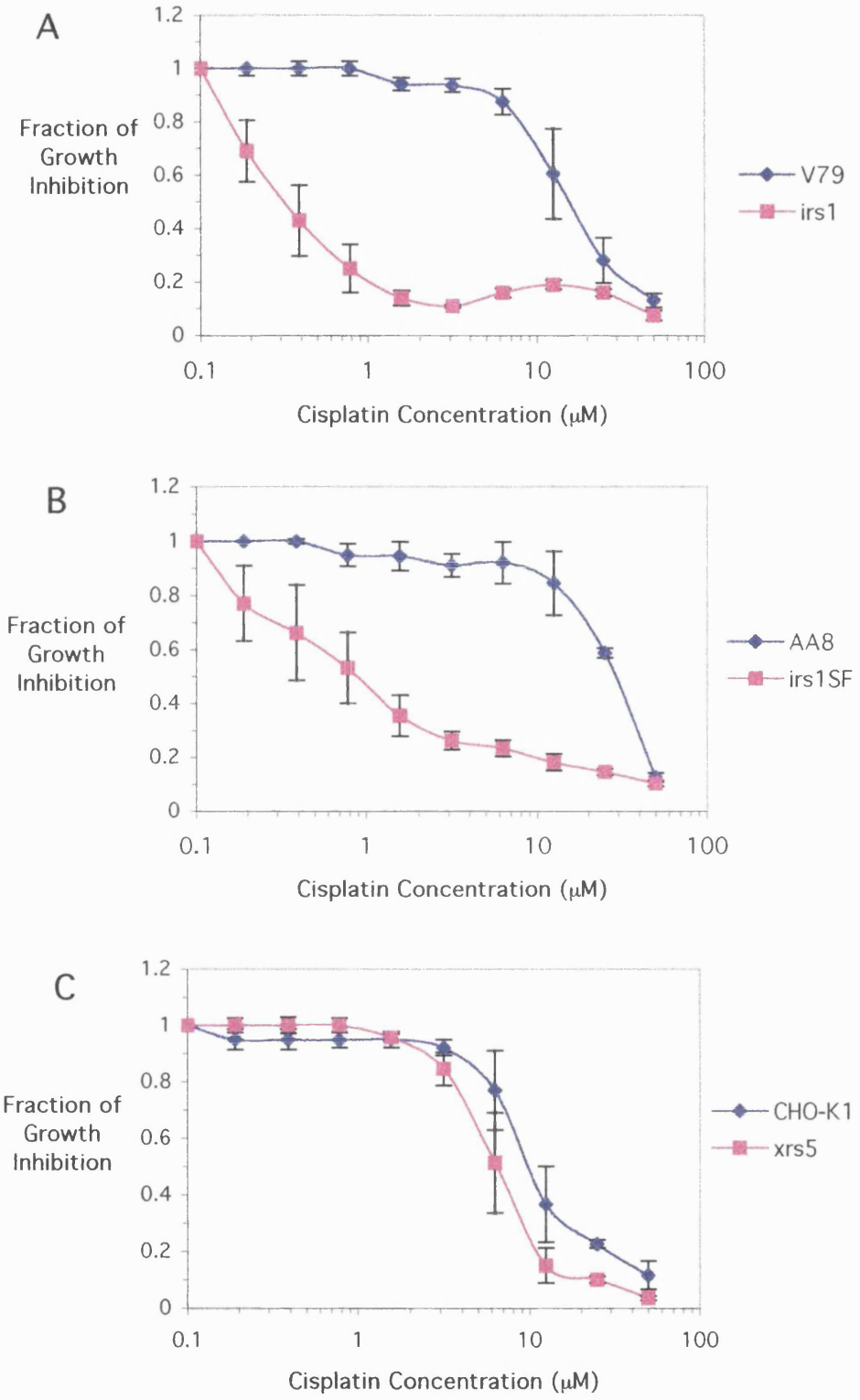


Figure 4.2. Cytotoxicity of cisplatin in HR mutants, A. XRCC2 (irs1) and the wild type cell line V79, B. XRCC3 (irs1SF) and wild type AA8 cell line, and C. NHEJ mutant XRCC5 (xrs5) and the wild type cell line CHO-K1. Cells were treated with increasing concentrations of cisplatin for 1 hour, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition for each dose was calculated as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

Schepman *et al.*, 1985), and the mechanism by which they are repaired is poorly understood. Using the modified comet assay, which permits detection of the level of ICLs at the single cell level, the induction and repair of cisplatin ICLs were measured.

Figure 4.3 shows comet images of AA8 cells treated with increasing doses of cisplatin for 1 hour and analysed immediately. No background strand breaks were observed in control unirradiated sample. The irradiated control sample, which received 12.5 Gy X-irradiation showed single-strand breaks, and quantitative analysis indicated an average mean tail moment of 12 μm. As evident from figure 4.3, this tail moment decreased with increasing concentration of cisplatin indicating a dose dependent induction of ICLs. Figure 4.4 shows the percentage decrease in tail moment (calculated as described in the Materials and Methods) with increasing concentrations of cisplatin. A decrease in tail moment occurs linearly up to 30 μM dose and then a plateau is observed at higher doses. No single-strand breaks were seen in un-irradiated samples treated with increasing doses of cisplatin suggesting that single-strand breaks are not induced by cisplatin (data not shown).

The repair kinetics of cisplatin ICLs was studied in the AA8 parent cell line. A dose of 50 μM was chosen for these experiments since this is the concentration which gives approximately 50% inhibition of growth in AA8 cells and it induces an easily detectable level of ICLs (~40% decrease in tail moment). Exponentially growing AA8 cells were treated with 50 μM cisplatin for 1 h, incubated in drug free medium for different time periods (post incubation time), and the level of ICLs determined. The

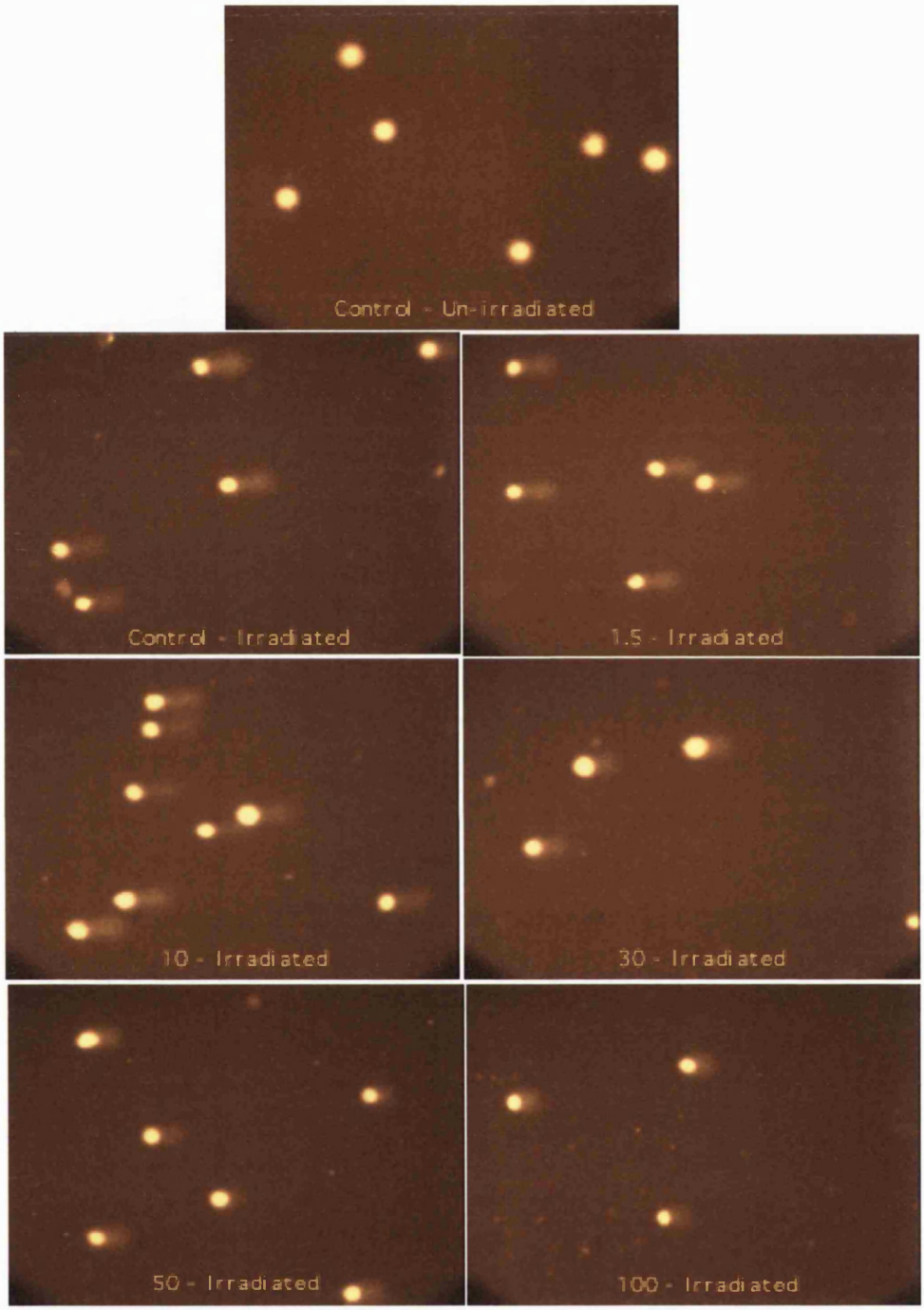


Figure 4.3. Comet images of AA8 cells treated with increasing concentrations of cisplatin for 1 h and analysed immediately. The concentrations stated above are in μM. Irradiated samples received 12.5 Gy dose of X-irradiation to induce single strand breaks.

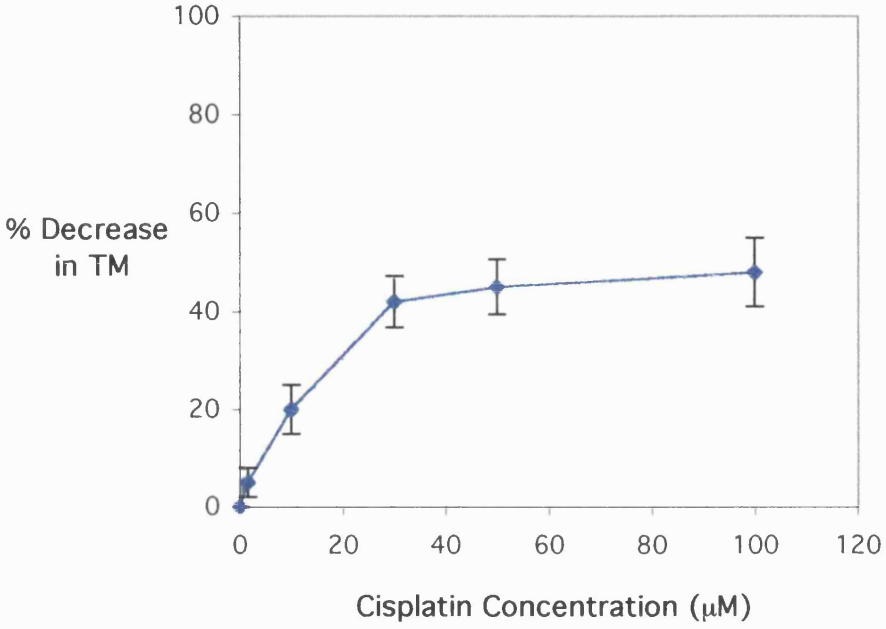
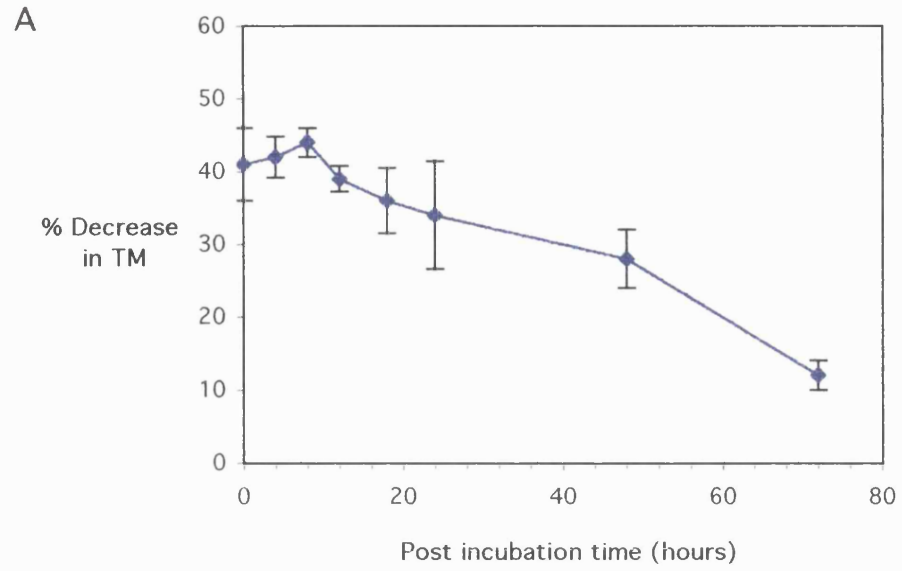


Figure 4.4. Quantitative data of decrease in TM with increasing concentration of cisplatin. AA8 cells were treated with increasing doses of cisplatin for 1 h and analyzed immediately using the comet assay. For each drug concentration, the TM of 50 comets was measured using Kinetic Imaging Komet Software and mean values were calculated.The percentage decrease in TM compared to untreated samples was then calculated as described in Materials and Methods. Results shown above are mean of three independent experiments, and error bars show the standard error of the mean

results are shown in figure 4.5A. The peak of ICLs was observed following 8 h of post incubation time. However, the level of cross-linking at this time point was not considerably different from the time of drug removal (44% decrease in tail moment at 8 h compared to 41% at 0 h). The decrease in tail moment then declined with time indicating repair of ICLs. The percentage repair of ICL over time, compared to the peak of ICL observed at 8 h post incubation time is shown in figure 4.5B. Following 24 h post incubation, 22% of the ICLs were removed, and at 72 h, 70% repair was observed.

4.2.3. Induction and repair of ICLs in mutant cell lines defective in XPB, XPF, XPG and ERCC1

To determine whether the difference in sensitivity to cisplatin displayed by NER mutants is due to differences in the level of induction of ICLs, or to subsequent repair of these lesions, induction and repair of ICLs following treatment with cisplatin was assessed in the NER mutant cell lines. Induction and repair kinetics of the highly sensitive XPF and ERCC1 mutants, and the moderately sensitive XPB and XPG mutants were compared with that of the AA8 parent cell line. All cell lines were treated with 50 μM cisplatin for 1 h, and the level of ICLs was assessed following 0, 24, 48 and 72 h of post incubation period in drug free medium to allow repair. The results are shown in figure 4.6. A similar level of ICL induction was seen in all cell lines studied, suggesting that difference in sensitivity is not due to difference in induction of ICL. Consistent with their extreme sensitivities, the XPF and ERCC1 mutants were defective in the unhooking step or incision of cisplatin-induced ICLs.



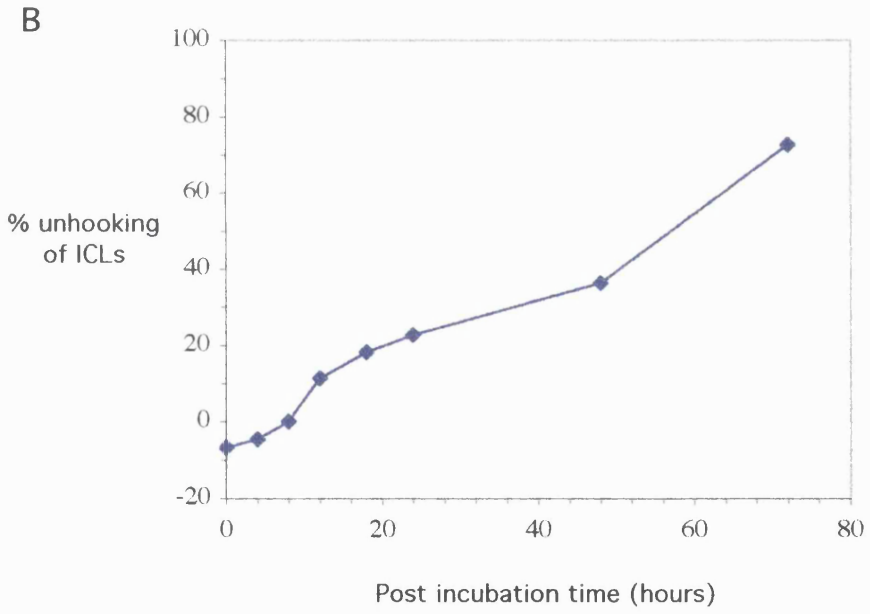


Figure 4.5. Kinetics of formation and repair of cisplatin-induced ICLs. AA8 cells were treated with 50 μM cisplatin for 1 h, incubated in drug free medium and the level of ICLs were analysed at different time points using the modified comet assay. A. Percentage decrease in TM compared to irradiated (12.5 Gy) untreated samples was calculated from 50 comets using Kinetic Imaging Komet software as described in Materials and Methods. Results presented are mean of three independent experiments, and error bars show the standard error of the mean. B. Percentage unhooking of ICLs compared to the peak level of ICLs seen at 8 h was calculated from data shown in A.

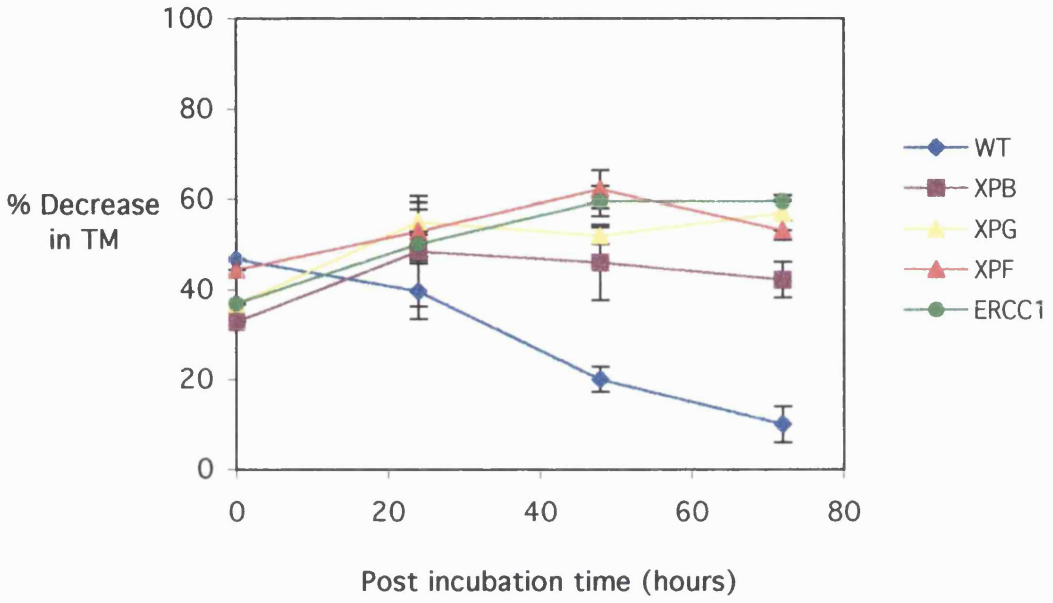


Figure 4.6. Induction and repair of cisplatin-induced ICLs in parent (AA8) and NER mutant cell lines; XPB (UV23), XPG (UV135), XPF (UV47) and ERCC1 (UV96). Cells were treated with 50 μM cisplatin for 1 h, and incubated in drug free medium for 24, 48, and 72 h to allow repair, and analyzed for ICLs using the modified comet assay. The percentage decrease in TM compared to irradiated (12.5 Gy) untreated samples was calculated from 50 comets using Kinetic Imaging Komet software as described in Materials and Methods. Results presented are mean of at least three independent experiments, and error bars show the standard error of the mean.

Surprisingly, the moderately sensitive XPB and XPG mutants were also deficient in unhooking of ICLs. These results indicate no clear correlation between cytotoxicity of cisplatin and ICL repair kinetics and suggest that the different level of sensitivities of NER mutants to cisplatin is not solely due to a difference in their ability to process ICLs.

4.2.4. Induction and repair of cisplatin intra-strand cross-links

Since no correlation was observed between cytotoxicity of cisplatin and repair of ICLs in NER mutants, induction and repair of intra-strand cisplatin adducts were also investigated in the parent and NER mutant cell lines. These adducts were measured using a competitive ELISA method developed by Tilby *et al.*, (1991) which specifically recognise cisplatin intra-strand cross-links. This technique is discussed in detail in the Materials and Methods (section 2.4). In summary, the sample to be assayed is mixed with a small amount of antibody which recognise cisplatin intra-strand cross-links. This mixture is then placed in an ELISA well that is coated with a standard, uniform amount of platinated DNA. The amount of antibody that binds to coated platinated DNA is inversely related to the amount of cisplatin adducts in the sample, which acts as a competing antigen.

The assay involves the serial dilution of the DNA sample to be measured. Each dilution is then treated as a separate sample and the mean fluorescence intensity for each dilution is plotted against the dilution factor. A curve can then be fitted to the set of points to calculate the dilution of the sample, which gives 50% inhibition of the

assay signal. Using a known amount of platinated DNA as the standard curve, the amount of cisplatin intra-strand adducts in the sample can then be calculated.

Figure 4.7 shows the standard curve. As indicated on the curve, 50% inhibition of the maximum fluorescence was observed at 2.5 fmol/ml cisplatin adduct concentration. This value was used to calculate the adduct levels in cisplatin treated cells. As shown in figure 4.8, when the AA8 parent cell line was treated with an increasing doses of cisplatin for 1 hour, a linear increase in the induction of cisplatin intra-strand adducts was observed.

In subsequent experiments to study the repair of these adducts, a 50 μM dose, the IC50 value of cisplatin in AA8 parent cell line was used which gave a measurable level of adducts (9.5 fmol/μg DNA). The repair kinetics of cisplatin intra-strand cross-links in the AA8 parent cell line is shown in figure 4.9. Between 0 - 4 h following drug removal, a slight increase in adduct level was observed which was followed by a time dependent repair. At 24, and 48 h following drug removal 64% and 74% of the adducts were repaired, respectively.

Figure 4.10 shows the repair of cisplatin intra-strand adducts in NER mutants compared to that of the parent cell line. A higher level of intra-strand adducts were observed in the CSB, XPG and XPF mutants immediately after the 1 h treatment. In addition, the CSB and XPG mutants were highly defective in the elimination of these adducts. The XPF mutant also showed a higher level of intra-strand adducts compared

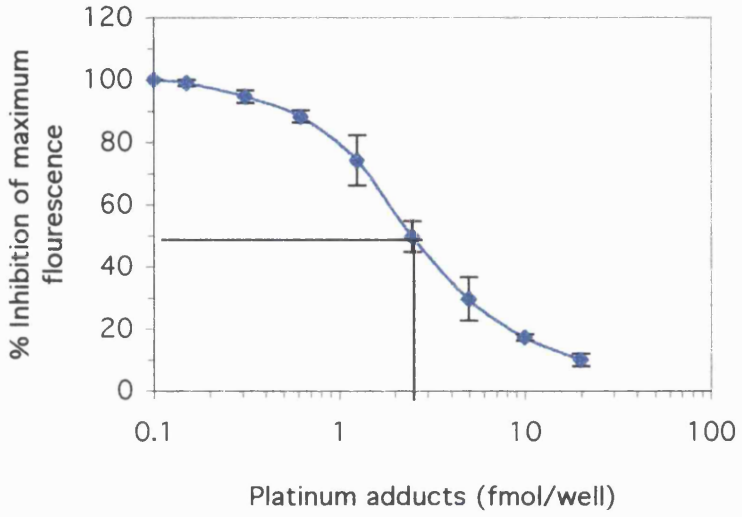


Figure 4.7. ELISA standard curve. Native calf thymus DNA (Lot 333) containing a known amount of cisplatin adducts was serially diluted, pre incubated with a constant amount of ICR4 antibody and assayed by competitive ELISA. The percentage inhibition of maximum flourescence compared to a control sample which was not pre-incubated with antibody was calculated and plotted against the platinum adduct concentration. 50% inhibition was observed at a cisplain adduct level of 2.5 fmol/well. Results are the mean of three independent experiments and error bars shows the standard error of the mean.

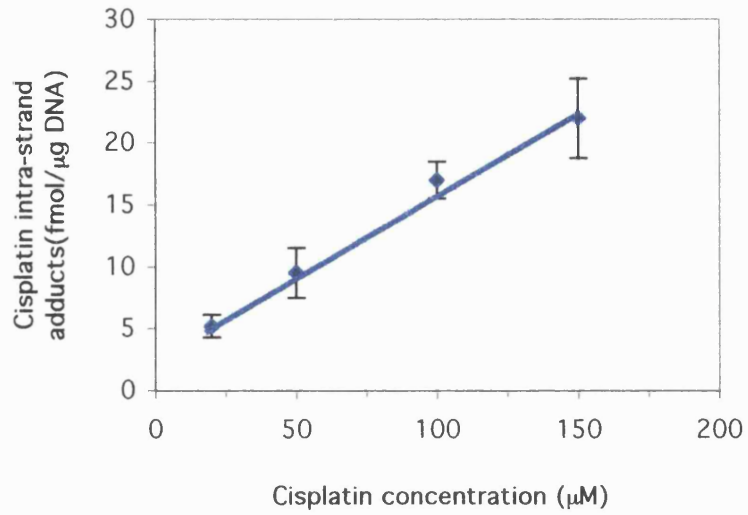


Figure 4.8. Induction of cisplatin intra-strand cross-links following treatment of AA8 cells with increasing doses of cisplatin. Exponentially growing AA8 cells were treated with cisplatin for 1 hour and the level of intra-strand adducts was analysed immediately using competitive ELISA.Results are the mean of 3 independent experiments, and the error bars show the standard error of the mean.

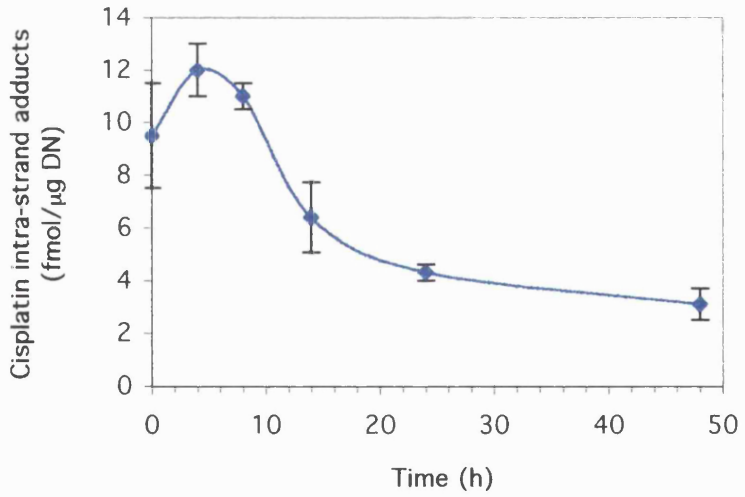


Figure 4.9. Repair kinetics of cisplatin intra-strand adducts in the AA8 parent cell line. Exponentially growing AA8 cells were treated with 50 µM cisplatin for 1 hour, incubated in drug free medium for various time points and the level of intra-strand adducts quantified using competitive ELISA. Results are the mean of 5 independent experiments, and the error bars show the standard error of the mean.

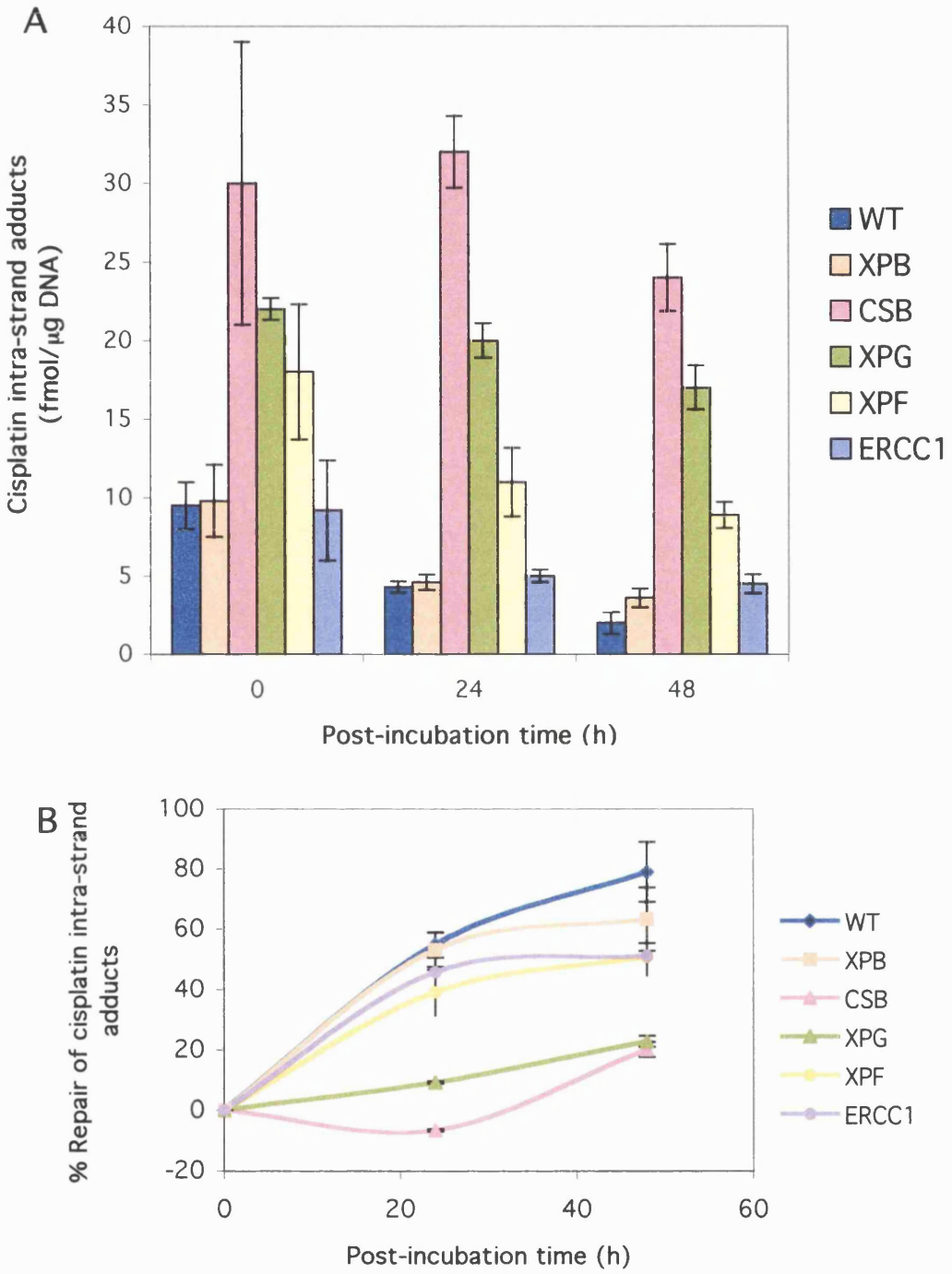


Figure 4.10. Induction and repair of cisplatin intra-strand cross-links. **A**. The level of cisplatin intra-strand cross-links in AA8 parent cell line and in NER mutants ; XPB (UV23), CSB (UV61), XPG (UV135), XPF (UV47), and ERCC1 (UV96). Exponentially growing cells were treated with 50 µM cisplatin for 1 hour and adduct levels were quanified immediately following drug removal (0 h), 24 h and 48 h using competitive ELISA. Results presented are mean of 3 - 5 individual experiments and error bars shows the standard error of the mean. **B**. Results shown in A presented as percentage repair.

to the parent cell line, but this was less pronounced than for the CSB and XPG mutants (fig. 4.10A). At 48 h following drug removal, CSB, XPG and XPF mutants contained 12, 8.5 and 4.4 times more adducts, respectively, than the parent cell line (fig. 4.10A). Figure 4.10B shows the percentage repair of these adducts compared to the adduct level seen following 1 h drug exposure. The data indicate that CSB and XPG mutants are highly deficient in the repair of intra-strand cross-links, but XPF, ERCC1, and XPB mutants were only slightly defective in the repair.

4.2.5. Induction of DSB as an intermediate in the repair of cisplatin ICLs

It has been clearly demonstrated in *S. cerevisiae* that the repair of ICLs, involves the formation of double-strand breaks (Magana-Schwencke *et al.*, 1982, Jachymczyk *et al.*, 1981). Recent studies in this laboratory (McHugh *et al.*, 2000) demonstrated the induction of DSBs in yeast during the processing of HN2-induced DNA damage. Similarly, in chapter 3, it was clearly demonstrated that DSBs arise also in mammalian cells during processing of HN2 induced DNA damage. Recent studies in *E. coli* (Zdraveski *et al.*, 2000) have also suggested that DSBs may occur in the repair of cisplatin induced DNA damage. However, despite these suggestions, the induction of DSBs during the processing of cisplatin-induced DNA damage has not been investigated in mammalian cells to date. The possible induction of DSBs following cisplatin treatment was therefore examined in CHO cells using the PFGE method.

Exponentially growing AA8 cells were treated with increasing doses of cisplatin for 1 h, and analysed for DSBs by PFGE as described in Materials and Methods. As shown

in figure 4.11A, no DSBs were observed even following a 150 μM treatment. To investigate whether DSBs arise over later times, AA8 cells were treated with 100 μM cisplatin for 1 h, and DSBs were monitored over a 24 h period. As shown in figure 4.11B, no DSBs were observed at any of the time points analysed.

Studies with nitrogen mustard (Chapter 3) showed that the XRCC2 and XRCC3 HR mutants were highly sensitive to HN2, and this correlated with an impaired ability to repair HN2-induced DSBs. XRCC2 and XRCC3 mutants are also extremely sensitive to cisplatin. If the level of induction of DSBs by cisplatin is very low, and if they are readily repaired, it is possible that the PFGE method utilised is not sensitive enough to detect these DSBs. If the high sensitivity of XRCC2 and XRCC3 mutants to cisplatin is due to impaired repair of these minute levels of DSBs, these mutants might accumulate DSBs following cisplatin treatment. To investigate this possibility, the induction of DSBs was examined in the XRCC2 and XRCC3 mutants over a 24 h time period, following 1 h treatment with 100 μM cisplatin. As evident from figure 4.12 these experiments failed to demonstrate the occurrence of any DSBs. These results strongly suggest that DSBs do not occur in the processing of cisplatin-induced DNA damage.

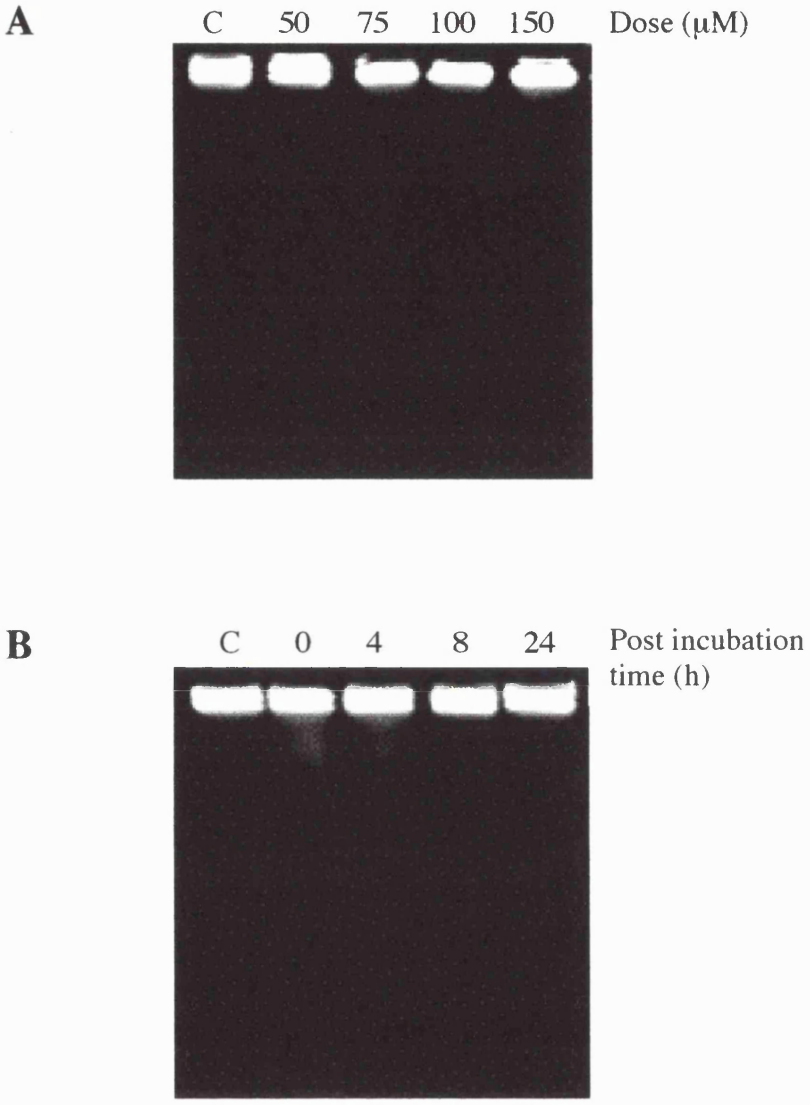


Figure 4.11. Analysis of induction of DSBs following treatment with cisplatin using PFGE method. A. Exponentially growing AA8 cells were treated with increasing concentrations of cisplatin for 1 h and immediately analysed for the presence of DSBs. B. Induction of DSBs with time. Exponentially growing AA8 cells were treated with 100 μM cisplatin for 1 hour, and incubated in drug free medium. Induction of DSBs was analysed at different time points.

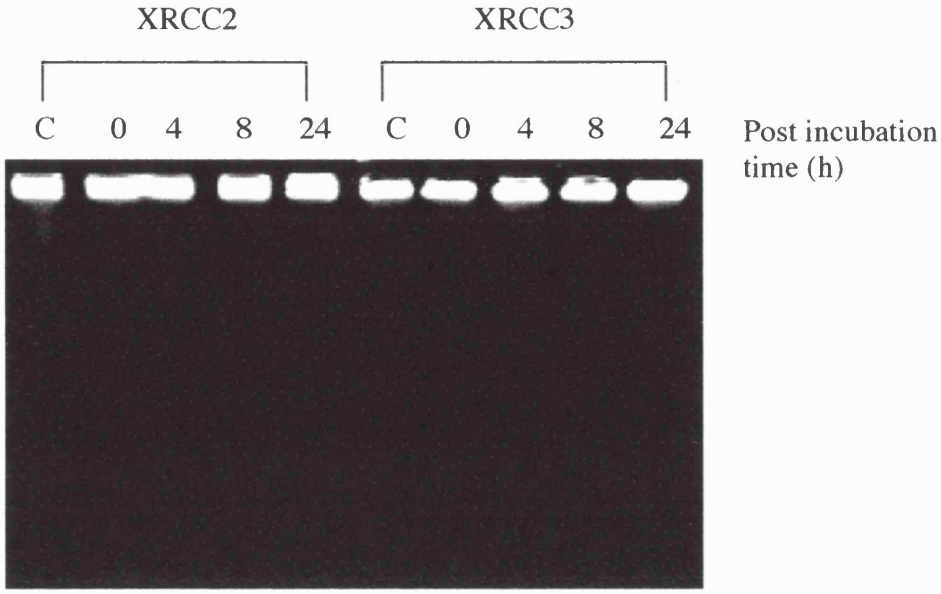


Figure 4.12. Analysis of DSBs in XRCC2 (irs1) and XRCC3 (irs1SF) mutant cell lines following treatment with cisplatin. Exponentially growing cells were treated with 100 μM cisplatin for 1 h and incubated in drug free medium. Induction of DSBs was analysed at the time points stated above.

4.3. DISCUSSION

It is believed that both the NER and recombination repair pathways are required for the elimination of cisplatin DNA adducts. However, the exact mechanism by which these pathways mediate repair still remains to be elaborated. In this study, a panel of CHO cell lines defective in NER, HR and NHEJ components were used to elucidate the role of these two pathways in the repair of cisplatin-induced DNA damage.

In agreement with previous reports (Hoy *et al.*, 1985, Damia *et al.*, 1996), the results obtained in this study confirmed that CHO cells defective in XPF and ERCC1 proteins which produce the 5' incision of DNA lesions during NER, are highly sensitive to cisplatin. In contrast, the other mutants defective in XPB, XPD, XPG and CSB which are also required to complete the NER process do not show extreme sensitivity. This suggests that the XPF and ERCC1 structure-specific nucleases plays a critical role in the repair of the major cytotoxic lesions induced by cisplatin, and that the other proteins involved in NER are less important.

As described earlier, cisplatin forms a variety of DNA lesions. Although it has been successfully used as an anti-cancer agent for many years, it is still unclear, which of the various cisplatin adducts formed in cellular DNA ultimately lead to cell death. Various studies have put forward d(GpG) 1,2-intra-strand (Chu *et al.*, 1994), d(ApG) 1,2-intra-strand d(ApG) (Fichtinger-schepman *et al.*, 1995) and ICLs (Zwelling *et al.*, 1979) as candidates for the major cytotoxic lesion.

Although the *trans* isomer of cisplatin, transplatin has no anti-tumour activity, it also binds to DNA but forms a spectrum of DNA-adducts distinct from that of cisplatin. Therefore, many studies have addressed the origin of the biological differences between cisplatin and transplatin, in an attempt to elucidate the mechanism of action of cisplatin. Using alkaline elution, Zwelling *et al.*, (1979) compared the level of interstrand cross-links produced by these two agents. Their results showed that a much higher concentration of transplatin than cisplatin was required to produce the same level of ICLs and cytotoxicity in L1210 cells. These studies lead to the suggestion that the ICL is the major cytotoxic lesion. However, the results presented in this study do not favour this suggestion. Both the highly sensitive XPF and ERCC1 mutants, and the slightly sensitive XPB and XPG mutants were unable to unhook ICLs, indicating that all these components are required for the removal of ICLs. No correlation was observed between cytotoxicity and either induction or subsequent repair of ICLs. It was surprising that, although the XPB and XPG mutants were completely defective in the unhooking step of ICL repair, they were only 1.4 and 3.1 fold more sensitive to cisplatin than the parent cell line, compared to 37-40 fold increase in sensitivity observed in XPF and ERCC1 mutants. A similar study by Meyn *et al.*, (1982) also showed that another ERCC1 mutant CHO cell line, UV20, was extremely sensitive to cisplatin and defective in the repair of ICLs, suggesting a direct correlation between cytotoxicity and ICL repair. However, the ICL repair ability of the less sensitive XPB and XPG cell lines was not investigated in this study. Results shown here demonstrate for the first time that the XPB and XPG components of the NER machinery are also essential for the unhooking of cisplatin ICLs *in vivo*.

These results are distinct from those obtained with HN2, where a correlation between the cytotoxicity and the ability to unhook ICLs was shown (Chapter 3). Although the slightly sensitive XPB and XPG mutant lines were proficient in the unhooking of HN2-induced ICLs, results presented here show that they are deficient in the unhooking of cisplatin induced ICLs. These results indicate that cisplatin induced ICLs are repaired by a very different mechanism to those induced by HN2. Previous studies have shown that ICLs comprise only a minority (~1%) of the adducts formed by cisplatin (Fichtinger-Schepman *et al.*, 1985). In agreement, the results presented in this study showed that the level of ICLs induced by cisplatin is much lower than that induced by an equimolar dose of HN2 (chapter 3). Following 1 h treatment of cells with 16 μM HN2 (IC50 value of HN2), nearly 90% decrease in tail moment was observed with the modified comet assay, compared to less than 30% decrease in tail moment seen with the same dose of cisplatin. When cells were treated with the IC50 dose of cisplatin (50 μM) for 1 h, only 45% decrease in tail moment was observed. Collectively, data presented here suggest that ICLs are not the major cytotoxic lesion induced by cisplatin.

If the d(GpG) 1,2-intra-strand adduct is the major cytotoxic lesion induced by cisplatin, according to cytotoxic data, XPB, XPD proteins cannot be expected to play a significant role in the repair of this lesion, since these mutants were only slightly more sensitive to cisplatin than the parent cell line. However, *in vitro* analysis of incision of d(GpG) 1,2-intra-strand cross-links by cell extracts and purified proteins have shown that both XPB and XPD are required for the incision of this adduct (Moggs *et al.*,

1997). These studies have been carried out in oligonucleotides containing site specific intra-strand cisplatin adducts, and the *in vitro* incision activities observed might be different to the repair processes occur in genomic DNA *in vivo*. To address these questions, the ability of highly sensitive and less sensitive NER mutants to remove d(GpG) 1,2-intra-strand cross-links were measured *in vivo* using a competitive ELISA technique. Results showed that CSB and XPG mutants were highly defective in the repair of these lesions. Interestingly, cytotoxicity data showed that a CSB mutant, defective in TCR, and XPG mutant defective in 3' incision activity are more sensitive to cisplatin than XPB and XPD mutants defective in helicase activity. CSB and XPG mutants were 5.5 and 3.1 fold more sensitive to cisplatin, respectively, compared to less than 1.5 fold higher sensitivity observed in XPB and XPD mutants. In agreement with the sensitivity data, the CSB mutant was more defective than the XPG mutant in the repair of intra-strand cross-links. These results suggest that the higher sensitivity of CSB and XPG mutants to cisplatin could be due to impaired repair of cisplatin intra-strand cross-links.

An interesting observation was that the XPF mutant, which showed a 40 fold higher sensitivity to cisplatin than the parent cell line, was defective in the repair of intra-strand cross-links, but the ERCC1 mutant which also showed 37-fold higher sensitivity was proficient. This was an unexpected result since the XPF and ERCC1 proteins make a heterodimer and are responsible for the 5' incision activity during the NER process (Brookman *et al.*, 1996, Sijbers *et al.*, 1996a). The fact that only the XPF mutant showed impaired repair, suggests that in the case of cisplatin intra-strand cross-

link repair, only the XPF subunit is required for the 5' incision activity. Supporting this suggestion, biochemical characterization of XPF protein has shown that XPF possesses endonuclease activity in the absence of ERCC1 protein (McCutchen-Maloney *et al.*, 1999). These results further suggest that the extreme sensitivity of XPF and ERCC1 mutants to cisplatin cannot be due to an inability to process intra-strand cross-links. Although defective removal of cisplatin intra-strand cross-links leads to about 5.5 fold higher sensitivity, the data do not suggest that the intra-strand cross-link is the sole cytotoxic lesion induced by cisplatin. It is possible that the cytotoxicty of cisplatin is not due to a single type of lesion, but due to an additive effect of many DNA and protein lesions and other cellular effects.

The results presented suggest that a considerable proportion of intra-strand cisplatin cross-links are preferentially repaired in the active regions of the genome, since the CSB mutant was defective in the repair of these lesions. However, the CSB mutant did not show an increase in sensitivity to other bifunctional agents studied, which include HN2 (Chapter 3), melphalan and SJG-136 (Chapter 5). Other studies have shown that CSB mutant cells are also sensitive to UV-irradiation (Vreeswijk *et al.*, 1998). Analysis of the two major UV induced photolesions, cyclobutane pyrimidine dimers (CPD) and pyrimidine 6-4 pyrimidone photoproducts (6-4 PP), have shown that TCR plays a role in the removal of CPD lesions from transcribed strands, but not 6-4-PP lesions (Vreeswijk *et al.*, 1998). Another study has demonstrated that TCR does not contribute to the repair of DNA lesions induced by N-acetoxy-2-acetylaminofluorene (NA-AAF) (van Oosterwijk *et al.*, 1996). These data suggest that TCR can only

remove certain types of lesions from transcribed strands. Therefore, the increased sensitivity of the CSB mutant to cisplatin, but not to the other agents tested must be due to the role of TCR in the removal of critical cisplatin induced DNA lesions but not HN2, melphalan or SJG-136 induced DNA lesions.

The XPG mutant also demonstrated greater sensitivity to cisplatin than to HN2, and was defective in the removal of intra-strand cisplatin cross-links, suggesting that both CSB and XPG may act together in the repair of cisplatin intra-strand cross-links. Interestingly, *in vitro* protein-protein interaction studies have shown that the XPG protein interacts with the TFIIH and CSB proteins (Iyer *et al.*, 1996). It is therefore possible that XPG may play a role in TCR. Therefore increased sensitivity of the XPG mutant to cisplatin might be a result of impaired TCR in these cells. To test this hypothesis it will be necessary to analyse the repair of cisplatin DNA adducts in both transcribed and non-transcribed strands in XPG and CSB mutants, possibly using single strand ligation (sslig) PCR (Grimaldi *et al.*, 1994).

The results presented indicate that in wild type cells, the induction of cisplatin ICLs continues during the 8 h period following drug removal and then the level of ICLs slowly decreases. The continuos formation of ICLs following drug removal is presumably due to conversion of cisplatin mono-adducts formed during drug exposure into ICLs (Fichtinger-Schepman *et al.*, 1995). After 8 h, the level of ICL in the parent cell line decreased with time, but in the mutant cell lines, it further increased. The difference between maximum cross-linking between parent and the NER mutant cell

lines can be explained by assuming that the repair process is active in AA8 cells starting from time 0 h, and can remove monoadducts as well as ICLs. Therefore two competing reactions occur in the parent cell line, one involving formation of ICLs from monoadducts and the second, involving repair of mono-adducts and ICLs. Since, no repair occurs in the mutant cell lines, more cross-links can be detected in mutant cell lines. Previous studies in L1210 mouse leukaemia cells have shown that the peak of cisplatin ICLs occur at 12 h after drug removal and at time 0 h, the ICLs are nearly undetectable (Zwelling *et al.*, 1979). The results presented here, show different kinetics in CHO cell lines. Considerable number of ICLs are detected at time 0 h and the peak level of ICLs at 8 h was only slightly higher than at 0 h. Factors including differences in cellular uptake of cisplatin, and efficiency of removal of adducts may account for the differences in kinetics of ICL formation seen in two types of cells.

Many studies have suggested that DSBs may arise as intermediates in the processing of ICLs. The results presented in chapter 3 confirmed that DSBs occur as a result of cellular activities during the processing of HN2-induced DNA damage in mammalian cells. It was shown that, consistent with the extreme sensitivities of XRCC2 and XRCC3 mutants to HN2, these mutants displayed impaired repair of HN2 induced DSBs. Confirming previously published data (Caldecott and Jeggo., 1991), the results shown here demonstrate that the XRCC2 and XRCC3 mutants are also hypersensitive to cisplatin. This suggest that homologous recombination plays a major role in the repair of cisplatin induced DNA damage. A recent study by Zdraveski *et al.*, (2000) demonstrated that *E. coli* mutants defective in daughter-strand gap and double-strand

break recombination pathways are hypersensitive to cisplatin, providing evidence that DSBs may arise in the repair of cisplatin ICLs. However, surprisingly, no DSB formation was observed in the processing of cisplatin induced DNA damage. DSBs were not detected even in the highly sensitive XRCC2 and XRCC3 mutant cell lines. These results imply that cisplatin ICLs are processed without the formation of a DSB intermediate. However, the homologous recombination pathway is required in the processing of cisplatin-induced DNA damage, as XRCC2 and XRCC3 mutants are extremely sensitive to cisplatin.

If the induction of DSBs following HN2 treatment is due to arrest of replication forks, the results presented here suggest that DNA adducts induced by cisplatin do not arrest replication forks. Interestingly, previous studies have shown that mammalian cells possess the ability to replicate DNA containing cisplatin adducts, suggesting that some DNA polymerases are capable of replicating Pt-containing DNA. DNA polymerase β has been suggested as a candidate, which can perform extensive bypass of Pt-DNA adducts *in vitro* (Vaisman *et al.*, 2000). Recent studies have demonstrated that DNA polymerase η, which is defective in XPV patients, can also bypass cisplatin intra-strand cross-links efficiently and with high fidelity (Masutani *et al.*, 2000). These studies therefore indicate that the absence of DSBs observed following cisplatin treatment might be due to the presence of DNA polymerases which can bypass cisplatin adducts. To date, replication bypass of HN2-induced DNA adducts has not been reported. Therefore, it is possible that the same polymerases that can bypass cisplatin lesions are not capable of bypassing HN2-induced DNA lesions.

Although cisplatin ICLs are processed without a DSB intermediate, the HR pathway is still required in the processing of cisplatin induced DNA damage, as the XRCC2 and XRCC3 mutants are extremely sensitive to cisplatin. Two other emerging HR components, BRCA1 and BRCA2, which also belong to the Rad51-related family of proteins, have also been implicated in the repair of cisplatin-induced DNA damage (Bhattacharyya *et al.*, 2000, Yuan *et al.*, 1999). Mutations in these genes render cells hypersensitive to cisplatin. Immuno-fluorescence studies have shown that BRCA1 promotes assembly of subnuclear Rad51 foci following treatment of mouse ES cells with cisplatin (Bhattacharyya *et al.*, 2000). In addition, another recently generated Rad51 family mutant, Rad51B(-/-), derived from chicken DT40 cells has also shown hypersensitivity to cisplatin (Takata *et al.*, 2000). Collectively, these studies suggest that the HR pathway is extremely important in the repair of cisplatin-induced DNA repair. Since DSBs are not induced following DNA damage by cisplatin, the HR pathway must be acting on cisplatin repair intermediates other than DSBs. The exact mechanism by which HR pathway process cisplatin lesions still remains to be discovered. These emerging HR factors may interact with each other and with XRCC2 and XRCC3 proteins in these processes. The sensitivity of the NHEJ mutant xrs5 to cisplatin was only slightly higher than that of the parent cells line. Therefore, the role of NHEJ in the repair of cisplatin appears to be minor.

In summary, the results presented in this study confirmed that XPF and ERCC1 proteins, which play a role in NER, and XRCC2 and XRCC3 proteins involved in homologous recombination are extremely important in the repair of the major

cytotoxic lesions induced by cisplatin. No correlation between cytotoxicity to cisplatin and ability to repair of ICLs was observed suggesting that ICLs are not the most cytotoxic lesion induced by cisplatin. In addition to XPF and ERCC1, the XPB and XPG proteins are also essential in the unhooking of ICLs induced by cisplatin, in contrast to what is seen with HN2. This suggest that different types of DNA adducts are dealt with by different mechanisms. In support of this, these results suggest that the processing of cisplatin lesions involves TCR, but HN2 rely less on this repair machinery. This study also demonstrated that DSBs do not occur in the processing of cisplatin-induced DNA damage. Since, HR is required to overcome cisplatin cytotoxicity, this repair pathway must be acting on cisplatin DNA lesions other than DSBs. Collectively, the data presented demonstrate that the mechanism of repair of cisplatin-induced DNA damage is significantly different to that of HN2.

CHAPTER 5: COMPARISON OF REPAIR OF DNA LESIONS INDUCED BY MONOFUNCTIONAL AND BIFUNCTIONAL, MAJOR AND MINOR GROOVE BINDING AGENTS.

5.1. INTRODUCTION

In the previous two Chapters, the repair of DNA adducts induced by the major groove binding alkylating agent HN2 and major groove binding platinum agent cisplatin was discussed. Recent studies have focused attention on the DNA minor groove as a target for alkylation. Novel anticancer drugs have been developed recently which bind exclusively in the minor groove of DNA in a sequence selective manner. These include the pyrrolobenzodiazepine (PBD) based compounds, DSB-120 and SJG-136, which were discussed in detail in Chapter 1.

In this Chapter, the repair of monofunctional and bifunctional, major and minor groove DNA alkylating agents are compared. Two major groove binding bifunctional alkylating agents HN2 and melphalan, and their monofunctional counterparts (mono-HN2 and mono-melphalan, respectively) were compared with the novel minor groove binding bifunctional drug SJG-136 and its monofunctional analogue MMY.

The availability of monofunctional analogues permits the study of the cytotoxic effects of ICLs in comparison to monoadducts. It also allows the elucidation of different mechanisms involved in the repair of ICLs and monoadducts. In this section, the

cytotoxicity of bifunctional vs. monofunctional, and major groove vs. minor groove agents is compared. Using a panel of CHO mutants defective in NER, HR and NHEJ pathways, the repair mechanisms involved in the elimination of ICLs and mono-adducts in the major and minor groove of DNA was studied.

5.2. RESULTS

5.2.1. Cytotoxicity of monofunctional and bifunctional, major and minor groove alkylating agents in AA8 parent CHO cell line

The cytotoxicity of mono- and bifunctional, major and minor groove agents was assessed in AA8 cells (wild type) following a 1 h drug exposure using the SRB assay. Figure 5.1 illustrates the IC50 values of each drug. As expected, both major and minor groove bifunctional agents demonstrated higher cytotoxicity than their monofunctional counterparts. The IC50 values of HN2, melphalan and SJG 136 were 190-, 4-, and 40-fold lower than their monomeric analogues respectively. These results indicate that some DNA adducts induced by the three bifunctional agents are more cytotoxic than the monoadducts induced by the monofunctional agents.

The novel minor groove binding bifunctional agent SJG-136 was far more cytotoxic than the bifunctional major groove binding agents. When IC50 values are compared, SJG-136 is 172, 53 and 110 times more cytotoxic than cisplatin, HN2 and melphalan, respectively. Similarly, the monofunctional counterpart of SJG-136, MMY also showed higher cytotoxicity than the major groove monofunctional agents. The level of

cytotoxicty of MMY was comparable with bifunctional HN2, and was higher than cisplatin and melphalan. These results imply that DNA mono-adducts in the minor groove are much more cytotoxic than DNA mono-adducts and ICLs in the major groove.

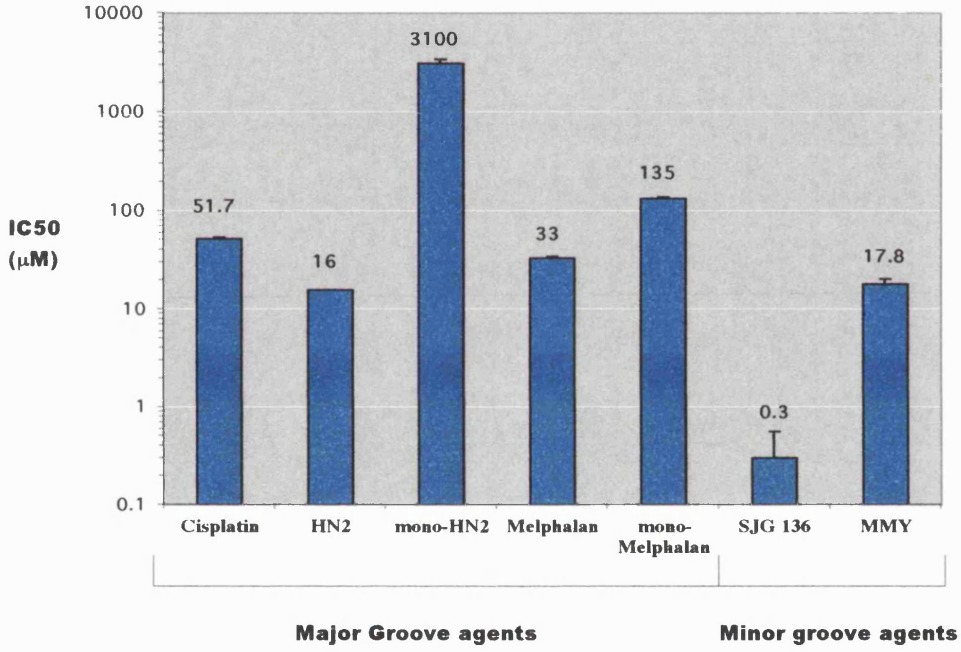


Figure 5.1. The cytotoxicity of mono- and bifunctional, major and minor groove agents in the AA8 parent cell line. Cells were treated with increasing doses of the drug for 1 h, incubated with fresh medium for 3 days, stained with SRB and the IC50 values were determined. The error bars show the standard error of the mean calculated from 3 independent experiments. The numbers show the IC50 value in μM calculated as described in Materials and Methods.

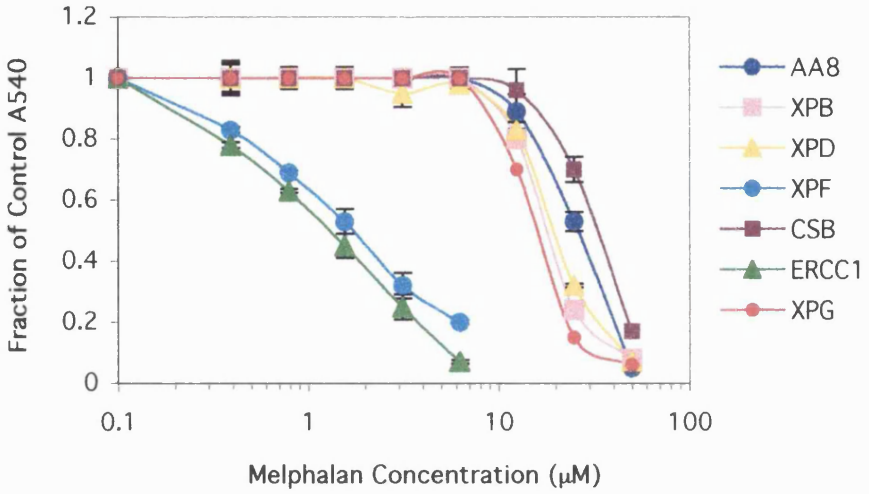
5.2.2. Sensitivity of NER defective CHO cell lines to major and minor groove mono- and bifunctional agents

The role of NER, HR and NHEJ in the repair of HN2 and cisplatin induced DNA damage was discussed in Chapters 3 and 4. The sensitivity of NER and recombination mutants to melphalan, mono-melphalan, SJG-136 and MMY are discussed in the following sections.

The sensitivity of NER mutants to melphalan is shown in figure 5.2A. The pattern of sensitivity of these mutants to melphalan is very similar to that observed for HN2 (Chapter 3). XPF and ERCC1 mutants were highly sensitive showing a relative sensitivity of 15 and 21, respectively, compared to the parent cell line. In contrast, the NER mutants defective in XPB, XPD and XPG were only 1.3 - 1.5 fold more sensitive than the parent cell line. Interestingly, the CSB mutant did not show increased sensitivity to melphalan, but showed resistant compared to the parent cell line, which was also observed with HN2 (figure 3.1, Chapter 3). All NER mutants tested, including XPF and ERCC1, showed only a slightly higher sensitivity (2-3 fold) to mono-melphalan (figure 5.2B).

XPF and ERCC1 mutants were also highly sensitive (7.5 fold higher sensitivity) to the minor groove cross-linker SJG-136 (figure 5.3A). However, the level of sensitivity of XPF and ERCC1 mutant cells to SJG-136 was less than what was observed with other major groove binding bifunctional agents tested in this study. The other NER mutants were only slightly more sensitive than the parent cell line. As for mono-melphalan, all

A



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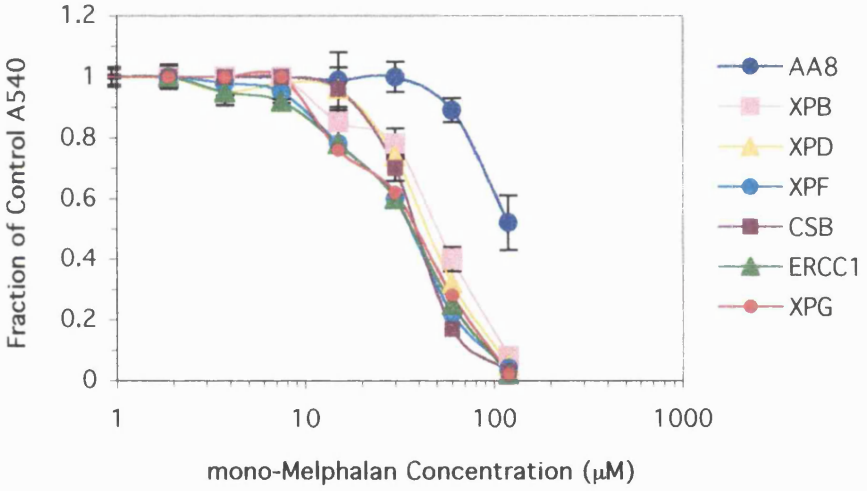

Figure 5.2. Cytotoxicity of melphalan (A) and mono-melphalan (B) in AA8 wild type cells and in NER mutants; XPB (UV23), XPD (UV42), XPF (UV47), CSB (UV61), ERCC1 (UV96) and XPG (UV135). Exponentially growing cells were treated with increasing concentrations of the drug for 1 h, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition was calculated for each dose as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

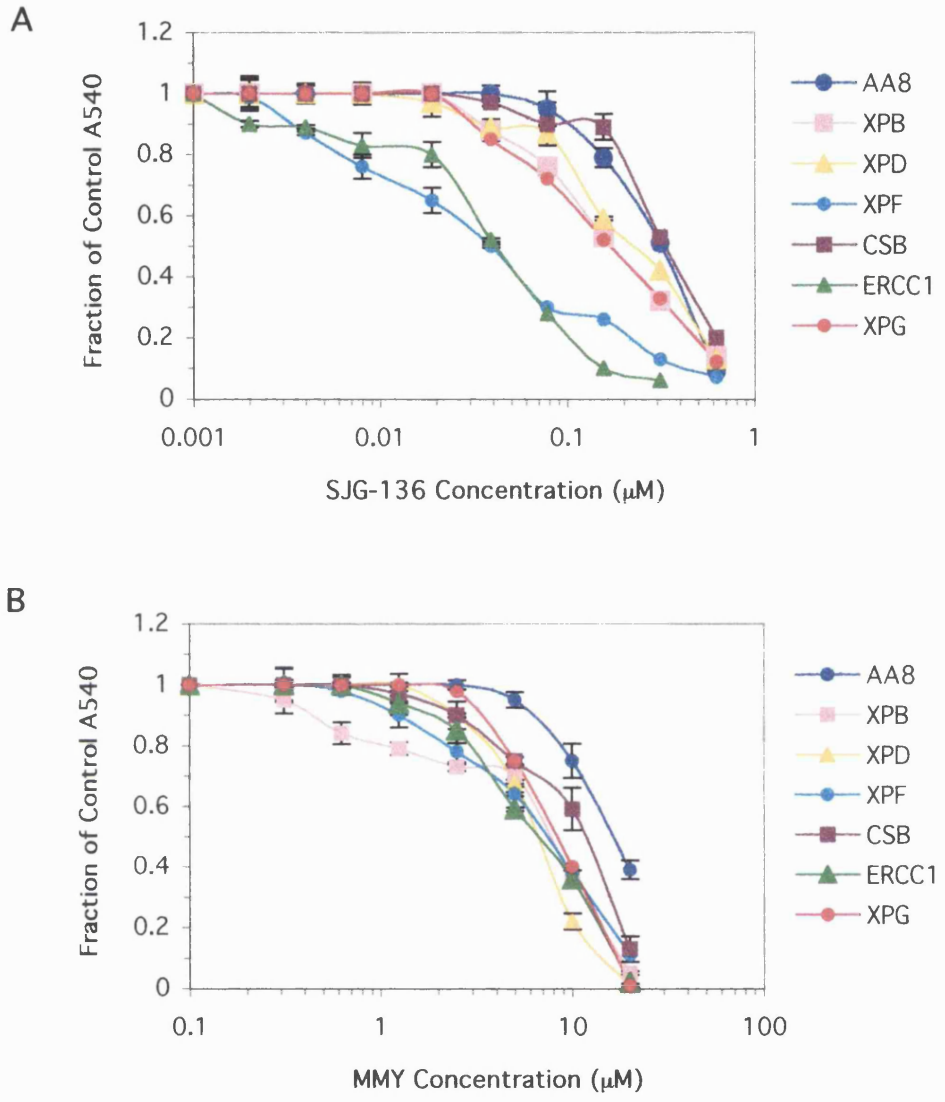

Figure 5.3. Cytotoxicity of SJG-136 (A) and MMY (B) in AA8 wild type cells and in NER mutants; XPB (UV23), XPD (UV42), XPF (UV47), CSB (UV61), ERCC1 (UV96) and XPG (UV135). Exponentially growing cells were treated with increasing concentrations of the drug for 1 h, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition was calculated for each dose as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

NER mutants demonstrated only a slightly higher sensitivity (1.6 - 3 fold) to the monofunctional version of SJG-136, MMY (figure 5.3B).

These results demonstrate that all the NER factors examined play a role in the repair of mono-adducts induced by major and minor groove mono-functional agents, and the repair of ICLs in both major and minor groove of DNA require a mechanism involving XPF and ERCC1.

5.2.3. Sensitivity of recombination defective CHO cell lines to major and minor groove mono- and bifunctional agents

As presented in figure 5.4A and B, both the XRCC2 and XRCC3 mutants were highly sensitive to melphalan. An 18 and 17 fold higher sensitivity was observed, respectively, compared to their parent cell lines. The NHEJ mutant, XRCC5 was only 2.1 fold more sensitive to melphalan than its parent cell line CHO-K1 (figure 5.4C).

Interestingly, XRCC2 and XRCC3 mutants were also highly sensitive to mono-melphalan (figure 5.5A and 5.5B), showing a 17 and 13 fold greater sensitivity than their parent cell line. The XRCC5 mutant was only 2.7 fold more sensitive than its parent cell line.

Both the XRCC2 and XRCC3 homologous recombination mutants also showed higher sensitivity to SJG-136 (figure 5.6A and 5.6B). However, when IC50 values were compared, the XRCC2 mutant demonstrated a two fold higher difference in sensitivity

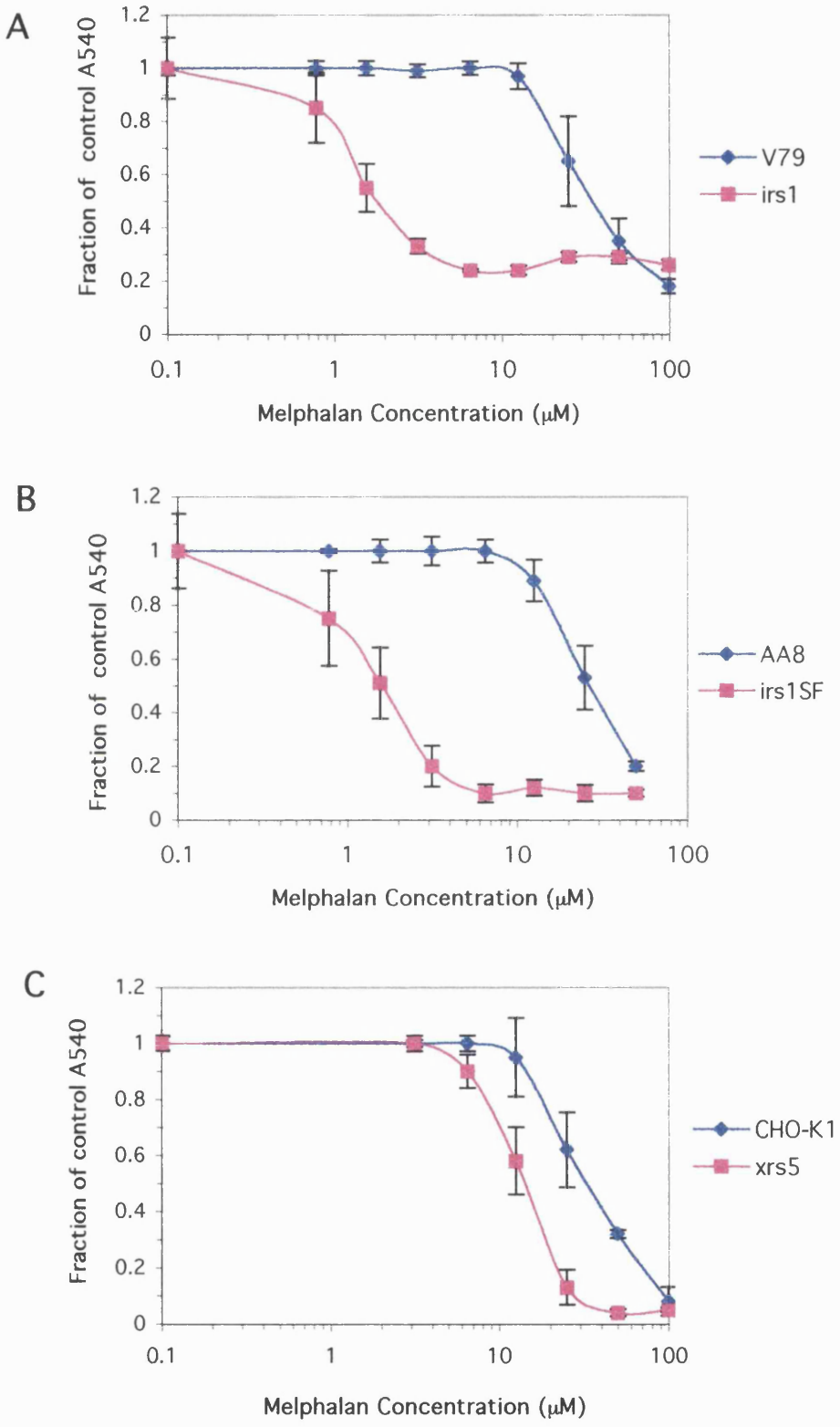

Figure 5.4. Cytotoxicity of melphalan in HR mutants, A. XRCC2 (irs1) and the wild type cell line V79, B. XRCC3 (irs1SF) and wild type AA8 cell line, and C. NHEJ mutant, XRCC5 (xrs5) and the wild type cell line CHO-K1. Cells were treated with increasing concentrations of melphalan for 1 hour, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition for each dose was calculated as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

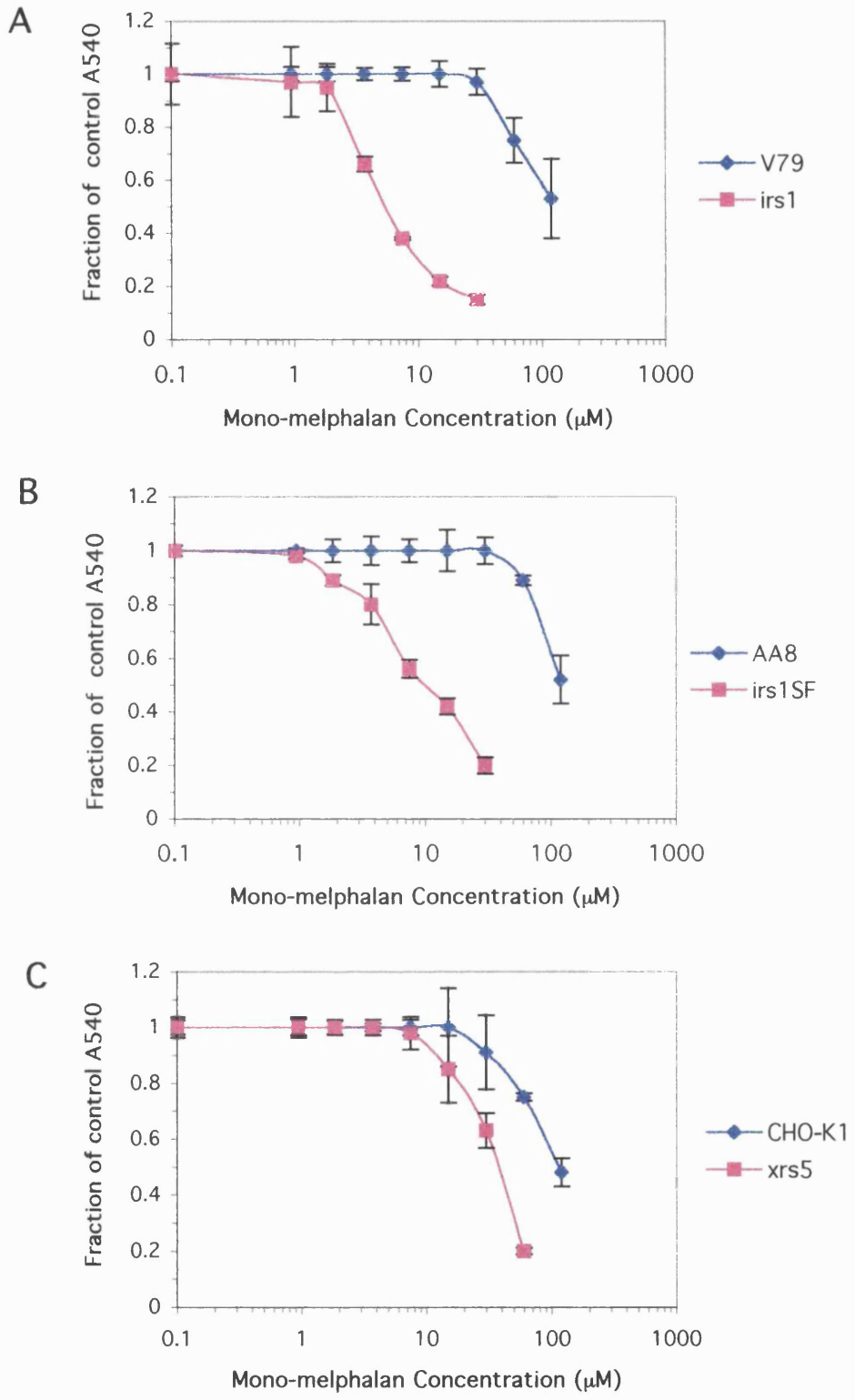

Figure 5.5. Cytotoxicity of mono-melphalan in HR mutants, A. XRCC2 (irs1) and the wild type cell line V79, B. XRCC3 (irs1SF) and wild type AA8 cell line, and C. NHEJ mutant, XRCC5 (xrs5) and the wild type cell line CHO-K1. Cells were treated with increasing concentrations of melphalan for 1 hour, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition for each dose was calculated as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

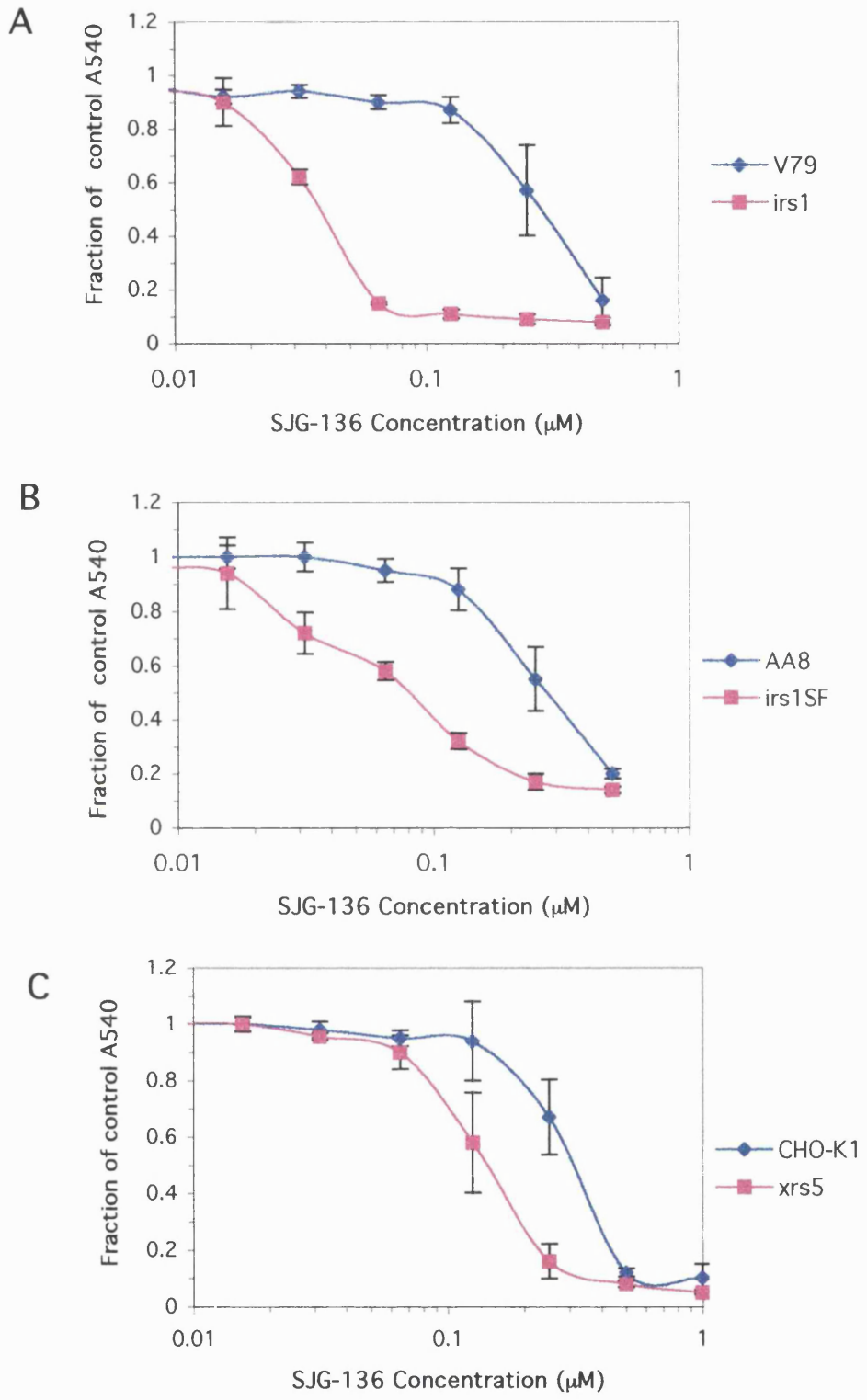

Figure 5.6. Cytotoxicity of SJG-136 in HR mutants, A. XRCC2 (irs1) and the wild type cell line V79, B. XRCC3 (irs1SF) and wild type AA8 cell line, and C. NHEJ mutant, XRCC5 (xrs5) and the wild type cell line CHO-K1. Cells were treated with increasing concentrations of SJG-136 for 1 hour, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition for each dose was calculated as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

than the XRCC3 mutant (7.5 fold and 3.5 fold, respectively) compared to the parent cell line. The NHEJ mutant, XRCC5, showed only a 2-fold higher sensitivity to SJG-136 than its parent cell line (figure 5.6C).

Similarly, both XRCC2 and XRCC3 mutants also showed a higher sensitivity to MMY. A 9-fold and 3.3-fold higher sensitivity was observed in XRCC2 and XRCC3 mutants, respectively, compared to their parent cell line. (figure 5.7A and 5.7B). This pattern of sensitivity is similar to that observed for SJG-136. The XRCC5 mutant showed only a 2.7 fold higher sensitivity to mmy (figure 5.7C) compared to its parent cell line.

The above results illustrate that the XRCC2 and XRCC3 homologous recombination components are not only important in the repair of ICLs, but also play a role in the repair of some DNA-lesions induced by mono-functional agents. In the case of the minor groove agents studied, XRCC2 seems to play a more important role than XRCC3.

5.2.4. Induction and repair of ICLs

To compare the level of induction of ICLs by melphalan and SJG-136, AA8 cells were treated with increasing concentrations of the drug for 1 h and the level of ICLs measured using the modified comet assay as described in Materials and Methods. As shown in figure 5.8, both melphalan and SJG-136 induced ICLs in a dose dependent manner. SJG-136 was much more efficient in the induction of ICLs than melphalan. A

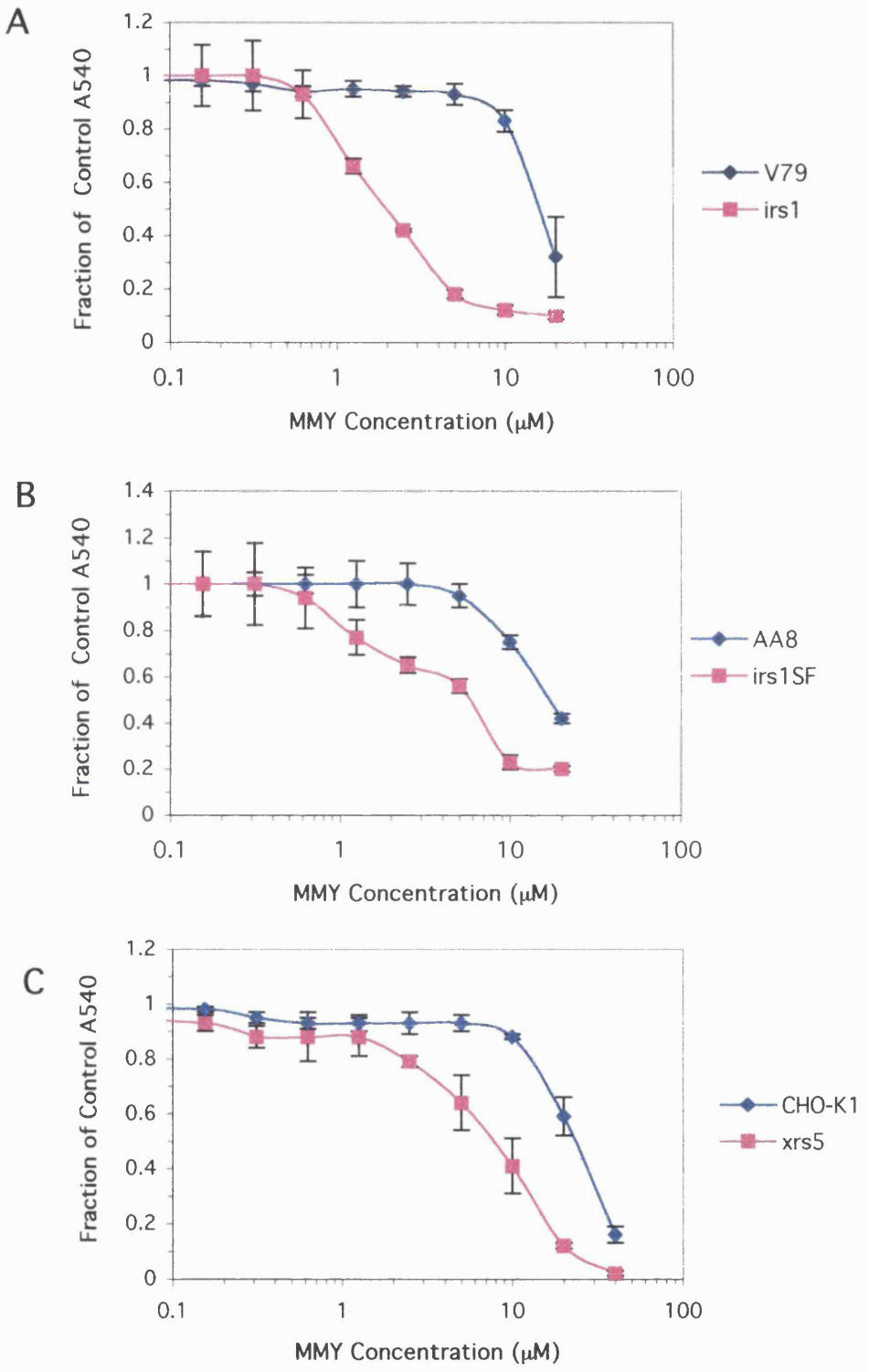

Figure 5.7. Cytotoxicity of MMY in HR mutants, **A.** XRCC2 (irs1) and the wild type cell line V79, **B.** XRCC3 (irs1SF) and wild type AA8 cell line, and **C.** NHEJ mutant, XRCC5 (xrs5) and the wild type cell line CHO-K1. Cells were treated with increasing concentrations of MMY for 1 hour, incubated in drug free medium for 3 days and stained with SRB. The fraction of growth inhibition for each dose was calculated as described in Materials and Methods. All results are the mean of at least three independent experiments, and error bars show the standard error of the mean.

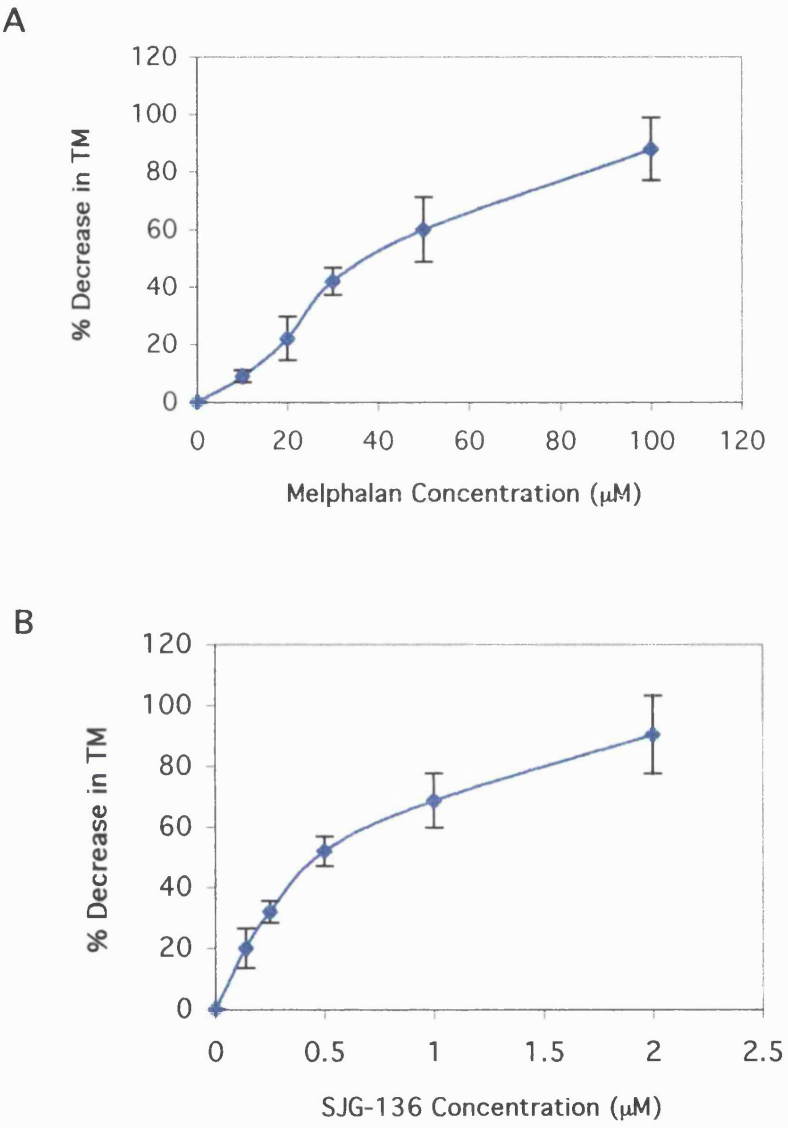

Figure 5.8. Induction of ICLs, represented as percentage decrease in TM following treatment of AA8 cells with melphalan (**A**) and SJG-136 (**B**) Exponentially growing AA8 cells were treated with increasing doses of the drug for 1 h and analysed immediately using modified comet assay. For each drug concentration, TM of 50 comets was mesured using Kinetic Imaging Software and mean values were calculated. The percentage decrease in the TM compared to the untreated sample was then calculated as described in Materials and Methods section. Results shown above are mean of three independent experiments, and the error bars represent the standard error of the mean.

50% decrease in TM was observed following treatment of cells with approximately 0.4 μM SJG-136. A dose of 35 μM was required to observe a similar level ICLs formation by melphalan.

In subsequent experiments to investigate the incision or unhooking of melphalan and SJG-136 induced ICLs, 30 μM and 0.3 μM doses were used, respectively. These concentrations were chosen as they produced 50% growth inhibition in cytotoxicity assays (IC50 values) and gave 40 - 55% inhibition of TM in the comet assay. To investigate the kinetics of ICL unhooking, cells were treated with the drug for 1 h, and incubated in fresh medium to allow repair, and the level of ICLs measured at various time points. The kinetics of unhooking of ICLs induced by melphalan and SJG-136 in AA8 parent cell line are compared in figure 5.9. The level of ICLs induced by both drugs remained relatively constant during the first 24 h period following drug removal. At 48 h and 72 h nearly 60% and 90% of the melphalan induced ICLs were unhooked. However, unhooking of SJG-136 induced ICLs was significantly less efficient. Only 30% and 44% of ICLs were unhooked at 48 h and 72 h following drug removal.

Figure 5.10A illustrate the kinetics of unhooking of melphalan induced ICLs by NER mutants compared to their parent cell line. In Chapter 3, it was shown that XPF and ERCC1, but not other NER mutants examined were defective in the unhooking of HN2 induced ICLs. Similarly, XPF and ERCC1 mutants were also defective in the unhooking of melphalan induced ICLs, and XPB and XPG mutants incised the ICLs with an efficiency similar to the parent cell line. Therefore, the unhooking of

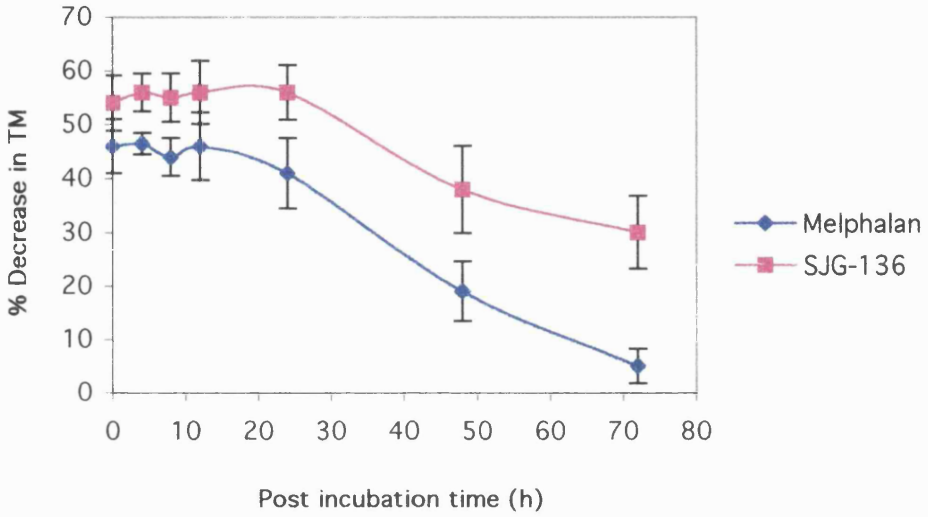

Figure 5.9. Kinetics of unhooking of melphalan and SJG-136 induced ICLs. Exponentially growing AA8 cells were treated with 30 μM melphalan or 0.3 μM SJG-136 for 1 hour, incubated in fresh medium and the level of ICLs was measured at various time points using modified comet assay as explained in Materials and Methods.Results presented are mean of three independent experiments and error bars shows the standard error of the mean.

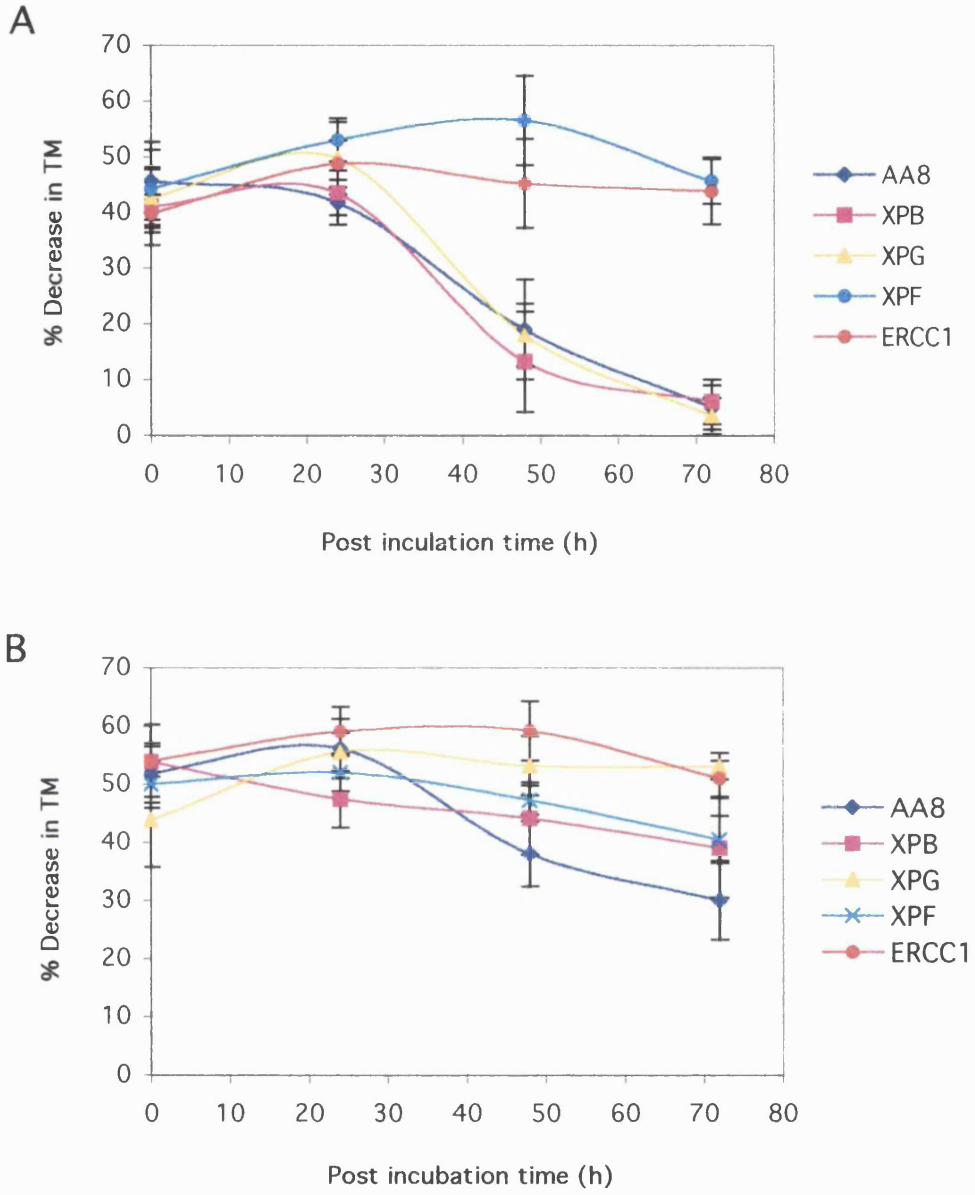

Figure 5.10. Kinetics of unhooking of melphalan (**A**) and SJG-136 (**B**) induced ICLs in parent (AA8) and NER mutant cell lines; XPB (UV23), XPG (UV135), XPF (UV47) and ERCC1 (UV96).Cells were treated with 30 μM melphalan (**A**) or 0.3 μM SJG-136 (**B**) for 1 hour, incubated in fresh medium and the level of ICLs was measured at various time points using modified comet assay as explained in Materials and Methods.Results presented are mean of three independent experiments and error bars show the standard error of the mean.

melphalan induced ICLs by NER mutants correlate well with their sensitivities to melphalan.

The unhooking kinetics of SJG-136 induced ICLs are presented in figure 5.10B. All NER mutants examined were defective in the unhooking of these ICLs. Suprisingly, these data do not show a clear correlation between the sensitivity of NER mutants to SJG-136 and ability to incise at ICLs.

5.2.5. DSB induction.

In chapter 3, it was shown that following treatment of cells with HN2, DSBs are induced. However, such an induction of DSBs was not observed in the case of cisplatin (Chapter 4). The induction of DSBs was also investigated following treatment with melphalan and SJG-136. Figure 5.11A shows that following treatment of AA8 cells with melphalan for 1 h, DSBs arise in a dose dependent manner. As shown in figure 5.11B these DSBs are repaired with time. The repair kinetics of melphalan-induced DSBs are slower than those of HN2 induced DSBs. In chapter 3 it was shown that majority of HN2 induced DSBs are repaired within 8 h and complete repair was observed at 24 h. In the case of melphalan, significant number of DSBs still remained un-repaired following 24 h post incubation time.

No DSBs were observed following treatment of AA8 cells with SJG-136 (figure 5.12A). To investigate whether DSBs arise at later time points, AA8 cells were treated with 0.5 μM dose of SJG-136 and induction of DSBs was assessed between 0 and 24 h

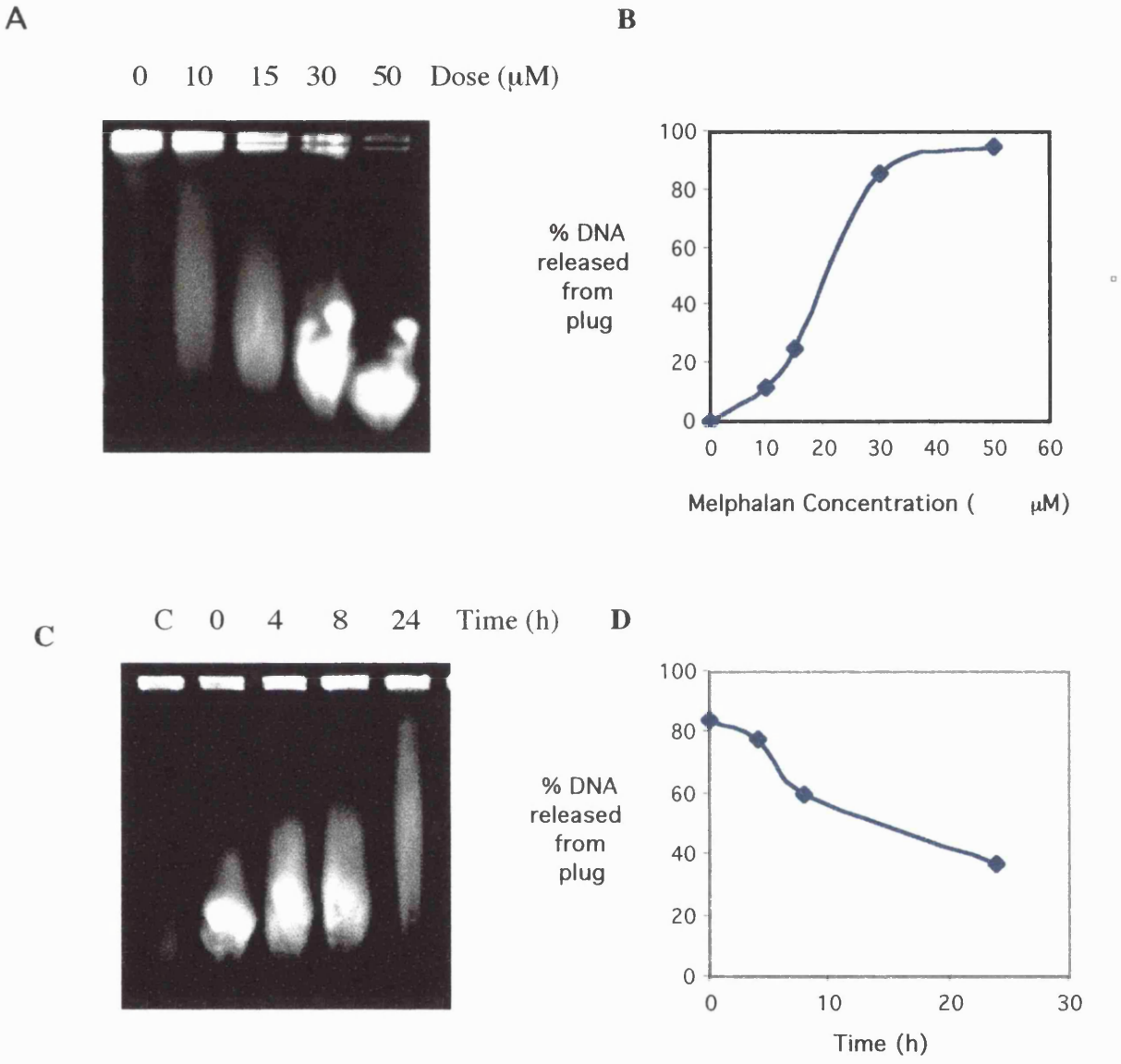

Figure 5.11. Induction (**A & B**) and repair (**C & D**) of DSBs following treatment with melphalan. **A.** Exponentially growing AA8 cells were treated with increasing doses of melphalan for 1 h and DSBs were analysed immediately using PFGE method. **B.** Quantitative analysis of the percentage of DNA released from the plugs for the gel shown in **A. C.** AA8 cells were treated with 30 μM melphalan for 1 h, incubated in drug free medium and DSBs were analysed at various time points. **D.** Quantitative analysis of the percentage of DNA released from the plugs for the gel shown in **C.**

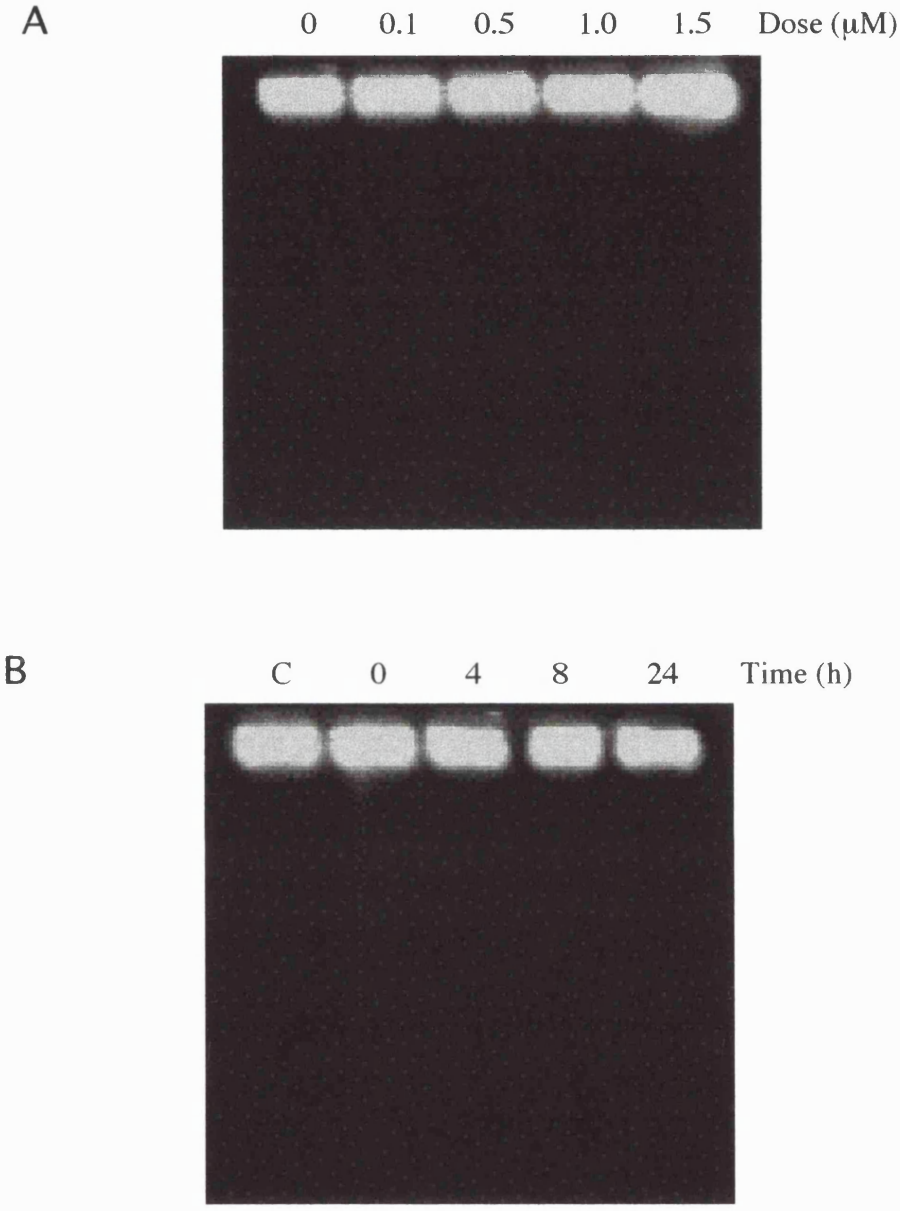

Figure 5.12. Induction (**A**) and repair (**B**) of DSBs following treatment with SJG-136 **A**. Exponentially growing AA8 cells were treated with increasing doses of SJG-136 for 1 h and DSBs were analysed immediately using PFGE method. **B.** AA8 cells were treated with 0.3 μM SJG-136 for 1 h, incubated in drug free medium and DSBs were analysed at various time points.

time points. As shown in figure 5.12B, DSBs were not observed at any of the time points tested. Therefore these results demonstrate that the processing of DNA damage produced by SJG-136 does not induce DSBs.

To investigate whether melphalan-induced DSBs arise as a result of ICL or mono-adduct processing, DSB induction was monitored following treatment of AA8 cells with a dose of mono-melphalan equivalent to the IC50 value of melphalan (30 μM). Figure 5.13 shows that no DSBs were induced. However, when cells were treated with the IC50 dose of mono-melphalan (120 μM), DSBs were induced. These results suggest that not only ICLs, but also a high number of DNA mono-adducts can lead to DSB formation.

It is possible that DSBs induced by melphalan and mono-melphalan, are a result of many closely opposed single strand breaks on both strands of DNA. Such single-strand breaks induced by melphalan would not be detected using the comet assay due to the presence of ICLs. However, the comet assay does permit the detection of single-strand breaks following treatment with mono-melphalan as ICLs are not induced. Therefore, the induction of single-strand breaks by mono-melphalan was investigated using the comet assay, omitting the irradiation step. As shown in figure 5.14, at 100 μM dose no detectable single strand breaks were observed. But at doses above 100 μM, single-strand breaks occurred in a dose dependent manner. However, at doses where DSBs were observed (120 μM), only a very low level of single-strand breaks was detected. These results do not support the hypothesis that the DSBs induced by mono-melphalan

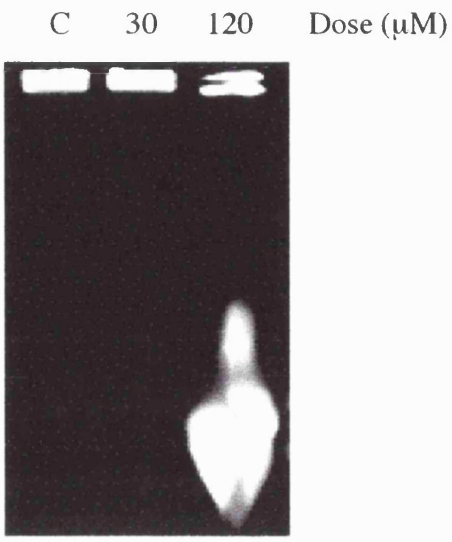

Figure 5.13. Induction of DSB following treatment with mono-melphalan. Exponentially growing AA8 cells were treated with 30 μM (equimolar dose) or 120 μM mono-melphaln (equitoxic dose) for 1 h and DSBs analysed immediately. C = control untreated sample.

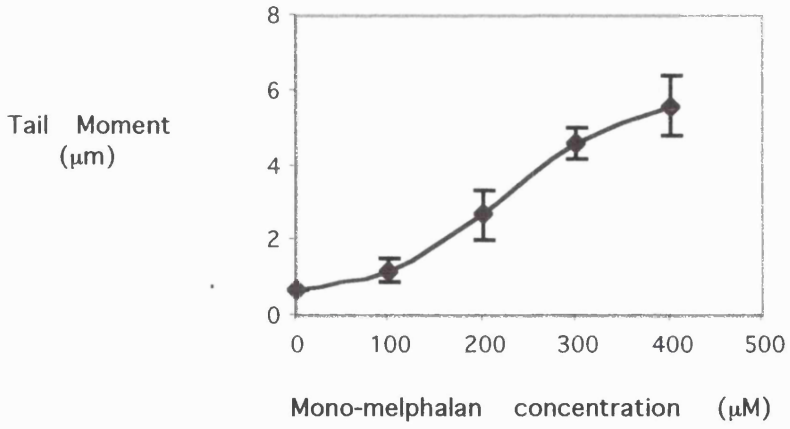

Figure 5.14. Induction of single strand breaks following treatment of AA8 cells with increasing doses of mono-melphalan. Exponentially growing AA8 cells were treated with increasing doses of mono-melphalan for 1 h and analysed immediately using comet assay omitting the irradiation step. Results are mean of three independent experiments and the error bars show the standard error of the mean.

are the result of many closely opposed single-strand breaks occurring on both strands of DNA.

5.3. DISCUSSION

Confirming previous reports (reviewed by Tew *et al.*, 1996, Hartley 2001), the results presented here indicate that bifunctional agents are more cytotoxic than their monofunctional analogues. The highly cytotoxic effects of bifunctional agents correlate well with their ability to induced DNA ICLs. The minor groove binding bifunctional agent SJG-136 showed extreme cytotoxicity and was highly efficient in the induction of ICLs. Its monomeric analogue MMY was also highly cytotoxic compared to the major groove agents tested. These results imply that minor groove binding alkylating agents are more potent than the major groove binding agents. Similarly, other minor groove agents such as tallimustine and CC-1065 have also shown higher cytotoxicity than more conventional major groove binding agents (Damia *et al.*, 1996).

The higher cytotoxicity of minor groove binders could be due to slower or absent repair of the lesions they induce. For example, human LoVo cells are unable to repair DNA damage induced by the minor groove alkylating agent tallimustine (Rossi *et al.* 1996). It is known that many minor groove alkylating agents do not cause as much DNA helix distortion as the major groove binders. Therefore, cellular DNA repair proteins may not recognise these lesions as well as those produced in the major groove binders. Supporting this hypothesis, the repair of SJG-136 induced ICLs has not been observed even at 48 h following drug removal in human fibroblasts (Peter Clingen,

personnel communication). Results presented here shows that these ICLs are repaired in CHO cells, but at a much slower rate compared to those produced by melphalan, HN2 or cisplatin. The level of SJG-136 induced ICLs remained relatively constant over 24 h and only 30 and 44% of ICLs were unhooked at 48 h and 72 h, respectively. Collectively these data suggest that both the high efficiency of ICL induction and inefficient repair may contribute to the high cytotoxic potency of SJG-136.

Previous studies using human melanoma cells (Hansson *et al.*, 1987) and murine L1210 cells (Ross *et al.*, 1978) have shown that the peak of melphalan-induced ICL formation occurs between 6 - 12 h following drug removal. In this study, considerable level of ICLs were observed immediately after drug removal, which remained relatively constant during a 24 h period. The reason for the difference in peak of ICL formation observed in this study and in previous studies could be due to an increased drug uptake or increased steady state repair of ICLs in the AA8 cells. At 48 h and 72 h following drug removal, nearly 60% and 90% of the melphalan induced ICLs were incised.

XPF and ERCC1 mutants were highly sensitive to melphalan and SJG-136, but not to their monofunctional counterparts mono-melphalan and MMY, respectively. In chapter 3 and 4 it was shown that these mutants are also highly sensitive to HN2 and cisplatin. The extent of sensitivity of XPF and ERCC1 mutants to SJG-136 was significantly lower than that for the other major groove binding agents tested (7.5 fold increase compared to $>$ 15 fold), suggesting that XPF and ERCC1 factors are less important in the repair of the ICLs in the minor groove of DNA than in the major

groove. One explanation for this observation could be that other unknown repair mechanisms may exist which act on the minor groove DNA lesions, in addition to an XPF and ERCC1 dependent mechanism.

All the NER mutants tested showed a similar level of sensitivity to both mono-melphalan and MMY, suggesting that all components of the NER machinery are important in the repair of bulky mono-adducts. The relatively small increased sensitivity of XPB, XPD, and XPG mutants to melphalan and SJG-136 could be due to an inability to repair the mono-adducts induced by these agents. *In vitro* studies employing cells extracts and DNA substrate containing melphalan mono-adduct have shown that the complete NER system is required to excise melphalan monoadducts (Grant *et al.*, 1998).

The highly sensitive XPF and ERCC1 mutants were defective in the incision of ICLs induced by melphalan, whereas the slightly sensitive XPB and XPG mutants were able to unhook ICLs normally. These results suggest that the mechanism of incision of melphalan induced ICLs is the same as that for HN2 (Chapter 3). Only the XPF and ERCC1 components are required for this step. In contrast, all the NER mutants tested were defective in the repair of SJG-136 induced ICLs, suggesting that all the NER components tested are required for the unhooking of this type of ICLs. This lack of correlation between cytotoxicity and incision of ICLs was unexpected as the major cytotoxic lesion induced by SJG-136 is thought to be ICLs. It is possible that the mono-adducts induced by this drug also contribute to cytotoxicity. Currently, not much

is known about the different types of lesions induced by SJG-136. These results suggest that lesions such as DNA protein cross-links, intrastrand cross-links may also contribute to the cytotoxicity of SJG 136.

In Chapter 3 it was demonstrated that DSBs arise following treatment of cells with HN2. In a similar manner, melphalan was also able to induce DSBs in dividing cells. Induction of DSBs has also been reported previously following treatment of A549 human lung epithelial cells with melphalan, which are subsequently repaired at a rate similar to that observed in this study (Vock *et al.*, 1998). Although DSBs were not induced following treatment of cells with an equimolar dose (30 μM) of mono-melphalan, they were induced at an equitoxic dose (120 μM). Similarly, in chapter 3 it was shown that when cells were treated with an equitoxic dose (IC50 value) of mono-HN2, DSBs are induced. These observations do not support the hypothesis that DSBs arise as a result of ICL processing as originally thought. An explanation for the mechanism of induction of DSBs by monofunctional nitrogen mustards is that occurrence of high frequency of single strand breaks on both strands of DNA may lead to formation of DSBs. However, although single-strand breaks were detected, very high doses of mono-melphalan were required to induce these single strand breaks. Therefore, these low levels of single strand breaks are unlikely to be the cause of DSBs.

Interestingly, DSBs were not induced by SJG-136, even following treatment with 4 times the IC50 dose. This observation again does not support the hypothesis that DSB

formation is a result of ICL processing. It seems that DSB formation is common to nitrogen mustards, but not to other agents. The exact mechanism by which these DSBs are induced is currently poorly understood and further studies are required to elucidate their origin.

XRCC2 and XRCC3 homologous recombination mutants were highly sensitive to melphalan, but the NHEJ mutant XRCC5 was only slightly sensitive. These results imply that melphalan-induced DSBs are mainly repaired by HR, and the contribution of NHEJ is minor. To date, XRCC2 and XRCC3 mutants were known to exhibit high sensitivity to bifunctional cross-linking agents (Caldecott *et al.*, 1991). Surprisingly, the results presented here show that both XRCC2 and XRCC3 mutants are also highly sensitive to mono-melphalan indicating that HR is also responsible for the repair of DNA damage induced by mono-melphalan. Since it was clear that DSBs are also induced following treatment with high doses of mono-melphalan, the greater sensitivity of XRCC2 and XRCC3 mutants to this agent could be due to impaired repair of the DSBs.

XRCC2 and XRCC3 mutants also demonstrated similar sensitivity to both SJG-136 and MMY, although, the level of sensitivity of XRCC2 mutant to both agents was twice than that of XRCC3 mutant. This observation suggests that XRCC2 plays an important role in the repair of DNA damage in the minor groove. It is possible that a sub-pathway of HR, which depends on XRCC2 is responsible for the repair of DNA damage in the minor groove. As the XRCC2 mutant showed higher sensitivity to both

SJG-136 and MMY, this XRCC2 dependent repair pathway is not only responsible for the repair of ICLs but also mono-adducts. Since DSBs are not induced by SJG-136 or MMY, the XRCC2 dependent repair pathway may be acting on other DNA lesions or repair intermediates. The equal level of increased sensitivity of XRCC2 mutant to both SJG-136 and MMY suggests that minor groove mono-adducts can be equally cytotoxic as ICLs when not repaired.

The sensitivity of the XRCC2 mutant to MMY was about 9 fold higher than the parent cell line. However NER mutants were only 1.6 - 3 fold more sensitive to MMY than the parent cell line. This novel finding implies that a XRCC2-dependent pathway is more important in the repair of DNA minor groove adducts induced by MMY than the NER pathway. Although it is known that NER is the main mechanism by which bulky DNA lesions are eliminated, these results provide initial evidence for the existence of a novel XRCC2-dependent pathway which still remains to be explored. Other novel mechanisms may also operate in the repair of minor groove adducts. Studies from this laboratory have shown that in *S. cerevisiae*, *RAD18* gene which belongs to *RAD6* epistasis group is highly important in the repair of DNA-adducts induced by benzoic acid mustard tethering 3 pyrrole units which selectively alkylate in the minor groove of DNA (Brooks *et al.*, 2000). Another recent study from this laboratory indicates that in *S. cerevisiae* RAD6 pathway also play a role in the repair of minor groove DNA lesions induced by CC-1065 (Konstantinos Kiakos, unpublished data). Collectively, these data suggests that possibly, RAD6 pathway may also play an important role in the repair of lesions induced by SJG-136 and MMY in mammalian cells.

CHAPTER 6: GENERAL DISCUSSION

Many clinically important chemotherapeutic agents induce DNA ICLs as the critical cytotoxic lesion. These adducts pose a particular challenge to DNA repair systems since they involve both strands of DNA and cannot therefore be repaired using the information in the complementary strand. Studies in *E.coli* and the yeast *S.cerevisiae* have shown that repair of ICLs involve a both NER and recombination pathways, and in the case of yeast, double strand breaks are induced as intermediates during this process (Cole *et al.*, 1973, Dardalhon *et al.*, 1995, McHugh *et al.*, 2000).

Much less in known about the biology of ICL repair in mammalian cells. This study aimed to understand the mechanism of repair of ICLs induced by nitrogen mustards, cisplatin and novel sequence specific minor groove cross-linking agents using a series of CHO cell lines mutated in NER, HR and NHEJ pathways. The availability of mono-functional analogues of these drugs (except for cisplatin) allowed the repair pathways acting on mono-adducts and ICLs to be distinguished.

Since mono-adducts damage only one strand of DNA, repair mechanisms for these lesions rely on the undamaged complementary DNA strand as the template. For instance, NER, BER and MMR pathways work in this way. Results presented here demonstrate that all the NER factors studied are equally important in the elemination of mono-adducts, since all NER mutants tested showed similar sensitivity to the mono-functional alkylating agents.

Repair of ICLs is more complicated, since it affect both strands of the double helix. As in yeast and bacteria, repair of these lesions in mammalian cells was thought to involve both NER and recombination. Confirming previously published data (Hoy *et al.*, 1985, Andersson *et al.*, 1996, Damia *et al.*, 1996), results presented in this study showed that XPF and ERCC1 mutants were extremely sensitive to cross-linking agents, but other NER mutants were only slightly sensitive. These observations indicate a unique role of XPF and ERCC1 in the elimination of cross-links. It has been suggested that the extreme sensitivity of XPF and ERCC1 mutants to cross-linking agents could be due to dual defects in NER and recombination (Weeda *et al.*, 1997, Thompson, 1999). Results presented in this study demonstrate that there is no unique repair mechanism that act on ICLs, but the pathway utilised depends on the type of the ICL. For instance, ICLs induced by the nitrogen mustards HN2 and melphlan are repaired by a different mechanism to that of ICLs induced by cisplatin or SJG-136.

Data presented illustrate that, consistent with their extreme sensitivity, ERCC1 and XPF mutants were defective in the incision or unhooking of ICLs induced by nitrogen mustards. Other NER mutants incised these ICLs proficiently. These findings do not support the suggestion that hypersensitivity of ERCC1 and XPF mutants to cross-linking agents is due to their dual roles in NER and recombination. Instead, it suggests that only ERCC1 and XPF repair factors are required for the incision of ICLs induced by nitrogen mustards, but that the whole NER mechanism does not play a role. In contrast to these findings, all NER mutants tested were deficient in the unhooking of ICLs induced by cisplatin and SJG-136. These observations suggest that ERCC1 and

XPF may depend on other NER factors for the incision of cisplatin and SJG-136 induced ICLs. Although XPG and XPB NER mutants were defective in the unhooking of the ICLs induced by cisplatin and SJG-136, these mutants were only slightly sensitive to these drugs. These results indicate that XPG and XPB mutant cells are capable of dividing even in the presence of ICLs.

Previous studies have clearly demonstrated that DSBs are generated at both psoralen and HN2-induced ICLs in the yeast *S. cerevisiae (*Jachymczyk *et al.*, 1981, Magana-Schwencke *et al.*, 1982, McHugh *et al.*, 2000). The results presented here demonstrated for the first time that DSB are induced during the processing of some ICLs in mammalian cells. A critical finding was that induction of these DSBs was highly reduced in non-dividing cells. These results are in agreement with studies in *S.cerevisiae.* In rapidly dividing yeast cells, DSBs were formed efficiently following treatment with HN2, but not in cells grown in stationary phase (McHugh *et al.*, 2000). This observation together, with data presented in this study, suggests that DSB are the result of arrest of replication folks at the sites of ICLs. Supporting this suggestion, a recent study by Akkari *et al.*, (2000) demonstrated that in human primary fibroblasts, psoralen ICLs induced during the G2 phase of the cell cycle, triggered extensive chromosomal breakage during passage through S phase of the cell cycle, but not during the mitosis immediately following treatment. These cells did not arrest in G2/M, but arrested in the next cycle following S phase. The authors suggest that ICLs may not be sensed, and hence not repaired in either G1 or G2 in human fibroblasts, and DNA replication is critical to induce cell cycle arrest and chromosomal breakage.

The exact mechanism of formation of replication-related nitrogen mustard-induced DSBs presented in this study is currently not clear. Results showed that they are not a result of the incision activities of ERCC1 and XPF at sites of ICLs, since DSB induction was also observed in ERCC1 and XPF mutants. Whether the breaks at the arrested replication forks are due to enzymatic cleavage of DNA, involving specific nucleases, or if the DNA breaks occur spontaneously at sites of ICLs, remains to be discovered.

A recent study in *E. coli* illustrated that mutants defective in double-strand break recombination pathways are hypersensitive to cisplatin, providing evidence that DSBs may arise in the repair of cisplatin ICLs (Zdraveski *et al.*, 2000). However, induction of DSBs following treatment with cisplatin had not been systematically explored. Results presented in this study show that, surprisingly, DSBs are not induced during processing of cisplatin-induced ICLs in mammalian cells. Similarly, induction of DSBs was not observed during the processing of ICLs induced by the minor groove cross-linking agent SJG-136. These observations lead to the suggestion that replication forks are not arrested at sites of ICLs induced by cisplatin or SJG-136, but are bypassed by DNA polymerases. This process is termed replication bypass (also called translesion replication, error prone repair or lesion bypass), which is defined as the ability of a replication complex to proceed past a DNA adduct known to block or stall the complex during synthesis (reviewed in Kunz *et al.*, 2000).

Studies by Hoffmann and co workers (1995) demonstrated that DNA polymerase β is capable of replication bypass of cisplatin d(GpG) intra-strand adducts *in vitro*, but DNA polymerases α, δ, and ε were blocked at the sites of these adducts. Interestingly, DNA polymerase β was also capable of elongating the arrested replication products of polymerase α ad δ, thus showing its capacity to successfully complete stalled replication. The products of translesion synthesis by polymerase β contain a high frequency of mutations in the vicinity of cisplatin-adducts (Hoffman *et al.*, 1996). On the basis of these data Hoffmann *et al.*, (1996) have suggested that polymerase β could be involved in error-prone translesion replication past DNA adducts *in vivo*. DNA polymerase β is the structurally simplest of the mammalian DNA polymerases, and is believed to function primarily on the repair of damaged DNA. In NIH 3T3 cells, down regulation of DNA polymerase β by an antisense RNA method, significantly enhanced the cytotoxicity of cisplatin, but not that of nitrogen mustards (Horton *et al.*, 1995), indicating the importance of DNA polymerase β specifically on the repair of cisplatin-induced DNA damage. Recent studies have shown that DNA polymerases η is also capable of translesion synthesis passed cisplatin adducts. Human polymerase η is the product of the XPV gene, which is mutated in XP variant patients (XP-V). XP-V cells are defective in replication of damaged DNA. In contrast to polymerase β, polymerase η can catalyse translesion synthesis past cisplatin d(GpG) intra-strand adducts with high fidelity (Masutani *et al.*, 2000, Vaisman *et al.*, 2000).

To date, translesion sythesis by either polymerase β or η passed cisplatin ICLs has not been studied. It is possible that these polymerases are also capable of bypassing

cisplatin and SJG-136-induced ICLs, which may explain why DSBs are not formed following treatment with these drugs. In contrast, these polymerases may not be able to bypass nitrogen mustard induced cross-links, and as a result, arrest of replication forks at sites of ICLs may lead to the induction of DSBs. The ability of these polymerases to bypass cisplatin and SJG-136 ICLs in comparison to that of nitrogen mustard induced ICLs has to be investigated to understand the origin of the DSBs observed by nitrogen mustards in this study.

As discussed above, the data presented showed that only XPF and ERCC1 were required for the incision of nitrogen mustard-induced cross-links, but all NER components were required for the incision of cisplatin and SJG-136 induced cross-links. One explanation for these observations is that induction of DSBs at the sites of ICLs may facilitate the access of XPF and ERCC1 to the sites of cross-links without the aid of other NER factors. But in the absence of DSBs, unwinding of the DNA double helix by the action of other components of the NER machinery may be required for XPF and ERCC1 to access the sites of ICLs.

The exact mechanism by which the XPF-ERCC1 endonuclease incises ICLs is not yet understood. This endonuclease is capable of incising many DNA structures, including junctions in stem loops, splayed arms, flaps, and single strand overhanging DNA (de Laat *et al.*, 1998). *In vitro* studies by Bessho *et al.*, (1997) indicated that during repair of psoralen-induced ICLs by CHO cell extracts, the NER system excises a 22-28 nucleotide oligomer from the 5' side of the ICL. The gap generated is filled by a futile

DNA synthesis reaction, but the ICL remains unrepaired (Mu *et al.*, 2000). The same group also demonstrated that in the presence of RPA, the XPF-ERCC1 complex could act as a 3' to 5' exonuclease on cross-linked DNA with high processivity (Mu *et al.*, 2000). This exonucleolytic digestion either terminates immediately past the cross-link, or can continue to the 5' terminus of the linear duplex substrates, completely removing one strand (Mu *et al.*, 2000). Interestingly, recent *in vitro* studies by Kuraoka *et al.*, (2000) have demonstrated that the XPF-ERCC1 endonuclease has the ability to cleave a psoralen-induced ICL placed in a duplex 4-6 bases from a junction with unpaired DNA, on both 5' and 3' side of the adduct. Which of these incision activities of XPF-ERCC1 complex is responsible for the unhooking of HN2, cisplatin and SJG-136 induced cross-links remains to be investigated.

The component of the XPF-ERCC1 complex which possesses the endonuclease activity is still not clear. This complex is found in human cells as a tight association of XPF and ERCC1 proteins (Biggerstaff *et al.*, 1993, van Vuuren *et al.*, 1993, Sijbers *et al.*, 1996, Gaillard *et al.*, 2001). Both proteins are undetectable, or very low, in cell extracts where the corresponding partner is mutated or absent (Biggerstaff *et al.*, 1993, van Vuuren *et al.*, 1993, Sijbers *et al.*, 1996, Gaillard *et al.*, 2001). However, recombinant XPF purified from *E. coli* has demonstrated endonuclease activity, and can bind to DNA in the absence of ERCC1 (McCutchen-Maloney *et al.*, 1999). The same study has shown that the DNA binding and endonuclease activity of XPF is located at the N-terminal domain of XPF and the c-terminal domain is responsible for XPF-ERCC1 complex formation. In agreement, another recent study by Kumaresan *et*

al., (2002) has demonstrated that two regions of XPF, one N-terminal region and one C-terminal region are highly important in the 5' incision at a psoralen ICL. It has been reported that the N-terminal region of ERCC1 can interact with XPA, therefore it is likely that a ternary XPF-ERCC1-XPA complex is formed. The exact mechanism of action of XPF-ERCC1 in monoadduct and cross-link repair still remains to be elucidated.

Although CHO mutants defective in XPF and ERCC1 are extremely sensitive to ICL inducing agents, human fibroblasts cell lines defective in XPF protein are only slightly sensitive to ICL inducing agents (Peter Clingen, unpublished data, Yagi *et al.*, 1998, Zhang *et al.*, 2000). To date, a clinical syndrome due to defective ERCC1 has not been reported. Homozygous disruption of the ERCC1 gene in mice led to severe runting and death (Weeda *et al.*, 1997). These mice exhibited unusual liver pathology, enlarged nuclei with both polyploidy and aneuploidy, absence of subcutaneous fat, early onset of ferritin deposition in the spleen, kidney malfunction, and defects in NER ICL repair. Authors have proposed that the phenotype of the ERCC1 -/- mice can be attributed to a deficiency in ICL repair by means of mitotic recombination pathways, since XPA -/- and XPC-/- mice are defective only in NER are not similarly affected (Weeda *et al.*, 1997).

If ERCC1 is highly important in the repair of ICLs, the only mild sensitivity of human XPF defective cell lines to cross-linking agents reported (Peter Clingen, unpublished data, Yagi *et al.*, 1998, Zhang *et al.*, 2000) is surprising, since XPF and ERCC1 act as

a complex. One explanation for this discrepancy is that the activities of XPF in NER and ICL repair are separable. In agreement with this suggestion, a recent study by Zhang *et al.*, (2000) has demonstrated that human XPF cells which showed only slight sensitivity to MMC, was deficient in NER but proficient in the repair of ICLs. In addition, another XPF defective CHO cell line, UV140 which was only mildly sensitive to MMC, were also defective in NER, but proficient in ICL repair (Zhang *et al.*, 2000). These results further suggests that activities of XPF in NER and ICL repair is separable and the mild sensitivity human XPF cells lines to ICL inducing agents may be due to their defects in NER but not in ICL repair. An impairment of XPF in ICL repair may be lethal, and therefore not reported to date.

In *S. cerevisiae* the Rad1/Rad10 endonuclease, the yeast homolog of the XPF-ERCC1 endonuclease, participates in the removal of non-homologous 3'-ends in the SSA pathway (Fishman-Lobell *et al.*, 1992). Similarly, there are evidence that XPF-ERCC1 may also play a role in SSA pathway. Studies in hamster and mouse cell lines indicate that deficiency in ERCC1 does not substantially alter the frequency of extrachromosomal or chromosomal homologous recombination (Sargent *et al.*, 1997, Melton *et al.*, 1998). However, ERCC1 deficiency resulted in a high frequency of rearrangements at the adenine phosphoribosyltransferase (APRT) locus, which occurred during homologous recombination between direct repeats (Sargent *et al.*, 1997). The reason for this high frequency of rearrangements was later found to be due to mis-processing of non-homologous 3' tails, indicating a role of XPF-ERCC1 in the SSA pathway of recombination (Sargent *et al.*, 2000).

The extreme sensitivity of XPF and ERCC1 CHO mutants to cross-linking agents has been suggested to be due to impairment in the repair of ICLs through recombination. Results presented here showed that in the repair of ICLs induced by nitrogen mustards, XPF and ERCC1 play a role in the incision of ICLs without the aid of other NER components, but XPF and ERCC1 do not play a role in the repair of the DSBs induced. These results imply that the high sensitivity of XPF and ERCC1 mutants to nitrogen mustards is due to their inability to incise the ICLs and not due to a defect in the recombination arm of repair. However, the results presented suggest that the high sensitivity of XPF and ERCC1 mutants to cisplatin and SJG-136, is not due to defective incision, since all NER mutants tested including the slightly sensitive mutants were deficient in the incision step. Since DSBs were not induced in the repair of cisplatin and SJG-136-induced ICLs, a different mechanism may operate to eliminate these ICLs. Therefore, it is possible that there is an additional role of XPF-ERCC1, maybe through the SSA pathway in the repair of cisplatin and SJG-136 induced ICLs.

DSBs induced during replication of cross-linked DNA are likely to represent a recombinogenic signal required to complete repair. In *S. cerevisiae*, DSBs induced during repair of psoralen ICLs and nitrogen mustard induced ICLs are predominantly repaired by HR (Averbeck *et al.*, 1998, McHugh *et al.*, 2000). In mammalian cells, IR induced DSBs are mainly repaired by NHEJ and it has been suggested that this pathway is predominantly responsible for DSB repair, in contrast to the situation in

lower eukaryotes (Weaver, 1995, Jeggo, 1998). However, during the last few years this concept has been revised and HR is now recognised as also being important mechanism for DSB repair in mammalian cells (Thacker, 1999). Results presented in this study demonstrated that the repair of DSBs induced by nitrogen mustards is substantially reduced in XRCC2 and XRCC3 CHO mutants, but not in NHEJ defective XRCC5 mutants. Therefore, these results imply that as in yeast, HR plays a critical role in the repair of ICL associated DSBs in mammalian cells and the role of NHEJ is minor. In agreement with these results, a recent study by Arnaudeau *et al.*, (2001) has shown that replication associated DSBs induced by campothesin are predominantly repaired by HR involving XRCC3 and NHEJ pathway is less important. Although DSBs induced by IR are mainly repaired by NHEJ these results imply that HR is highly important in the repair of replication associated DSBs. An important question to be answered is whether either one pathway or the other repairs only particular forms of DSBs. A DSB in the context of a replication fork probably differs from a replication independent DSB and the pathway employed to resolve these lesions might depend on the structure of the DSB.

Confirming previously published data (Caldecott and Jeggo, 1991), this study showed that XRCC2 and XRCC3 mutants were extremely sensitive to cisplatin, but a XRCC5 mutant was only slightly sensitive, indicating a role of HR in the repair of cisplatin induced DNA damage. Several other emerging HR components, including BRCA1, BRCA2, and RAD51 have also been implicated in the mammalian response to cisplatin-induced DNA damage (Yuan *et al.*, 1999, Takata *et al.*, 2000, Bhattacharyya

et al., 2000) and cells defective in these genes are sensitive to cisplatin. Together with these factors, the XPF-ERCC1 complex may also play a role in recombinational repair of cisplatin damage, since the results presented indicate that excision repair defects of cells lacking these activities do not account for the extreme sensitivity of these cell lines. Since DSBs are not formed following cisplatin treatment, repair intermediates induced by these ICLs must be different to those of nitrogen mustard induced ICLs. Future work must focus on the identification and comparison of ICL repair intermediates induced by different drugs and endpoints resulting from loss of XPF and ERCC1.

To understand the intermediates involved in the repair of psoralen-induced ICLs, Greenberg and co-workers (2001) characterised the repair products of site-specifically placed psoralen crosslinks, and compared the endpoints with those of psoralen monoadducts and DSBs. Psoralen ICLs induced gene conversion and crossing over at frequencies similar to DSBs, confirming homologous recombination as the major repair pathway. ICLs also induced targeted mutations, implicating error-prone post-replication repair as an alternate pathway for ICL repair. Therefore, it is possible that a similar mechanism may also operate in the repair of ICLs induced by cisplatin. To explore this area of ICL repair, similar studies would need to be performed with plasmids containing cisplatin and nitrogen mustard-induced ICLs.

The exact role of XRCC2 and XRCC3 in HR is still not well understood. But recently, several studies have shown that these proteins interact with other RAD51 paralogs,

suggesting that the five proteins might function as a complex. In human cells RAD51 paralogs are found in two distinct complexes. One complex contains RAD51B, RAD51C, RAD51D and XRCC2 (defined as BCDX2) and whereas the second contains RAD51C with XRCC3 (Masson *et al.*, 2001a, Wiese *et al.*, 2002, Liu *et al.*, 2002). BCDX2 can bind single stranded DNA and to single stranded gaps and nicks in duplex DNA (Masson *et al.*, 2001). RAD51C-XRCC3 bind preferentially to single strand DNA and promote DNA aggregation reactions that can lead to the annealing of complementary DNAs (Kurumizaka *et al.*, 2001, Masson *et al.*, 2001b). These studies imply that XRCC2 and XRCC3 together other RAD51 paralogs may play a role at early stages of recombination. It is possible that interaction of BCDX2 with nicks may be important for the repair of DNA ICLs. Since results presented demonstrated that XRCC2 is more important than XRCC3 in the repair of DNA lesions in the minor groove, it is possible that BCDX2 and RAD51-XRCC3 may act on different types of lesions and BCDX2 is highly important in the repair of minor groove DNA adducts. All five RAD51 paralogs have been disrupted in chicken DT40 cells and mutants shown to exhibit similar recombination defective phenotype (Takata et al., 2000, 2001). All show reduced growth rates, chromosomal instability, and accumulate spontaneous breaks, presumably due to an inability to repair broken replication forks. These studied further support the suggestion that XRCC2 and XRCC3, together with other RAD51 paralogs play an important role in HR recombination and in the repair of DSB and ICLs.

6.1. A Model for ICL repair in mammalian cells

Surveying the data available, it can be concluded that there is no single pathway responsible for all types of ICL repair. Different mechanisms are utilized depending on the type of ICL and the cell cycle phase. In accordance with the results presented in this study and those of several other studies, a model showing a possible pathway by which ICLs could be repaired or bypassed is proposed.

Data presented in this study suggests that nitrogen mustard-induced ICLs and cisplatin and SJG-136 induced ICLs are repaired by different mechanisms. There are two main differences between these repair pathways. Firstly, in the case of nitrogen mustards, DSBs are induced, but cisplatin and SJG-136 induced ICLs are repaired without such an intermediate. Secondly, only XPF and ERCC1 are required for unhooking of nitrogen mustard induced ICLs, but all NER factors are required for unhooking of cisplatin and SJG-136 induced ICLs. Taking into consideration the data presented in this study and other studies, any model for ICL repair must also account for the following observations:

(i) The observation of Mu *et al.*, (2000) that the XPF and ERCC1 heterodimer (in the presence of RPA) acts as a 3' to 5' exonuclease able to digest DNA past a cross-link.
(ii) XPF and ERCC1 and other NER factors are not required for the repair of ICL associated DSBs.
(iii) Homologous recombination but not the NHEJ pathway is required for the repair of ICL-induced DSBs.

(iv) The strand exchanges stimulated by ICLs require XPF, ERCC1, XRCC2, XRCC3 and RPA to proceed efficiently (Li *et al.*, 1999).

A proposed model for the repair of nitrogen mustard-induced ICLs is outlined in figure 6.1. In dividing cells, when replication forks encounter ICLs, they become inactivated and DSBs are generated. The DSBs formed initiate HR, which involves strand invasion mediated by XRCC2 and XRCC3. This is an early event and possibly it may precede the ERCC1-XPF excision event. Migration may be stalled at the site of a ICL, since it may require ICL excision to proceed further. Alternatively, it may extend past the cross-link, which has been demonstrated for RuvAB branch migration past a site specific psoralen cross-link in *E. coli* (George *et al.*, 2000). The cross-link is unhooked by XPF-ERCC1 heterodimer via its 3' to 5' exonucleolytic activity in the presence of RPA (Mu *et al.*, 2000). The gap generated as a result of XPF-ERCC1-RPA digestion is filled using the invading strand as the template. The incised cross-link moiety is subsequently removed in a second XPF-ERCC1 excision event, and the recombination intermediates are resolved.

The origins of cross-link induced DSBs are currently not known and require further investigation. It appears likely that in both yeast and mammalian cells they are the product of activities which act to process a stalled replication fork. This is an area of intense interest (Cox *et al.*, 2000, Flores-Rozas *et al.*, 2000), and although the biochemical details of the processes involved are emerging in *E.coli*, very little is known about these events in eukaryotes. A detailed understanding of the mechanism of

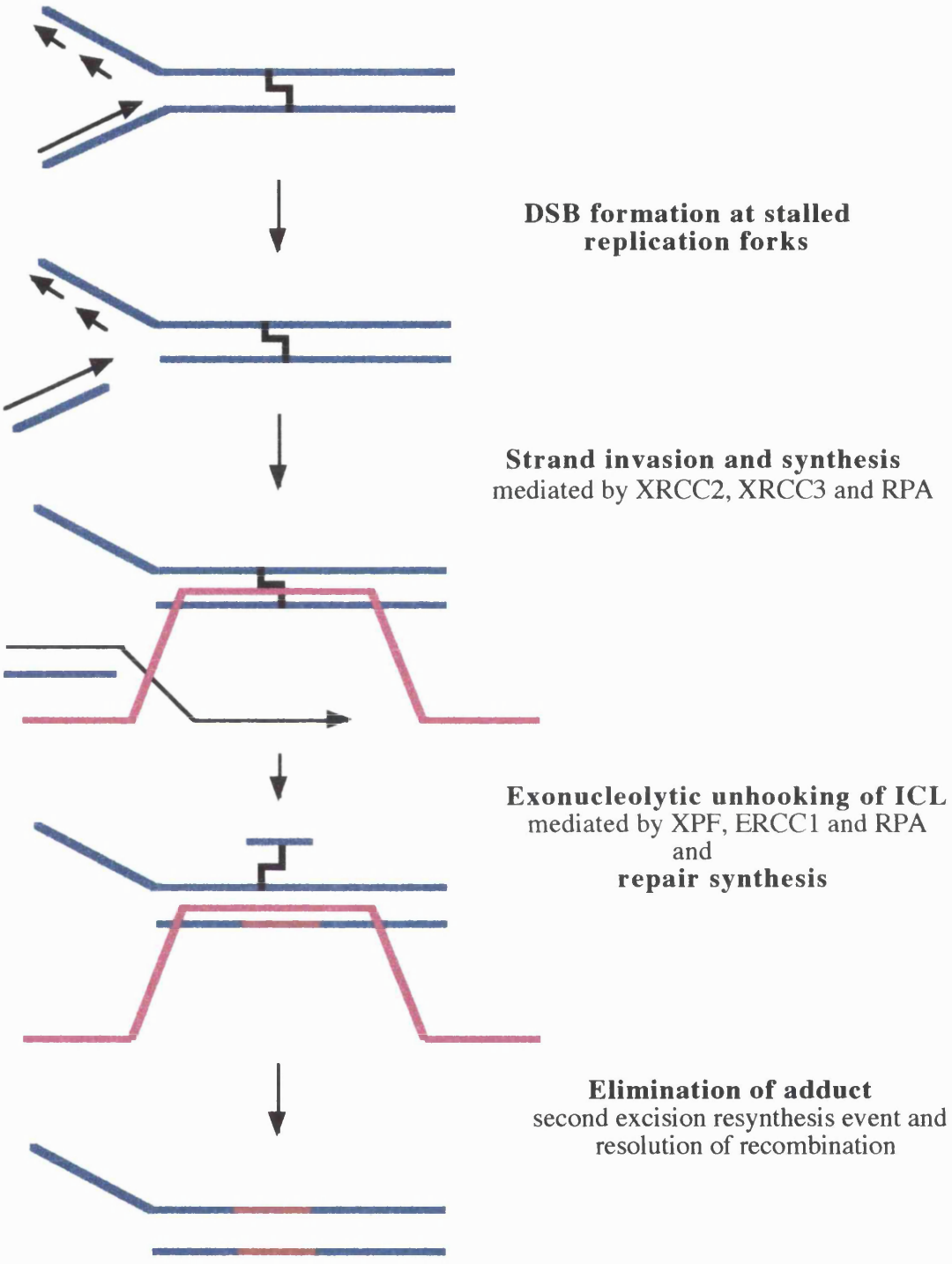

Figure 6.1. Proposed model for the repair of HN2-induced ICLs in mammalian cells. In dividing cells the arrest of replication forks near the site of cross-links generates DSBs. These DSBs initiate XRCC2 and XRCC3 dependent recombination. The XPF-ERCC1 heterodimer unhooks the cross-links through it's 3'-to-5' exonuclease activity in the presence of RPA. The gap generated is then filled, using the invading strand as the template. The second incision activity by the XPF-ERCC1 heterodimer then completely removes the unhooked cross-linked adduct. The gap generated is then filled using the newly synthesized complementary strand as the template.

DSB induction at stalled replication forks is necessary to develop a more detailed picture of the cellular processes acting to eliminate ICLs in higher eukaryotes.

Since DSBs are not observed during the repair of the ICLs induced by cisplatin and SJG-136, it is possible that the repair mechanism is similar to that in *E.coli*. A proposed model for the repair of these ICLs is outlined in figure 6.2. Results presented in this study suggest that in addition to XPF and ERCC1, other NER factors (XPG, XPB and XPD) also play a role in the incision of ICLs induced by cisplatin and SJG-136. Therefore it is possible that the incisions 5' to the ICL requiring the whole NER system identified by Bessho *et al.*, (1997) initiate this pathway. As the authors proposed, the incised substrate can be further degraded by the action of the 3' to 5' exonuclease activity of the XPF-ERCC1 complex in the presence of RPA (Mu *et al.*, 2000). This yields a single-stranded DNA region, which is necessary for recombination. Invasion and strand exchange then takes place with intact homologous DNA resulting in heteroduplex DNA encompassing the region containing the cross-linked oligonucleotide. Together with other homologoues recombination proteins, XRCC2, XRCC3 and possibly XPF-ERCC1 may play a role in this process. A second incision by NER proteins then releases the ICL-containing oligonucleotide, and the resulting gap is filled by DNA synthesis and ligation. Alternatively, the gap generated following incision can be filled in by a translesion polymerase possibly polymerase β or η.

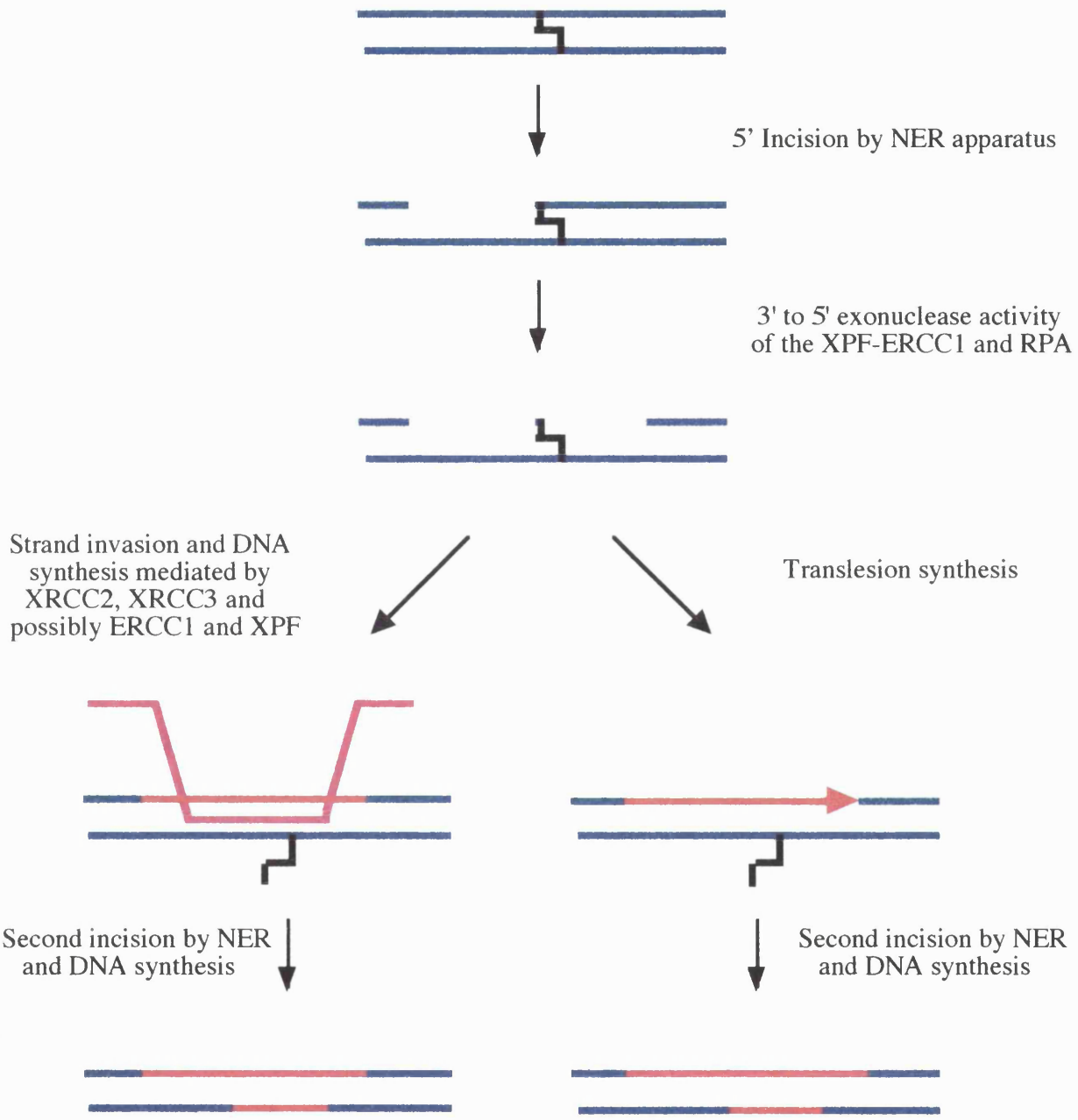

Figure 6.2. Proposed model for cisplatin or SJG-136 induced ICLs in mammalian cells. ICL repair is initiated by 5' incision requiring the whole NER system. The 3' to 5' exonucleolitic activity of XPF-ERCC1 possibly in the presence of RPA further degrade the DNA 3' to the ICL. The incised substrate can then be processed by either homologous recombination or translesion synthesis. Homologous recombination involves strand invasion and synthesis and XRCC2, XRCC3, ERCC1 and XPF may be required for this process. Alternatively, the gap generated following incision and exonucleolitic digestion can be filled in by translesion synthesis possibly involving DNA polymerase β or η.

6.2. Conclusions and future prospectus

Generally, ICL repair in mammalian cells has some similarities to ICL repair in bacteria and yeast, but as with most other cellular processes, mammalian ICL repair is more complex. As several mammalian homologs of *S. cerevisiae* ICL repair genes have a number of paralogs, it could be a sign of specialization of proteins in the repair of different types of ICLs, or at different stages of the cell cycle. The results presented in this study clearly demonstrated that the repair mechanism involved depends on the type of ICL.

Data presented showed for the first time that, in mammalian cells, repair of ICLs induced by nitrogen mustards involve a DSB intermediate. In contrast, ICLs induced by cisplatin and SJG-136 are resolved without such an intermediate. It was also shown that the HR pathway plays a role in the repair of DSB and other ICL repair intermediates but the role of NHEJ pathway was minor. Results also indicated that only XPF and ERCC1 NER factors are required for the unhooking of ICLs induced by nitrogen mustards, but all NER factors studied are required for the unhooking of cisplatin and SJG-136 induced ICLs. The extreme sensitivity of XPF and ERCC1 mutants to cisplatin and SJG-136 suggests that they may play an additional role in ICL repair, possibly in the recombination arm of repair or an additional incision or exonucleolytic activities.

To date, only a few ICL repair genes have been identified, which have been mainly discovered using cell lines hypersensitive to ICL inducing agents, and by searching for

homologs of yeast repair genes. Many other relevant repair genes may still remain to be discovered. Future studies must focus on the identification of novel genes involved in ICL repair pathways, and biochemical studies to focus on characterization of ICL repair intermediates.

Since many ICL agents are currently used in cancer chemotherapy, and novel minor groove cross-linking agents such as SJG-136 are in clinical development, knowledge about the formation and repair of these ICLs enables us to understand their mechanism of action. A recent study in this laboratory has clearly demonstrated that repair of melphalan-induced ICL may be a major mechanism for the development of acquired resistance to melphalan in multiple myeloma patients (Spanswick *et al.*, 2002). This study showed a marked difference between melphalan-induced ICL repair between melphalan naïve and melphalan treated patients. While naïve patients showed no repair, treated patients showed an increased repair of melphalan-induced ICLs. In these patients, *in vitro* sensitivity to melphalan in plasma cells correlated well with ICL repair. Another study has also shown a significantly increased repair of melphalan-induced ICLs in lymphocytes taken from chronic lymphocytic leukaemia (CLL) patients known to be resistance to nitrogen mustards (Torres-Garcia *et al.*, 1989). Although these studies demonstrate a correlation between ICL repair and resistance to chemotherapy, the exact mechanism that leads to increased ICL repair following chemotherapy still remains to be elucidated. A detailed knowledge about ICL repair pathways is not only important to understand the mechanisms of resistance to chemotherapy, but it will also contribute to the development of improved chemotherapeutic agents which can overcome resistance to combat cancer. The key

proteins involved in ICL repair can be considered as novel therapeutic targets, whose inhibition could increase sensitivity to cross-linking drugs.

In an attempt to develop more selective and less toxic drugs, novel cross-linking agents such as SJG-136 continue to be developed (Gregson *et al.*, 1999, 2001). The ability to deliver such potent drugs to tumour sites could lead to an effective treatment and reduced toxicity. Antibody directed enzyme prodrug therapy (ADEPT) is a two stage therapeutic approach with the objective of selectively generating a cytotoxic agent from its non toxic prodrug form at the tumour site (Springer and Niculescu-Duvas, 1997). Gene directed enzyme prodrug therapy (GDEPT) is a related approach but relies on the selective delivery of a gene coding for the relevant enzyme to the tumour site (Connors, 1995). Recently, minor groove binding PBD prodrugs using DC-81 and DSB-120 have been developed and evaluated for potential use in ADEPT and GDEPT therapies (Sagnou *et al.*, 2000). These preliminary studies have shown that PBD prodrugs can be activated 13-100 fold in the human adenocarcinoma cell line LS174T. Based on these results a design and synthesis of a second generation of PBD prodrugs with potentially higher activation factors is currently underway. Such treatments combined with strategies to inhibit ICL repair at tumour sites may not only enhance the therapeutic effectiveness but will also reduce the dose limiting toxicity which remains one of the major drawback in cancer chemotherapy.

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